NEUROTROPHIN TRAFFICKING IN DEVELOPMENT AND DISEASE

by
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ABSTRACT

Down syndrome is the most common chromosomal disorder affecting the nervous system in humans. All studies of neuronal defects in Down syndrome have focused on the central nervous system. However, Down syndrome individuals commonly display autonomic dysfunctions, the molecular mechanisms of which are unknown. The sympathetic nervous system is an important branch of the autonomic nervous system that if impaired, contributes to these defects. In this study, I examined the sympathetic nervous system in Down syndrome and the molecular mechanisms that disrupt its development.

Normal function of the sympathetic nervous system requires a precise, coordinated developmental program. Using human tissue samples and a trisomic mouse model, I found that the development of the sympathetic nervous system is disrupted in Down syndrome. I found that reduced target innervation and neuronal survival were a result of impaired trafficking of the neurotrophin nerve growth factor (NGF) and its cognate receptor tyrosine kinase, TrkA. The retrograde trafficking of the NGF-TrkA signaling endosome is essential in the development of the sympathetic nervous system.

The trafficking of NGF-TrkA is regulated by the serine-threonine phosphatase calcineurin. Calcineurin activity is regulated by the Down syndrome protein Regulator of Calcineurin 1 (RCAN1). Using *in vitro* adenoviral expression and a transgenic RCAN1 mouse *in vivo*, I showed that excess RCAN1 inhibits calcineurin activity, impairs TrkA trafficking, and disrupts NGF trophic functions in sympathetic neurons. By reducing RCAN1 dosage to wild type in a Down syndrome mouse model, I showed that RCAN1 overexpression is
necessary for TrkA trafficking defects found in DS. Reduction of RCAN1 dosage also ameliorated innervation and neuronal survival defects. This mechanism may also be present in other systems.

Basal forebrain cholinergic neurons (BFCNs) also require NGF trafficking for normal development and undergo degeneration in Down syndrome. I provide preliminary evidence that calcineurin also plays a role in BFCN development, likely through regulating NGF-TrkA trafficking. The overexpression of RCAN1 likely inhibits calcineurin activity in this mechanism as well, and contributes to degeneration of the BFCNs in Down syndrome. This study gives insight into how neurotrophins are trafficked during development and how defects in trafficking can lead to disease.

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PREFACE

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Chapter 1: Introduction
Down syndrome (DS) is the most common chromosomal disorder affecting the nervous system, occurring in about 1 out of 700 births in the United States (Parker, 2010). Initially characterized as a single disorder by Langdon Down in 1866, this genetic disease presents with a well-established set of identifiable characteristics including delayed development, craniofacial abnormalities and intellectual disability (Down, 1866). The cause of this disorder was not understood until 1959, when Jérôme Lejeune and colleagues attributed the phenotypes to trisomy of the 21st human chromosome (Lejeune, 1959). Trisomy most often occurs as a result of a non-disjunction event during gametogenesis (Shin, 2009). Once considered a life-threatening diagnosis, recent advances including improved treatment of congenital heart defects and social inclusion have doubled the average lifespan of DS individuals in 30 years (Yang, 2002; Presson, 2013). Despite these advances, DS individuals still suffer from a variety of conditions, many of which have a neuronal basis.

Down syndrome patients suffer from several conditions of the central nervous system that have been characterized and studied in great depth. Hypotonia and motor function delays in DS infants, for example, have been linked to the disrupted development of the cerebellum (Morris, 1982; Shumway-Cook, 1985; Spanò, 1999; Moldrich, 2007; Lyle, 2009). There is also an increased occurrence of epilepsy throughout infancy and young adulthood in DS individual (Stafstrom, 1991; Stafstrom, 1993; Bowley, 2000; McGrother, 2006). Mild to moderate cognitive impairment is found in all individuals, and is a hallmark of the disorder (Down, 1866; Pulsifer, 1996; Anderson, 2013). There is a delay in skill acquisition followed by a decline. These impairments include delayed language development, slow learning, and problems with memory.
Other psychiatric problems associated with this DS include behavioral issues and autism-related disorders (Kasari, 1990; Kent, 1999; Roizen et. al, 2003; Capone, 2006; Lowenthal, 2007; Molloy, 2009). Many studies have teased out the morphological changes and even changes in cellular function that are associated with these phenotypes (Coyle, 1986; Aylward, 1997; Siarey, 1999; Cooper, 2001; Bell, 2003; Kurt, 2004; Dang, 2014). However, very few have began to dissect the contribution of a single gene in the disease (Altafaj, 2008; Cossec, 2012). One such study found that the imbalance of excitatory and inhibitory synapses thought to underlie many of the functional defects in DS were due to Olig-mediated neurogenesis. An extra copy of Olig1 and Olig2 increased the neurogenesis of inhibitory neurons, altering prefrontal and cerebellar brain functions (Chakrabarti et. al, 2010). Another study linked the early onset memory loss in DS mice to the overexpression of APP, leading to the degeneration of basal forebrain cholinergic neurons (BFCNs), a cell population thought to play a key role in memory (Salehi et. al, 2006). These studies give basis to the idea that the overexpression of a single gene in DS can be largely responsible for a phenotype. As strides have been made to understand the changes in the central nervous system though, defects of the peripheral nervous system have been largely ignored.

Though autonomic and sensory defects in Down syndrome patients have been documented since the 1980s, the peripheral nervous system of these patients has not been investigated in any detail. Impaired somatosensory function has been documented, with DS patients reporting delayed and less precise reaction to painful stimuli (Brandt, 1995; Hennequin, 2000; Guerra, 2003;
Aguilar Cordero, 2015; Valkenburg, 2015). DS patients also suffer from autonomic dysfunctions including cardiac regulation during stress and exercise (Eberhard, 1991; Pitetti, 1991; Fernhall, 1996). Specifically, it was found that individuals with Down syndrome have reduced work capacity and chronotropic incompetence, functions regulated by the autonomic nervous system (Iellamo, 2005). Down syndrome individuals also have reduced response to sympathoexcitatory tasks and overall reduction in sympathetic tone, suggesting that aberrant sympathetic nervous system function is a major factor of this disorder (Fernhall, 2003; Fernhall, 2013). However, there have been very few studies investigating the mechanisms underlying autonomic dysfunctions, and no investigation into the neuronal components of these phenotypes.

The sympathetic nervous system is the branch of the autonomic nervous system that regulates the “fight or flight” response in stressful situations. The release of norepinephrine by sympathetic neurons dilates pupils, regulates blood sugar levels, and regulates cardiac function such as work capacity and chronotropic incompetence (LeLorier, 1976; Wallenstein, 1979; Matthews, 1980; Iverson, 2000; Roche, 2001). In times of stress, the brain activates preganglionic sympathetic neurons of the spinal cord that form cholinergic synapses on post-ganglionic neurons. Post-ganglionic sympathetic neuron cell bodies reside in the sympathetic chain, organized as bilateral pairs of ganglia that are connected by axonal fibers. These neurons project axons along vasculature proximally to reach final targets, which consist of almost every peripheral gland and organ including the nasal epithelium, salivary glands, spleen, pancreas, and heart. A robust, synchronized sympathetic response to stress is important to prepare the body for the proper response (Iverson, 2000). Full functioning of the sympathetic nervous
system relies on a precisely controlled development process that is well understood.

The progression of sympathetic nervous system development and many of the molecular players involved have been elucidated in great detail. Trunk neural crest cells integrate intrinsic transcriptional programs and extrinsic signals to generate sympathetic precursors. Under the guidance of semaphorins, neuropilins, ephrins, and adhesion molecules, these precursors migrate dorsally towards the aorta, and coalesce to form discrete ganglia (Britsch, 1998; Maina, 1998; Kawaski, 2002. The adrenergic cell fate is then acquired by expression of transcriptional regulators such as Mash1, Phox2b, Hand2 and Gata3 (Francis, 1999; Anderson 2000; Ernsberger, 2001; Goridis, 2002, Howard 2005). Precursors will proliferate and begin to extend neurites. Later stages of sympathetic development such as axon extension, dendrite and synapse formation, target innervation, and neuronal survival rely heavily on target-derived signals (Glebova, 2005).

Though many growth factors have been implicated in later stages of sympathetic development, the best characterized is the neurotrophin Nerve Growth Factor (NGF). NGF, secreted by target tissues, binds to its cognate receptor tyrosine kinases, TrkA, on the axonal terminal of sympathetic neurons. Upon binding to TrkA, NGF initiates several signaling cascades that result in axon growth, target innervation, and suppression of cell death pathways (Snider, 1994; Reichardt, 1997; Kaplan 1997; Sofroniew, 2001; Glebova, 2005). Globally knocking out the TrkA receptor in mice results in the complete loss of post-ganglionic sympathetic neurons and nociceptive sensory neurons. These mice only survive 2-3 weeks (Smeyne, 1994). Interestingly, NGF knockout mice die
shortly after birth, with an almost completely absent sympathetic nervous system. NGF/−/− mice that are also knockouts for the pro-apoptotic factor BAX, and therefore retain sympathetic cell number, have dramatically reduced innervation of target organs (Crowley, 1994; Fagan, 1996; Glebova, 2004). As a key regulator of sympathetic development, the NGF-TrkA signaling may be one of the main pathways that is affected in DS.

The signaling pathways that mediate NGF-dependent growth and survival have been largely worked out. Upon binding to NGF, TrkA receptors will dimerize and autophosphorylate. Similar to the other Trk receptors, phosphorylation at tyrosine residues will induce binding to signaling effectors such as SHC, PLCγ1, PI3K, FRS-2 and SH2-B (Kaplan, 1994; Green, 1995; Segal, 1996; Kaplan 1997). This binding will activate CREB transcription through the MAPK pathway, regulate apoptosis through the PI3K/AKT pathway, and release intracellular calcium through the PLCγ pathway (Riccio, 1997; Kuruvilla, 2000; Atwal, 2000; Azhou, 2009; Mok, 2009; Bodmer, 2011). The change in intracellular calcium levels can lead to the activation of Ca2+-calmodulin dependent pathways. As these effectors are present at the growth cone where NGF-TrkA binding occurs, cytoskeletal dynamics, axon growth and synapse formation can be regulated locally (Ginty et al., 2002). However, for full trophic function to occur, the NGF-TrkA signal must be retrogradely propagated to the cell body where it will induce a transcriptional response that is necessary for long-term axon growth, dendritic arborization, and overall neuronal survival (Watson, 1999; Watson 2001; Ye, 2003). This is made possible by the retrograde transport of the NGF-TrkA ligand receptor pair in a signaling endosome, an
organelle that allows continued signaling when it is transported from the axon to the cell body. The evidence for this signaling endosome is discussed below.

The idea that there is transport of NGF-TrkA stems from the observation that NGF protein, but not mRNA can be found in abundance at the sympathetic ganglia (Shelton, 1984; Bandtlow, 1987; Thoenen, 1988). At targets however, both the protein and mRNA can be found. This suggests that the NGF at the cell body is target-derived, and taken up by the neuron at the axonal terminal. Further, when labeled NGF is injected at the periphery, it can be detected at the cell body after several days (Hendry, 1974; Johnson, 1978). The significance of this finding was further dissected out using compartmentalized chamber system in vitro (Figure 1.1). In this system, sympathetic neurons whose bodies reside in one compartment project axons into a separate compartment. A grease barrier allows axons to reach this compartment, but prevents media in the two compartments from mixing (Campenote, 1977). By adding NGF to only the distal axon compartment, recapitulating the in vivo system, researchers were able to find activated Trk and downstream effectors at the cell body (Delcroix, 2003). This was also sufficient to promote neuronal axon growth and survival (Riccio, 1997; Senger, 1997; Tsui-Pierchala, 1999, Kuruvilla, 2000). When internalization is blocked at the axon terminal however, this signal is lost and neuronal death takes place. Labeling of Trk receptors at the axon terminal upon the addition of NGF also revealed that these receptors accumulate in the cell body. Furthermore, cross-linking experiments and isolation of an endosome consisting of activated TrkA, downstream effectors, and NGF indicate that this is the primary mode of retrograde propagation of the NGF-TrkA signal (Riccio, 1997; Kuruvilla, 2001;
Ye, 2003). The retrograde trafficking of this signaling endosome is vital for neurotrophins, and the disruption of this process has been implicated in disease.

Several studies have shown that the disruption of neurotrophin trafficking contributes to neuronal degeneration in a disease state (Bronfman, 2007; Bucci, 2014). One study showed that the transport of another neurotrophin, BDNF, is promoted by the protein huntingtin. Polyglutamine expansion of huntingtin, which is associated with Huntington’s disease, inhibits association of BDNF-containing vesicles with microtubules, resulting in cell death (Gauthier et al., 2004). In another study, a mutation in Alsin2 (AL2), implicated in familial amyotrophic lateral sclerosis (ALS), disrupted BDNF trafficking (Devon et al., 2006). BDNF trafficking is also disrupted in Alzheimer’s disease where β-amyloid oligomers impair trafficking of the activated ligand-Trk pair (Poon et al., 2011). Interestingly, disruption of NGF-TrkA trafficking leads to degeneration of the basal forebrain cholinergic neurons (BFCNs) in Down syndrome mice. This is due in part to excess amyloid precursor protein (APP) which prevents the signaling endosome from being transported to the cell body (Salehi et al., 2004). In all of these studies, neurotrophin trafficking is disproportionately affected. However, the cellular processes that are disrupted are general and may impact trafficking in general. To better understand how trafficking of a neurotrophin alone may contribute to disease, it is important to understand the unique mechanism by which neurotrophin trafficking occurs. This is best understood in sympathetic neurons, where NGF regulates its own internalization.

NGF itself modulates the formation of the NGF-TrkA signaling endosome in sympathetic neurons. This discovery stems from the differences between TrkA
trophic functions when binding to two different neurotrophins, NT-3 and NGF. While both neurotrophins are required for sympathetic neuronal development, NT-3 is only able to promote axon growth, whereas NGF can promote growth and survival. This is because NGF binding to TrkA can induce signaling at the cell body, whereas NT-3 signaling remains local. The addition of NT-3 to sympathetic neurons is not sufficient to induce internalization of TrkA. Further investigation revealed that NT-3 and NGF binding induces differential phosphorylation of the TrkA receptor. Phosphorylation of the tyrosine residue at 794 on TrkA by NGF is able to activate PLCγ, whereas NT-3 cannot (Figure 1.2). Phosphorylation of PLCγ and the resulting increase in the intracellular Ca^{2+} levels by release from intracellular stores activates the serine threonine phosphatase calcineurin. Calcineurin (CN) will then dephosphorylate the small GTPase, dynamin1. Dynamin1 is a neuronal protein that plays an important role in endocytosis of vesicles: it is required for the final scission step releasing the endosome from the cell membrane. Dynamin1 has two alternative splicing sites that result in at least 7 different isoforms. Only those isoforms that contain a PxIxIT motif are required for NGF-TrkA internalization. This motif is a common calcineurin interacting motif found in many proteins that bind to calcineurin (Bodmer et al, 2011). Though calcineurin is a ubiquitous protein expressed in all cell types and interacts with many proteins, the specificity of the dynamin1 isoform allows this system to be regulated locally and temporally. Normal calcineurin activity is required for proper trafficking to occur.

One protein important in normal functioning of calcineurin is regulator of calcineurin 1 (RCAN1). RCAN1 binds the catalytic subunit of CN and regulates
its phosphatase activity through a PxIxIT-like motif. Overexpression of RCAN1 inhibits CN-dependent processes, such as NFAT activation (Fuentes, 1995; Rothermal, 2000; Fuentes, 2000). Some studies also suggest that RCAN1 stimulates CN activity (Vega, 2003). Initially annotated as Down syndrome critical region 1 (DSCR1), this gene is found on Hsa21 and was one of a small number of genes thought to be part of the critical region that when trisomic, conferred the DS phenotype (Fuentes, 1995). RCAN1 has since been shown to recapitulate behavioral changes when it is individually over-expressed (Chang, 2003; Bhoiwala, 2013). RCAN1 has also been studied in the context of many diseases.

RCAN1 has been correlated with many diseases and developmental defects in several different tissue types. In the cardiovascular system, excess RCAN1, through its regulation of CN, has been found to be important for heart valve development, artherosclerosis, and other cardiovascular diseases (Méndez-Barbero, 2013). Overexpression has also been shown to disrupt immune responses and macrophage activation (Bhoiwala, 2011). RCAN1 also plays a role in growth plasticity in adult pancreas and may be contribute to diabetes through hypoinsulinemia. Most interestingly, RCAN1 has been found in high levels in the brains of patients with Alzheimer’s disease, as well as the brains of Down syndrome patients with symptoms of dementia (Ermak, 2001; Cook, 2005). In these diseases, decreased calcineurin activity and disrupted endocytosis is thought to contribute to overall neuronal dysfunction.

This study provides new insights into the molecular mechanisms underlying the previously unknown defects in sympathetic nervous system
development in Down syndrome. I found disrupted sympathetic nervous system
development in a Down syndrome mouse model and reduced innervation of
Down syndrome human tissue samples. I showed that these defects are a result
of disrupted NGF-TrkA trafficking. Trafficking defects are due to overexpression
of the Down syndrome gene *Regulator of Calcineurin 1* (RCAN1). When
individually overexpressed, RCAN1 is sufficient to block NGF-dependent TrkA
endocytosis, retrograde signaling and trophic functions *in vitro*. Mice with 3
copies of *RCAN1* also recapitulate sympathetic defects seen in DS mice.
However, when RCAN1 is knocked down in a Down syndrome mouse, Trk
endocytosis, sympathetic innervation, and neuronal survival are rescued. I also
suggest that a CN-dependent mechanism for NGF-TrkA trafficking may also be
present in BFCNs by showing reduced innervation of the hippocampus and
soma atrophy of mice with calcineurin knocked out of cholinergic neurons.
Figure 1.1. Compartmentalized chamber system used to physically separate cell body and proximal axons from distal axons.

Using a teflon-grease barrier, this compartmentalized culture system allows for the addition of distinct media to the cell body and distal axon. It also allows for harvesting of protein separately from each compartment.
Figure 1.2. Calcineurin is required for NGF-dependent TrkA internalization, axon growth, and neuronal survival.

NGF-dependent activation of TrkA initiates the PLCγ effector pathway, activating the calcium-dependent serine threonine phosphatase calcineurin. Calcineurin dephosphorylates PxIxIT motif-containing isoforms of the GTPase dynamin1 to promote TrkA endocytosis. Calcineurin-mediated endocytosis of TrkA locally promotes axon outgrowth, while retrograde trafficking of the endocytic vesicle induces neuronal survival.
Chapter 2: Developmental deficits in the sympathetic nervous system in Down syndrome

A portion of the work in this chapter has been submitted for publication at Nature Neuroscience:

Ami Patel, Naoya Yamashita, Maria Ascaño, Daniel Bodmer, Erica Boehm, Chantal Bodkin-Clarke, Yun Kyoung Ryu, and Rejji Kuruvilla. RCAN1 links impaired neurotrophin trafficking to aberrant development of the sympathetic nervous system in Down syndrome.
INTRODUCTION

To date, investigations of anomalies in the nervous system in Down syndrome have predominantly focused on the central nervous system (CNS), where cognitive impairment has been proposed to stem, in part, from abnormal brain development and an imbalance between excitatory and inhibitory neurotransmission (Haydar, 2012). However, Down syndrome individuals also exhibit marked dysfunction of the peripheral nervous system, including impaired autonomic and sensory functions, the molecular and cellular bases of which remain undefined (Brandt, 1995; Fernhall, 2003; Iellamo, 2005; Fernhall, 2013). The sympathetic nervous system is a branch of the autonomic nervous system that is essential for organ homeostasis. Postganglionic sympathetic neurons innervate diverse peripheral organs and tissues to govern fundamental physiological processes including regulation of blood glucose levels, cardiac output and body temperature. An emergent concept is that a dysfunctional sympathetic nervous system might be an instigating factor in the pathogenesis of congestive heart failure and diabetes (Saravia, 2003; Hasan, 2013), diseases that are more prevalent in Down syndrome individuals than in the general population (Roizen, 2003). Additionally, Down syndrome individuals have blunted cardiovascular responses to autonomic tasks including stress and exercise tests, and reduced secretion of the sympathetic neurotransmitter, norepinephrine (O'Driscoll, 2012; Fernhall, 2013). Notably, Down syndrome individuals without congenital heart defects exhibit impaired sympathetic regulation of cardiovascular functions such as heart rate and blood pressure (Iellamo, 2005). Autonomic dysfunction in Down syndrome individuals has been associated with reduced physical work capacity, chronic incompetence,
manifested as attenuated heart rate responses to exercise, and overall quality of life (Fernhall, 2013). In particular, chronotropic incompetence has been postulated to be predictive of coronary heart disease and early mortality. Although impaired autonomic functions are manifested in infants and young children with Down syndrome (O'Driscoll, 2012), it remains unknown whether functional deficits originate, in part, from aberrant development of the sympathetic nervous system. Towards understanding the contribution of aberrant development to autonomic dysfunction, this study examined human peripheral tissue samples from Down syndrome infants and matched controls, as well as a Down syndrome mouse model.

Though many different mouse models have been used for Down syndrome research, this study uses the $Dp(16)1Yey/+\) mouse. This mouse is trisomic for all genes on Mmu16 that are syntenic to Hsa21, and was created through homologous recombination (Yu, 2010). This was chosen over the more widely used Ts65Dn mouse, which is trisomic for fewer syntenic genes, and also contains an extra centromeric region of Mmu17 that is not relevant to Down syndrome (Figure 2.1). The Ts65Dn mouse was generated through reciprocal translocation in 1995 (Reeves, 1995). It has been used to study DS as it has many of the same learning and memory deficits as DS patients (Yu, 2010, Zhang, 2014). Less commonly used mouse models contain fewer trisomic genes. These include the Ts1Cje mouse, which has a smaller portion of Mmu16 that has been translocated to Mmu12 through homologous recombination (Sago, 1998). A mouse trisomic for what was once considered the “Down syndrome critical region,” the Ts1Rhr mouse was generated and found not to recapitulate a full DS phenotype (Olson, 2007). Though each of these models is useful for research, the
Dp(16)1Yey is by far the most relevant, and has many of the same phenotypes as DS individuals.

The development of the sympathetic nervous system was studied in the $Dp(16)1Yey/+ \,$ mice to understand sympathetic defects in DS. Similar to human tissue samples, the DS mouse model has deficits in sympathetic target innervation. This reduction in innervation precedes neuronal survival deficits observed at birth (P0.5). However, early stages of sympathetic development including migration, specification, proliferation, and differentiation remain unperturbed.
RESULTS

Reduced sympathetic innervation in Down syndrome human tissue samples

To determine if there are defects in sympathetic development in Down syndrome individuals, we assessed sympathetic innervation of peripheral organs from Down syndrome infants (see Table 1 for specimen details). Human spleen and pancreatic tissues were sectioned and subjected to TH immunostaining. Compared to the control tissues, we observed a pronounced decrease of TH-positive fibers in the Down syndrome spleen (Fig. 2.2a-c) and pancreatic (Fig. 2.2d-f) tissues. Quantification revealed a significant reduction in TH immunoreactivity in the Down syndrome tissues compared to controls (p=0.01 for spleen, p=0.026 for pancreas, t-test, n=3 each for Down syndrome and normal donor tissues). Co-labeling with antibodies against smooth muscle actin to mark the spleen vasculature (Fig. 2.2a,b and Fig. 2.3a,b), and insulin and glucagon to identify pancreatic islets (Fig. 2.2d,e and Fig. 2.3d,e), showed no overall disruptions in tissue morphology in the Down syndrome samples. Furthermore, Hematoxylin and Eosin (H&E) staining revealed intact tissue structure and similar histology between the Down syndrome and control tissues (Fig. 2.4). To address if the innervation defect in Down syndrome tissues was specific to sympathetic axons, we performed immunostaining for neurofilament, a pan-neuronal marker. We observed a trend toward decreased neurofilament immunoreactivity in the Down syndrome spleen (Fig. 2.3a-c) and pancreas (Fig. 2.3d-e), although these deficits were not as severe as the decreases in TH immunoreactivity, and were not statistically different from control tissues (p=0.10 for spleen, p=0.31 for pancreas, t-test, n=3 each for Down syndrome and normal donor tissues). That the decrease in neurofilament immunoreactivity did
not reach statistical significance is likely due to sympathetic nerves constituting only a fraction of total peripheral innervation. Double labeling revealed that approximately 30% of the neurofilament-immunoreactive fibers innervating the human spleen (Fig. 2.5a-c,g) and pancreatic tissues are TH-positive (Fig. 2.5h-j,n). Notably, we observed a significant reduction in the amount of TH/NF-double-positive axons in the Down syndrome tissues (Fig. 2.5d-g,k-n; p=0.0013 for spleen and p=0.0458 for pancreas, t-test, n=3 Down syndrome and 3 control donor tissues), consistent with the decrease in sympathetic innervation in Down syndrome tissues.

**A Down syndrome mouse model has defects in sympathetic development**

To investigate the developing sympathetic nervous system in Down syndrome, we employed a mouse model of Down syndrome, *Dp(16)1Yey/+* mice, that harbor a 22.9 Mb duplication spanning the entire region of mouse chromosome 16 syntenic with human chromosome 21 (Li, 2007). *Dp(16)1Yey/+* mice have the advantage of being trisomic solely for the human 21q11-q22.3 syntenic region compared to the widely used Ts65Dn model (Figure 2.1), and exhibit cognitive, cardiovascular and gastrointestinal phenotypes that recapitulate that observed in humans with Down syndrome (Li, 2007; Yu, 2010; Das, 2011). We performed whole mount immunostaining for Tyrosine Hydroxylase (TH), a marker for noradrenergic sympathetic neurons, in embryonic mice to examine the formation of the entire sympathetic chain ganglia and their axonal projections. We examined mice at embryonic day 16.5 (E16.5), a stage when neurogenesis, migration and noradrenergic specification are completed in the murine sympathetic nervous system (Fagan, 1996). We observed that sympathetic chain ganglia had coalesced into discrete
condensations, with axonal projections coursing along the intercostal arteries in both \textit{Dp(16)1Yey/+} and litter-mate embryos (Fig. 2.6a,b). In addition, TH immunohistochemistry and quantification of cell numbers using Nissl staining in tissue sections revealed no significant differences in size, shape and neuronal numbers in the superior cervical ganglia (SCG), the rostral-most ganglia in the sympathetic chain, between E16.5 \textit{Dp(16)1Yey/+} and wild-type embryos (Fig. 2.6c-e). Therefore, early developmental processes including neuronal production, migration and specification in the sympathetic nervous system are unaffected in Down syndrome mice.

Examination of sympathetic innervation of distal target fields, however, revealed a marked impairment in E16.5 \textit{Dp(16)1Yey/+} embryos. Sympathetic fibers reached and innervated end-organs such as the spleen, heart and nasal epithelium in \textit{Dp(16)1Yey/+} embryos, but the axons were shorter, sparser and less branched within the target fields (Fig. 2.7a-i). Further examination of sympathetic innervation at post-natal day 0.5 (P0.5), revealed far fewer sympathetic fibers in the nasal epithelium (Fig. 2.8a-c) and salivary glands (Fig. 2.8e-f) in \textit{Dp(16)1Yey/+} mice compared to control litter-mates. Quantification of neuronal numbers in the SCGs revealed a substantial 45% decrease in \textit{Dp(16)1Yey/+} mice at P0.5 (Fig. 2.9a-c). Concomitant with a decrease in SCG cell number, we found a significant increase in apoptotic profiles in P0.5 \textit{Dp(16)1Yey/+} ganglia, as assessed by immunostaining for cleaved caspase-3 (Fig. 2.9e-f).

Together, these findings provide evidence of aberrant sympathetic nervous system development in human Down syndrome tissues, analogous to the situation in the \textit{Dp(16)1Yey/+} mouse model of Down syndrome.
Figure 2.1. Most commonly used mouse models for Down syndrome

The genes from human chromosome 21 are distributed throughout mouse chromosomes 10, 16, and 17. The most commonly used Down syndrome mouse model (Ts65Dn) is trisomic for only a portion of Mmu16, in addition to a non-DS related centromeric region. The Ts1Cje and Ts1Rhr are trisomic for subsequently smaller portions of Mmu16. Dp(16)1Yey mice have three copies of all the Down syndrome genes found on Mmu16.
Figure 2.2. Diminished sympathetic innervation of Down syndrome peripheral tissues.

(a-f) TH immunostaining (in green) shows reduced sympathetic innervation of the spleen (a,b) and pancreatic tissues (d,e) from Down syndrome children relative to normal individuals. Spleen tissue sections were immunostained with smooth muscle actin (SMA, red) to reveal blood vessels, and pancreatic tissues were immunostained with insulin (in red) and glucagon (in orange). Scale bar: 50 μm. (c,f) Quantification of TH-positive sympathetic fibers by measuring the integrated fluorescent density per unit area using ImageJ. Results were expressed as fluorescence units per 100μm². Human tissues were obtained from 3 Down syndrome children and 3 age-and gender-matched controls. *p<0.05, one-tailed t-test.
Figure 2.3. Overall innervation is slightly reduced but gross morphology of human tissues in Down syndrome is comparable to matched controls.

(a-f) Neurofilament immunostaining (in green) in the spleen (a-c) and pancreas (d-f) shows a slight decrease in overall innervation of Down syndrome tissues. Spleen tissue sections were immunostained with smooth muscle actin (SMA, red) for blood vessels, and pancreatic tissues were immunostained with insulin (in red), and counter-stained for DAPI (blue). Scale bar: 50 μm (c, f) Quantification of neurofilament immunoreactivity in the spleen and pancreatic tissues. Results are expressed as fluorescence units per 100 μm². n=3 Down syndrome and 3 control donors. P values are 0.10 and 0.31 for spleen and pancreas, respectively, using one tailed t-test.
Figure 2.4 H&E staining reveals no gross morphological differences between Down syndrome tissues and matched controls

Gross morphology of human tissues from Down syndrome individuals is comparable to matched controls, but overall innervation is slightly reduced. The gross morphology of Down syndrome spleen (b) and pancreas (d), as visualized by H&E staining, appear similar to control tissues. bv=blood vessels, w=white pulp, i=islet. Scale bar: 50 μm.
Figure 2.5. The proportion of axonal fibers costained for TH and neurofilament is reduced in human tissue from Down syndrome patients.

(a-f, h-m) Costaining of axonal fibers with TH and neurofilament in human spleen (a-f) and pancreas (h-m) samples. (a-c, h-j) Normal tissue reveals that about 30% of neurofilament fibers are TH positive. This is significantly reduced in DS patients (d-f, k-m). (g,n) Quantification was performed by averaging the Scale bar: 50 μm (c, f) Quantification of neurofilament immunoreactivity in the spleen and pancreatic tissues. Results are expressed as percent of neurofilament fibers that are TH positive. n=3 Down syndrome and 3 control donors. P values are 0.10 and 0.31 for spleen and pancreas, respectively, using one tailed t-test.
Figure 2.6. Early stages of sympathetic development are normal in Dp(16)1Yey/+ mice.

(a,b) Whole-mount tyrosine hydroxylase immunostaining shows normal segmented organization of sympathetic chain ganglia and TH expression in Dp(16)1Yey/+ mice at E16.5. Arrows indicate proximal projections from the sympathetic ganglia. Scale bar: 1 mm. (c-e) SCG morphology and cell numbers are normal in E16.5 Dp(16)1Yey/+ mice, as well. SCGs were visualized by TH immunohistochemistry and cell counts were performed on Nissl stained tissue sections. Results are means ± SEM from n=3 mice per genotype. Scale bar: 100 μm. (f-h) However, sympathetic innervation of a target tissue, the nasal epithelium, is significantly reduced in E16.5 Dp(16)1Yey/+ embryos. Scale bar: 100 μm.
Figure 2.7. Sympathetic innervation of target organs is decreased in E16.5

*Dp(16)1Yey/+* embryos.

(a-f) Sympathetic innervation of target organs is decreased in *Dp(16)1Yey/+* embryos. Whole mount tyrosine hydroxylase (TH) immunostaining of the spleen (a,b), and heart (c,d) reveals that axons are shorter, thinner and less branched in E16.5 *Dp(16)1Yey/+* mice compared to litter-mate controls. Higher magnification images of the heart are shown in (e,f). Terminal extension and branching within the target fields are indicated by arrowheads and arrows, respectively. Representative images are shown from at least 3 animals per genotype that were analyzed. Scale bar: 200 mm for (a,b), 500 mm (c,d) and 200 mm (e,f). (h,i) Sympathetic innervation of a target tissue, the nasal epithelium, is significantly reduced in E16.5 *Dp(16)1Yey/+* embryos. Scale bar: 100 μm.
Nasal Epithelium

Innervation density of NE (% WT)

WT Dp(16)1Yey/+ TH+DAPI E16.5

TH TH

Heart

WT Dp(16)1Yey/+ TH E16.5

TH

Spleen

WT Dp(16)1Yey/+ TH E16.5

TH

Nasal Epithelium

WT Dp(16)1Yey/+ TH+DAPI E16.5

TH

i

Innervation density of NE (% WT)

WT Dp(16)1Yey/+ *
Figure 2.8. Sympathetic nervous system development is perturbed in a
Dp(16)1Yey/+ animals at birth.

(a-c) Reduced sympathetic innervation of nasal epithelium in Dp(16)1Yey/+ mice compared to wild-type litter-mates at birth (P0.5), determined by TH immunohistochemistry of tissue sections. DAPI staining is included to reveal the cellular material in the entire tissue section. Values are the mean ± SEM, n=3 for each genotype, **p<0.01, Scale bar: 50 mm. (d-f) Reduced sympathetic innervation of salivary glands in Dp(16)1Yey/+ mice compared to wild-type litter-mates at birth (P0.5). Scale bar: 100 μm. Innervation density in the nasal epithelium and salivary glands was quantified from n=3 mice per genotype, and expressed as a percentage of wild-type values. Results are the mean ± SEM. *p<0.05 using unpaired two-tailed t test.
Figure 2.9 Decreased sympathetic ganglia size and cell numbers in P0.5

Dp(16)1Yey/+ mice.

(a,b) SCGs were visualized by TH immunohistochemistry and cell counts were performed on Nissl stained tissue sections. Values are the mean ± SEM, n=3 mice for wild-type and n=4 for Dp(16)1Yey/+ mice. **p<0.01. Scale bar: 100 mm. (d-f) Increased apoptosis in P0.5 Dp(16)1Yey/+ SCGs, assessed by cleaved caspase-3 immunostaining. SCGs are outlined in dashed lines. Values are the mean ± SEM, n=4 mice for each genotype. *p<0.05. Scale bar: 100 mm. Statistical analyses by unpaired two-tailed Student’s t test for (c,f).
METHODS

Human Tissues

Human Down syndrome peripheral tissues were obtained from the NIH NeuroBioBank (NBB). Details of the specimens are provided in Supplementary Table 1.

Animals

All procedures relating to animal care and treatment conformed to institutional and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. Mice were maintained on a C57BL/6 background. Generation and genotyping of Dp(16)1Yey/+ mice have been described previously (Li, 2007), and the mice were generously provided by Dr. Eugene Yu and Dr. Roger Reeves.

Antibodies

The antibodies used in this study were previously validated for the following applications: TH (Millipore; AB152, immunohistochemistry), cleaved caspase-3 (Cell Signaling; 9661, immunohistochemistry), NF200 (Sigma-Aldrich; 4142, immunohistochemistry), insulin (Dako; A0564, immunohistochemistry), glucagon (Abcam; ab10988, immunohistochemistry) and α-smooth muscle actin-FITC (Sigma-Aldrich; F3777, immunohistochemistry).

Histological analyses of human tissues

Human tissues were fixed at 4°C for 3-4 hr in 4% paraformaldehyde (PFA), incubated in 30% sucrose overnight and embedded in OCT. Tissue sections (20 mm) were washed with PBS, blocked in 5% BSA and 0.3% Triton X-100 for 1 hr. Primary antibody incubation (TH, NF200, insulin, glucagon, α-smooth muscle actin-FITC) was performed overnight at room temperature in Can Get Signal enhancer solution from Toyobo. Following incubation with secondary antibodies
(2 hr, room temperature) and washes, slides were mounted with Prolong Antifade and DAPI. Images were acquired by confocal microscopy as 3-dimensional reconstructions from z-stacks. Quantification of innervation density in spleen and pancreatic tissues was done by measuring the integrated density of fluorescent pixels for TH or neurofilament immunostaining (ImageJ). Density was normalized to unit area, and at least 50 images per tissue were averaged. Results were expressed as arbitrary fluorescence units per 100 µm².

H&E staining was performed by the Johns Hopkins Oncology Histology Laboratory. The histological, imaging and quantification analyses were performed such that the investigator was blinded to the group allocations.

**Whole-mount tyrosine hydroxylase immunohistochemistry**

Whole-mount tyrosine hydroxylase immunohistochemistry was performed as previously described (Kuruvilla, 2004). Briefly, whole E16.5 mouse embryos were subjected to diaminobenzidine (DAB)-TH immunohistochemistry, using a rabbit anti-TH (Millipore, AB152) at 0.5 mg/ml incubated for 72 hr at 4°C. Detection was performed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare) at 4 mg/ml incubated overnight at 4°C. Visualization was accomplished with DAB (Sigma-Aldrich), followed by clearing in 2:1 benzyl benzoate/benzyl alcohol (Sigma-Aldrich).

**Immunohistochemical analyses of mouse tissues**

Mice at various developmental ages were fixed in 4% PFA at 4°C for 3-4 hr, cryoprotected in 30% sucrose in PBS, frozen in OCT and serially sectioned (12 mm). For immunofluorescence, sections were washed in PBS, permeabilized in PBS containing 1% Triton X-100, and blocked using 5% goat serum in PBS + 0.1% Triton X-100. Sections were incubated in the following primary antibodies
overnight: rabbit anti-TH (1:200; Millipore) and rabbit anti-cleaved caspase 3 (1:200; Cell Signaling). Following PBS washes, sections were incubated with anti-rabbit Alexa-488 secondary antibodies (1:200; Life Technologies). Sections were then washed in PBS and mounted in VectaShield (Vector Laboratories) containing 100 mg/ml DAPI.

Quantification of sympathetic innervation density in the salivary glands and nasal epithelium was done by calculating integrated TH fluorescence density per unit area (ImageJ) from multiple random images.

**Neuronal cell counts**

E16.5 and P0.5 mice for neuronal counts were prepared as described (Bodmer, 2009). Briefly, mouse torsos were fixed in PBS containing 4% PFA, and then cryoprotected overnight in 30% sucrose-PBS. SCG sections (12 mm) were stained with a solution containing 0.5% cresyl violet (Nissl). Cells with characteristic neuronal morphology and visible nucleoli were counted in every fifth Nissl stained section.

**Statistical Analyses**

Samples sizes were similar to those reported in previous publications (Kuruvilla, 2004 #2223; Ascano, 2009 #2312; Bodmer, 2011). Data were collected randomly and the assessment of human tissues was done in a manner blinded to the group allocation. For practical reasons, analyses of innervation in mouse tissues were done in a semi-blinded manner such that the investigator was aware of the genotypes prior to the experiment, but conducted the immunostaining and data analyses without knowing the genotypes of each sample. InStat software was used for statistical analyses. All Student’s t tests were performed assuming Gaussian distribution, two-tailed, unpaired, and a confidence interval of 95%.
For peripheral innervation of human tissue analyses, we used a one-tailed $t$ test, based on the prediction of directionality obtained from analyses in mice. Statistical analyses were based on at least 3 independent experiments, and described in the figure legends. All error bars represent the standard error of the mean (s.e.m).
DISCUSSION

Our findings support the idea that autonomic defects seen in Down syndrome patients are due to previously undefined defects in sympathetic nervous system development. There is minimal work in the literature defining the normal morphology of human sympathetic innervation in a developmental time course. In this study, it was found that during early infancy, most TH positive fibers are observed in close apposition to blood vessels, with some fibers branching out into the red pulp of the spleen. In the pancreas, TH-positive fibers are more branched, coming in close proximity to islets. At the developmental ages analyzed, not all islets are juxtaposed with TH-positive fibers, as is seen in adult tissue samples. We find that peripheral tissue samples from Down syndrome patients contain fewer TH-positive sympathetic fibers than samples from non-DS counterparts. Overall innervation to organs such as the spleen and pancreas is reduced when assayed using the pan-neuronal marker neurofilament. How these innervation defects may progress during early childhood and adolescence in Down syndrome remains unknown.

Developmental defects in the sympathetic nervous system can also be found in a Down syndrome mouse model, the \textit{Dp(16)1Yey/+} mouse. These mice have reduced sympathetic innervation of target organs as early as embryonic day 16.5 (E16.5). However, early stages of migration and proliferation are normal, suggesting that sympathetic precursors are not affected by previously described neural crest deficits. Innervation deficits precede neuronal survival deficits that are first observed at P0.5. The timeline and magnitude of these deficits are reminiscent of those found in mice that lack the neurotrophin nerve growth factor (NGF) (Glebova, 2004; Kuruvilla, 2004) or its cognate tyrosine kinase...
receptor TrkA. Indeed, there is precedence for NGF-TrkA deficits in Down syndrome. In the TrkA-expressing basal forebrain cholinergic neurons (BFCNs), perturbed NGF signaling has been implicated in learning and memory defects in Down syndrome. Given the necessary role of NGF and TrkA in sympathetic target innervation and neuronal survival, it is likely that the defects in sympathetic nervous system development in DS is due to impaired NGF-TrkA signaling. However, the mechanism by which NGF-TrkA signaling is perturbed in sympathetic neurons in DS remains unknown. In addition, the full impact of disrupted sympathetic development on DS individuals needs further study.

Though we found innervation defects, immunostaining and histology showed no changes in the gross morphology of the target tissues in both human tissue and the mouse model. Interestingly, we have previously found that genetic ablation of sympathetic innervation during early development resulted in altered pancreatic islet architecture and functional deficits in insulin secretion and glucose metabolism in mice. Several peripheral organs are abundantly innervated by sympathetic fibers, initiated during development, yet, the role of innervation as a contributing mechanism to perturbations in organogenesis and dysfunction in Down syndrome has received little attention. Delayed or aberrant development of peripheral organs such as the heart, thymus and gastrointestinal tract are manifested in Down syndrome. Our current findings provide a platform for further investigations to determine if developmental deficits in autonomic innervation underlie the well-established impairments in cardiovascular, endocrine and immune functions in Down syndrome.

Previously, we found that genetic ablation of sympathetic innervation during early development resulted in altered pancreatic islet architecture and
functional deficits in insulin secretion and glucose metabolism in mice (Borden, 2013). Several peripheral organs are abundantly innervated by sympathetic fibers, initiated during development, yet, the role of innervation as a contributing mechanism to perturbations in organogenesis and dysfunction in Down syndrome has received little attention. Delayed or aberrant development of peripheral organs such as the heart, thymus and gastrointestinal tract are manifested in Down syndrome (Roizen, 2003; Antonarakis, 2006). Our current findings provide a platform for further investigations to determine if developmental deficits in autonomic innervation underlie the well-established impairments in cardiovascular, endocrine and immune functions in Down syndrome.
Chapter 3: Disrupted neurotrophin trafficking in sympathetic neurons in Down syndrome

A portion of the work in this chapter has been submitted for publication at Nature Neuroscience:

Ami Patel, Naoya Yamashita, Maria Ascaño, Daniel Bodmer, Erica Boehm, Chantal Bodkin-Clarke, Yun Kyoung Ryu, and Rejji Kuruvilla. RCAN1 links impaired neurotrophin trafficking to aberrant development of the sympathetic nervous system in Down syndrome.
INTRODUCTION

The mechanism by which the previously described perturbations in sympathetic development occur in Down syndrome remains unclear. Based on animal studies, the best characterized molecular player in sympathetic nervous system development is Nerve Growth Factor (NGF), a neurotrophin that is secreted by peripheral tissues (Glebova, 2005). In new-born mice, genetic ablation of NGF or its cognate receptor, TrkA, results in diminished innervation of peripheral target tissues and loss of post-mitotic sympathetic neurons (Crowley, 1994; Fagan, 1996; Glebova, 2004), while transgenic over-expression of NGF in target tissues enhances growth of sympathetic nerves into final target fields (Edwards R. H., 1989; Hassankhani, 1995; Albers, 1994). Since NGF is released by neuronal targets, a salient feature of NGF signaling in highly polarized neurons is the regulation of endocytic trafficking of its TrkA receptors and intracellular signaling from internalized receptors (Cosker, 2014). NGF promotes endocytosis of its TrkA receptors in distal axons into NGF:TrkA-containing signaling endosomes that are retrogradely transported back to neuronal cell bodies to exert transcriptional control of neuronal survival and long-term growth (Ye, 2003; Delcroix, 2003; Heerssen, 2004; Zweifel, 2005). While the functional relevance of neurotrophin trafficking has been most appreciated during normal development, a corollary view is that dysregulation of endocytic trafficking could be the basis for decreased neurotrophic support in developmental disorders and late-onset neurodegenerative diseases (Salehi, 2003; Bronfman, 2007; Yap, 2013; Cosker, 2014).

Disrupted endocytic trafficking has been implicated in a variety of neurodegenerative diseases, including Down syndrome. TrkA-expression basal
forebrain cholinergic neurons (BFCNs) have been show to specifically have perturbed trafficking of the neurotrophin nerve growth factor (NGF). Given the essential role of NGF-TrkA in sympathetic nervous system development and previously described impairment in NGF-TrkA trafficking defects in Down syndrome, we asked if sympathetic neurons from a mouse model had defects in NGF-TrkA trafficking. We found that there is no change in the levels of TrkA or NGF expressed in sympathetic neurons or their targets respectively. However, there is decreased retrograde accumulation of NGF in sympathetic ganglia. This is likely due to impaired Trk receptor internalization found in cultured sympathetic neurons from the $Dp(16)1Yey/+ $ mice. This impairment provides a mechanism by which sympathetic nervous system development is perturbed in DS.
RESULTS

Reduced retrograde accumulation of NGF in Dp(16)1Yey/+ sympathetic ganglia

The deficits in sympathetic innervation in Dp(16)1Yey/+ embryos at E16.5 were reminiscent of phenotypes observed in mice lacking the target-derived neurotrophin, NGF (Glebova, 2004 #2389; Kuruvilla, 2004), which is known to control axonal extension and arborization when sympathetic axons have reached their final destinations (Glebova, 2005 #2387; Kuruvilla, 2004). The neuronal loss observed at P0.5, when sympathetic axons are actively engaged in a developmental survival competition for limiting amounts of NGF (Glebova, 2005), and the magnitude of the loss, similar to that in new-born TrkA−/− mice (Fagan, 1996), further implicate a failure in NGF signaling in Dp(16)1Yey/+ mice.

Using an ELISA-based immunoassay, we found a significant reduction in NGF protein levels in superior cervical ganglia (SCG) lysates from Dp(16)1Yey/+ mice, although NGF levels in SCG target tissues, the salivary glands, were similar to that in wild-type mice (Fig. 3.1a,b). The salivary glands are the primary source of NGF for the SCG; NGF produced in the salivary glands binds to TrkA receptors on sympathetic axons, and following endocytosis, is retrogradely transported to neuronal cell bodies located within the SCG. Similar to salivary glands, normal NGF levels were also found in another sympathetic target organ, the heart, in Dp(16)1Yey/+ mice (Fig. 3.1c). These results suggest that the diminished innervation and neuronal loss in Dp(16)1Yey/+ mice arise from deficits in NGF uptake and/or retrograde transport in sympathetic neurons, rather than decreased NGF production in target tissues.

Trk receptor endocytosis is perturbed in Dp(16)1Yey/+ neurons
NGF-mediated survival and axon growth of sympathetic neurons is critically dependent on endocytosis of TrkA receptors, the primary event in retrograde NGF signaling (Ascano, 2012; Harrington, 2013). Given the decrease in NGF protein accumulation in sympathetic neuronal cell bodies in Dp(16)1Yey/+ mice in vivo, we examined endocytic trafficking of Trk receptors using a chimeric Trk receptor-based, live-cell antibody feeding assay in cultured neurons (Ascano, 2009). Cultured sympathetic neurons isolated from P0.5 Dp(16)1Yey/+ and wild-type mice were infected with an adenoviral vector expressing FLAG-tagged chimeric receptors that have the extracellular domain of TrkB and the transmembrane and intracellular domains of TrkA (FLAG-TrkB:A). Sympathetic neurons do not normally express TrkB receptors, and the chimeric Trk receptors respond to the TrkB ligand, BDNF, but retain the signaling properties of TrkA (Ascano, 2009). Using live-cell immunocytochemistry with an antibody directed against the extracellular FLAG epitope, we observed prominent receptor internalization in response to neurotrophin stimulation in both the cell bodies (Fig. 3.2a,b) and axons (Fig. 3.2,f) in wild-type neurons. In contrast, ligand-dependent internalization was markedly attenuated in cell bodies (Fig. 3.2c,d) and axons (Fig. 3.2e,f) in Dp(16)1Yey/+ neurons. While neurotrophin treatment enhanced Trk receptor internalization by 2.2 ± 0.2-fold in cell bodies, and 1.6 ± 0.15-fold in axons, compared to unstimulated conditions in wild-type neurons, there were no significant differences between the un-stimulated and ligand-treated conditions in Dp(16)1Yey/+ neurons (Fig. 3.2i,j). Additionally, we did not observe differences in surface receptor expression between wild-type and Dp(16)1Yey/+ sympathetic neurons in the absence of ligand (compare Fig. 3.2a and 3.2c). Together, these
results indicate that ligand-dependent endocytosis of Trk receptors is attenuated in \( Dp(16)1Yey/+ \) sympathetic neurons.
Figure 3.1. Reduced NGF levels in SCG of Dp(16)1Yey/+ mice, but not targets.

(a,b) Dp(16)1Yey/+ mice show a significant decrease in NGF protein levels in sympathetic cell bodies located in superior cervical ganglia (SCG) (a), but not in the salivary glands or heart (b,c), target tissues innervated by sympathetic axons. NGF levels in SCGs were normalized to TH in SCGs and represented as picograms of NGF/mg of TH. NGF levels in salivary glands and heart were normalized to total protein. Results are the mean ± SEM from n=3 Dp(16)1Yey/+ mice and 4 control litter-mates for SCGs, n=5 mice per genotype for salivary glands, and at least n=3 mice per genotype for heart. *p<0.05, unpaired two-tailed Student’s t test.
**Figure 3.2. Impaired ligand-dependent endocytosis of Trk receptors in Dp(16)1Yey/+ neurons.**

(a-j) Ligand-dependent Trk receptor internalization is impaired in Dp(16)1Yey/+ sympathetic neurons. Sympathetic neurons from P0.5 Dp(16)1Yey/+ and wild-type sympathetic neurons were established in microfluidic chambers and infected with an adenovirus expressing FLAG-TrkB:A chimeric receptors. Neurons were labeled with FLAG antibody under non-permeabilizing conditions at 4°C for 30 min, followed by BDNF treatment for 30 min. FLAG immunoreactivity (red) was assessed in cell bodies (a-d) and axons (e-h). Scale bars: 5 and 10 mm for axons and cell bodies, respectively. (i,j) Quantification of internalized Trk in cell bodies and axons after treatments described in (a-h). Internal accumulation of chimeric receptors under the various conditions was determined by assessing the proportion of co-localization of FLAG immunofluorescence with that of GFP, which is co-expressed in infected neurons and is cytoplasmic. At least 40-50 neurons were analyzed per condition. Quantification is represented as fold-change relative to wild-type neurons with no neurotrophin. Results are the mean ± SEM from 3 independent experiments. *p<0.05, **p<0.01, n.s. not significant, two-way ANOVA followed by Bonferroni post-hoc test.
Flag+GFP

(a) -neurotrophin  (b) +neurotrophin

WT

Dp(16)1Yey/+ 

(c) -neurotrophin  (d) +neurotrophin

WT

Dp(16)1Yey/+ 

(i) Internalized receptors in cell bodies

WT Dp(16)1Yey/+  

[Graph showing internalized receptors in cell bodies]

(j) Internalized receptors in axons

WT Dp(16)1Yey/+  

[Graph showing internalized receptors in axons]
METHODS

Animals
All procedures relating to animal care and treatment conformed to institutional and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. Mice were maintained on a C57BL/6 background. Generation and genotyping of \textit{Dp(16)1Yey/\textplus} mice have been described previously (Li, 2007), and the mice were generously provided by Dr. Eugene Yu and Dr. Roger Reeves.

Reagents and antibodies
The generation of the FLAG-TrkB/A adenovirus has been previously described (Ascano, 2009). The antibodies used in this study were previously validated for the following applications: FLAG M2 (Sigma-Aldrich; H9658, antibody feeding assays). NGF levels were determined by ChemiKine NGF Sandwich ELISA kit (cat #: CYT304) from Millipore. TH levels were determined by TH Sandwich ELISA Kit from Biomatik (cat #: EKU08003).

NGF and TH ELISA assays
NGF levels were assessed using a NGF Sandwich ELISA Kit (Millipore). Briefly, SCGs, salivary glands and hearts were dissected from P1 mouse pups, homogenized, and centrifuged. Salivary gland and heart supernatants were diluted, and all tissue lysates were incubated in ChemKine wells overnight at 4°C. With the ChemiKine NGF assay system, sheep polyclonal antibodies generated against mouse NGF are coated onto a microplate and are used to capture NGF from a sample. After washing, samples were incubated with NGF-specific mouse monoclonal antibodies for 2hr at room temperature to detect the captured NGF, followed by incubation with peroxidase-conjugated secondary antibody (2 hr, room temperature), and TMB/E substrate (5 min, room
temperature). The reaction was stopped and development was assessed using a plate reader.

TH levels were assessed using a TH Sandwich ELISA Kit (Biomatik EKU08003). SCGs dissected from P1 mice were homogenized and centrifuged. Lysates were incubated in antibody-coated wells for 3 hr at 37°C. After washing, samples were incubated with a biotin-conjugated antibody specific to TH for 1 hr at at 37°C, followed by incubation with avidin conjugated to Horseradish Peroxidase (HRP) for 30 min at 37°C, TMB/E substrate for 15 min at 37°C, and assessed using a plate reader. NGF protein levels were normalized to TH protein levels in SCGs, and to total protein concentrations as determined by a BCA assay in salivary gland and heart lysates.

**TrkA receptor internalization assays**

Live cell antibody feeding assays to monitor Trk receptor internalization were performed as previously described (Ascano, 2009). Briefly, sympathetic neurons harvested from P0.5 wild-type and Dp(16)1Yey/+ mice were grown in microfluidic chambers for 2-4 days in vitro (D.I.V.) to allow axons to project into the outer chambers. Neurons were infected with an adenoviral vector that expresses GFP and FLAG-TrkB:A chimeric receptors. Infected neurons were identified by GFP expression. 48 hr post-infection, cultures were washed to remove all NGF, and incubated with mouse anti-FLAG antibody (M2, 4.2µg/ml Sigma-Aldrich) for 30 min at 4°C in PBS. Excess antibody was washed off followed by incubation with either control medium or medium containing BDNF (100 ng/ml) and the cells moved to 37°C for 30 min to allow for internalization. Cells were then washed quickly with PBS and immediately fixed with 4%
paraformaldehyde in PBS for 30 min at room temperature. Cells were then permeabilized with 0.1% Triton X-100/1% BSA/PBS and incubated with fluorescently conjugated anti-mouse secondary antibody for 1 hr, and then mounted on slides with Aquamount (Invitrogen). Images representing 0.8 µm optical slices were acquired using a Zeiss LSM 510 confocal scanning microscope equipped with Ar (458-488 nm) and He/Ne (543-633) lasers. The same confocal acquisition settings were applied to all images taken from a single experiment. Cell bodies were analyzed by taking z-stacks through the entire cell, and creating a 3-dimensional reconstruction using Image J. Axons were analyzed using single images with an aperture of 0.8 mm. Threshold settings for green and red scans were determined, and the integrated fluorescence values for each channel were quantified. Internalization was quantified as the ratio of anti-FLAG immunofluorescence (red) that co-localized with cytoplasmic GFP (green) relative to the total anti-FLAG immunofluorescence. Weighted coefficients of co-localization between the anti-FLAG and GFP fluorescence were determined by Image J software.

**Statistical Analyses**

Samples sizes were similar to those reported in previous publications. Data were collected randomly. InStat software was used for statistical analyses. All Student’s \( t \) tests were performed assuming Gaussian distribution, two-tailed, unpaired, and a confidence interval of 95%. Two-way ANOVA analyses were performed when more than two groups were compared. Statistical analyses were based on at least 3 independent experiments, and described in the figure legends. All error bars represent the standard error of the mean (s.e.m).
DISCUSSION

Our findings support the view that the sympathetic defects in \textit{Dp(16)1Yey/+} mice arise from a failure in target-derived NGF signaling. The sympathetic deficits in \textit{Dp(16)1Yey/+} mice (Chapter 2) are reminiscent of the phenotypes observed in mice lacking NGF or TrkA (Crowley, 1994; Fagan, 1996; Glebova, 2004; Kuruvilla, 2004). While several cellular processes could potentially contribute to the enhanced apoptosis and diminished innervation of sympathetic neurons in \textit{Dp(16)1Yey/+} mice, our findings provide evidence in support of disruption of TrkA endocytosis as a key mechanism. In \textit{Dp(16)1Yey/+} mice, we found a significant decrease in the retrograde accumulation of NGF protein in neuronal cell bodies in sympathetic ganglia, despite normal NGF production in the target tissues. NGF-dependent endocytosis of TrkA receptors in nerve terminals is well-established to be an essential step in the retrograde propagation of the NGF trophic signal and the ability of target-derived NGF to support the survival and long-term growth of sympathetic neurons (Ye, 2003; Kuruvilla, 2004; Zweifel, 2005). However, little is known about molecular mechanisms that selectively impair neurotrophin trafficking in a disease state. Though the overexpression of APP specifically has been shown to disrupt NGF-TrkA trafficking in the BFCNs, whether the same mechanism is present in sympathetic neurons is not known.

Though NGF-TrkA trophic support is required for both BFCNs and sympathetic neurons, its role and mode of action may differ greatly. For example, global knockout of NGF or TrkA results in reduced sympathetic innervation followed by almost complete loss of sympathetic ganglia. However, reduced NGF-TrkA signaling in the BFCNs causes loss of innervation, but not
necessarily cell death. Some studies argue that these cells reduce cholinergic gene expression and atrophy, without cell death. The mechanism of trafficking may also differ, as Salehi and colleagues found that there is no change in internalization of NGF-TrkA in the synapses of BFCNs in the DS brain, only retrograde trafficking. Our results show that in sympathetic neurons, the initial step of internalization is disrupted. Different molecular players may account for this discrepancy. However, which DS genes specifically play a role in NGF-TrkA internalization is not yet known.
Chapter 4: Inhibition of calcineurin activity by RCAN1 overexpression impairs NGF-dependent TrkA trafficking and trophic functions

A portion of the work in this chapter has been submitted for publication at Nature Neuroscience:

Ami Patel, Naoya Yamashita, Maria Ascaño, Daniel Bodmer, Erica Boehm, Chantal Bodkin-Clarke, Yun Kyoung Ryu, and Rejji Kuruvilla. *RCAN1 links impaired neurotrophin trafficking to aberrant development of the sympathetic nervous system in Down syndrome.*
INTRODUCTION

Regulator of Calcineurin 1 (RCAN1) is the founding member of a family of calcineurin (CN) regulators that is expressed in all eukaryotes (Kingsbury, 2000; Strippoli, 2000; Davies, 2007). In vertebrates, this family consists of two other members, RCAN2 and RCAN3, which are found on Hsa6 and Hsa1 respectively (Canaidar, 2006; Mulero, 2007; Facchin, 2008; Serrano-Candales, 2014). RCAN1 is found on Hsa21 and has been implicated in Down syndrome pathogenesis. RCAN1 was initially identified and described by Fuentes and colleagues in 1995 due to its chromosomal location (Fuentes, 1995). Initially named Down syndrome critical region 1 (DSCR1) RCAN1 was identified as part of a small cluster of genes that was thought to be necessary and sufficient to confer the DS phenotype (Nikolaienko, 2005; Olson, 2007). RCAN2 and RCAN3 were subsequently identified due to a common FLISPP motif (Strippoli, 2000). Though RCAN3 protein function has not been assessed, the main function of RCAN1 and RCAN2 is CN regulation, mainly through interactions with PxIxIT, PxIxIT-like and LxxP motifs (Fuentes, 2000; Kingsbury, 2000; Rothermal, 2000; Mehta, 2009). All RCANs are expressed in the mouse brain throughout development and adulthood, though relative levels and sub-cellular location varies. RCAN1 is also expressed in skeletal muscle, heart, and pancreas (Fuentes, 1997; . Relatively little is known about the other RCANs, but RCAN1 has been studied to great extent and implicated in disease.

Most of our understanding of RCAN1 comes through work done in the context of Down syndrome (DS). Genetic analyses of RCAN1 indicate that it is found on the minus strand of the long arm of the 21st chromosome and consists of 7 exons (Figure 4.1) (Fuentes, 1997). These exons give rise to two known
alternatively spliced transcripts that share exons 5-7. The first isoform, RCAN1.1 makes use of the 1st exon, splicing out exons 2-4, and is transcriptionally regulated by glucocorticoids. This isoform gives rise to two proteins, RCAN1.1-L, which is described as the predominantly expressed RCAN1 protein in the mouse brain, and RCAN1.1-S. The second isoform begins at exon 4 and is regulated by the calcineurin-regulated transcription factor NFAT (Fuentes, 1997; Yang, 2000; Casa, 2001; Genesca, 2003; Hesser, 2004; Minami, 2004; Shen, 2004; Cano, 2005; Mammucari, 2005; Ermak, 2006). All of these isoforms contain the protein motifs that are required for CN interactions.

RCAN1’s affects on CN are mediated by with several motifs. Similar to dynamin1, RCAN1 has been shown to interact with CN through a PxIxIT-like motif, PSVVVH. This sequence binds to the substrate-docking location in the catalytic unit of CN, CNA. Binding at this location suggests that RCAN1 is likely a competitive inhibitor, displacing other PxIxIT-containing proteins such as NFAT and dynamin1 (Hogan, 2003). A separate motif, the LxxP motif has also been shown to be required for inhibitory activity, but the mode of action of this domain remains to be determined. Other motifs have been found to be required the stimulate calcineurin activity, including an ExxP domain and a TxxP domain (Fuentes, 2000; Rothermal, 2000; Vega, 2002; Aubareda, 2006; Mehta, 2009). In addition, RCAN1 interactions with calcineurin, and other proteins, are regulated by phosphorylation (Genesca, 2003; Hilioti, 2004; Mehta, 2009). Though not all RCAN1 protein interactions are understood, it is known that the overexpression of RCAN1 in vivo recapitulates DS phenotypes.

To better understand the role of RCAN1 in disease, RCAN1 has been overexpressed in animal models with varying results. When human RCAN1.1
cDNA is over-expressed in the mouse brain under the control of the PDGFβ promoter, sensorimotor and learning behaviors are not disrupted: only slight hyperactivity is observed (Dierssen et. al, 2011). However, under the control of its own promoter, RCAN1.1 overexpression causes DS-like learning and memory deficits in mice (Keating et. al, 2007, Martin et. al, 2007). Overexpression of cDNA from the RCAN1.4 isoform under the NSE promoter revealed gender-specific differences in anxiety and motor behaviors, though learning behaviors using the Morris water maze were unchanged (Bhoiwala et al, 2013). The Drosophila homologue of RCAN1, nebula, however, produced significant learning and memory deficits (Chang et. al, 2003). A BAC-transgenic RCAN1 mouse showed significant hippocampal learning behavior (Xing et. al, 2013). Despite some disparities, all of these studies suggest that RCAN1 overexpression is sufficient to recapitulate some aspects of the Down syndrome phenotype. This led us to believe that the overexpression of RCAN1 may disrupt TrkA trafficking and sympathetic nervous system development, similar to our DS mouse model.

Given our findings that a DS mouse models has defects in NGF-TrkA trafficking in peripheral neurons and the known role of CN in trafficking, it is likely that overexpression of RCAN1 alone is sufficient to induce trafficking and sympathetic defects similar to DS. To this end, we overexpressed RCAN1 in vitro using an adenovirus and in vivo using a transgenic mouse model. We find that in vitro overexpression of RCAN1 attenuates TrkA internalization, retrograde signaling, and trophic functions. In vivo, the presence of an extra copy of RCAN1 is sufficient to disrupt sympathetic nervous system development. This suggests that sympathetic defects in DS mice are due to overexpression of RCAN1.
RESULTS

Excess RCAN1 inhibits TrkA endocytosis by altering dynamin1 phosphorylation

*Dp(16)1Yey/+* mice are trisomic for 113 genes orthologous to human chromosome 21 (Li, 2007; Das, 2011). The increased dosage of one or more of these genes could serve as the molecular locus for impaired Trk receptor trafficking and the aberrant development of the sympathetic nervous system. A clue to the identity of the responsible gene came from our previous observations that the calcium-calmodulin-activated phosphatase, calcineurin, is necessary for NGF-dependent endocytosis of TrkA receptors, and for sympathetic innervation of target tissues (Bodmer, 2011). Among the 113 trisomic genes in *Dp(16)1Yey/+* mice is RCAN1, originally named *Down’s Syndrome Candidate Region 1* (DSCR1) because of the gene locus within human chromosome 21 (Fuentes, 1995; Fuentes, 1997). RCAN1 belongs to a family of endogenous calcineurin inhibitors that are highly conserved from yeast to humans (Kingsbury, 2000; Fuentes, 2000; Rothermel, 2001; Ryeom, 2003; Chan, 2005; Hoeffer, 2007; Baek, 2009). RCAN1 expression is enriched in tissues that are particularly vulnerable in Down syndrome, including the nervous system, heart and skeletal muscle, and its levels are significantly elevated in individuals with Down syndrome and mouse models (Fuentes, 1995; Fuentes, 1997; Fuentes, 2000). We observed a significant (58%) decrease in calcineurin phosphatase activity in sympathetic ganglia lysates from P0.5 *Dp(16)1Yey/+* mice (see Figure 5.). Thus, we hypothesized that dysfunctional calcineurin signaling, through increased gene dosage of RCAN1, is a potential link between deficits in TrkA trafficking and decreased neurotrophic support in Down syndrome.
As a first step toward defining the role of RCAN1 in NGF-dependent trafficking and functions, we assessed the expression of endogenous RCAN1 in sympathetic neurons. Differential promoter usage results in two different RCAN1 transcripts, RCAN1.1 and RCAN1.4, where the first exon is either exon 1 or exon 4, followed by the shared last three exons (Fig. 4.1a) (Fuentes, 1997; Rothermel, 2003). RT-PCR analyses revealed that both RCAN1.1 and RCAN1.4 isoforms are present in developing SCGs in newborn mice (Fig. 4.1b). In situ hybridization using a probe directed against a common region in the two RCAN1 isoforms showed RCAN1 expression in the SCG and DRG at postnatal day 0.5 (P0.5) (Fig. 4.2a,c). To monitor the endogenous localization of RCAN1 protein, we performed immunostaining in dissociated sympathetic neurons employing a RCAN1 antibody. These analyses revealed that RCAN1 protein is localized throughout the neuron, including the distal axons (Fig. 4.2d-f). Together, these results demonstrate the presence of endogenous RCAN1 in sympathetic neurons, and notably, in distal axons during a stage when these neurons are responsive to target-derived NGF.

We next reasoned that increased RCAN1 levels in neurons would interfere with TrkA endocytosis. To test this prediction, a cell surface biotinylation assay was performed to measure NGF-dependent internalization of endogenous TrkA receptors in sympathetic neurons infected with an adenoviral vector expressing HA-tagged human RCAN1.4. Adenovirus-mediated expression resulted in an elevation of RCAN1 protein levels by 2.8-fold (Fig. 4.3). Treatment of sympathetic neurons with NGF for 30 minutes elicited robust internalization of TrkA receptors in control neurons expressing green fluorescent protein (GFP). In contrast, NGF-dependent internalization of biotinylated TrkA receptors was
markedly reduced in neurons over-expressing RCAN1 (Fig. 4.4a,b). A similar
defect in TrkA internalization is seen when RCAN1 was over-expressed in
sensory neurons (Fig. 4.4c,d). To determine if the endocytosis defect with excess
RCAN1 was specific to TrkA receptors, we examined the endocytosis of the
Transferrin receptor (TfR), a prototypical constitutively internalized receptor
(Dickson, 1983). To follow internalization of TfR, sympathetic neurons were
incubated with biotin-transferrin or Alexa-555-labeled-transferrin at 4°C and
intracellular transferrin accumulation at 37°C was either monitored
biochemically by streptavidin precipitation followed by transferrin
immunoblotting, or by following uptake of the fluorescent label, respectively.
There were no significant differences in TfR internalization between RCAN1 and
GFP-expressing sympathetic neurons (Fig. 4.5a-g). Similarly, the uptake of Alexa-
555-labeled Epidermal Growth Factor (EGF) was also unaffected in RCAN1-over-
expressing neurons (Supplementary Fig. 2.5h-l), suggesting normal
internalization of EGFR, a receptor tyrosine kinase that undergoes ligand-
induced internalization (Dickson, 1983). We conclude that RCAN1 over-
expression selectively interferes with ligand-dependent endocytosis of TrkA
receptors in NGF-responsive neurons.

To identify the molecular mechanisms by which RCAN1 over-expression
impairs TrkA endocytosis, we focused on the endocytic GTPase, dynamin1.
Previously, we found that calcineurin dephosphorylates neuron-specific splicing
isoforms of dynamin1 to drive TrkA internalization in sympathetic neurons
(Bodmer, 2011). Therefore, we assessed the phosphorylation status of dynamin1
in response to NGF in neurons over-expressing RCAN1 or GFP. Sympathetic
neurons were exposed to NGF for 30 min, and levels of phosphorylated
dynamin1 assessed using a phospho-specific antibody that specifically recognizes dynamin1 phosphorylated on Ser-778, one of two sites dephosphorylated by calcineurin (Clayton, 2009). NGF treatment induced a significant decrease in dynamin1 phosphorylation (by 22%) in control GFP-expressing neurons (Fig. 4.6). This decrease in dynamin1 phosphorylation was abolished by RCAN1 over-expression. Together, our findings indicate that excess RCAN1 disrupts internalization of TrkA receptors by inhibiting calcineurin-mediated dephosphorylation of dynamin1.

**RCAN1 over-expression attenuates long-distance retrograde NGF signaling**

The finding that RCAN1 over-expression abrogated TrkA endocytosis led us to predict that excess RCAN1 would impact NGF-mediated retrograde communication between axon terminals and distal neuronal cell bodies, a process that relies on TrkA endocytosis within nerve terminals. To assess retrograde NGF signaling in neurons, we used a compartmentalized culture system that allows for the separation of neuronal cell bodies and proximal axons from the distal axons by a teflon-grease diffusion barrier, and the application of neurotrophins exclusively to distal axons, thus recapitulating the in vivo situation. In compartmentalized cultures, NGF treatment of distal axons (100 ng/ml, 8 hr) resulted in the robust phosphorylation of TrkA receptors and of canonical signaling effectors, Erk1/2 and Akt, locally within distal axons in both RCAN1- and GFP-expressing neurons. However, while NGF promoted the retrograde accumulation of P-TrkA, P-Erk1/2 and P-Akt in cell bodies of control neurons, there was a pronounced reduction in the levels of these signaling molecules in the cell bodies of RCAN1-expressing neurons (Fig. 4.7).
If RCAN1 effects are mediated via its modulation of calcineurin activity, then local inhibition of calcineurin activity in distal axons should perturb retrograde NGF signaling similar to that elicited by RCAN1 over-expression. Indeed, we found that addition of the pharmacological calcineurin inhibitors, Cyclosporin A (CsA) (2 mg/ml) plus FK506 (0.2 mg/ml), exclusively to distal axons attenuated NGF-dependent retrograde signaling in compartmentalized cultures (Fig. 4.8). The pharmacological inhibition of calcineurin activity in neurons required the use of both CsA and FK506 since partial inhibition was observed with either reagent alone (Bodmer, 2011). Analogous to the findings with RCAN1 over-expression, axonal application of calcineurin inhibitors had no effect on NGF-dependent phosphorylation of TrkA, Erk1/2 and Akt locally within distal axons. Together, these results indicate that RCAN1 over-expression or localized calcineurin inhibition in axons interferes with the long-distance retrograde propagation of the NGF signal to neuronal cell bodies.

**Excess RCAN1 perturbs NGF-dependent neuronal survival and axon growth**

Retrograde propagation of a NGF signal from axon terminals to neuronal cell bodies is a prerequisite step in the ability of target-derived NGF to support neuronal survival (Ascano, 2012 Harrington, 2013). Thus, we asked if RCAN1 over-expression would compromise the ability of axon-applied NGF to retrogradely support neuronal survival. We monitored neuronal survival in response to NGF (100 ng/ml) added exclusively to distal axons in compartmentalized cultures of sympathetic neurons infected with the RCAN1 or a control LacZ adenovirus. In LacZ-infected neurons, NGF was sufficient to support the survival of the majority of neurons with only about 22.7 ± 1.2% undergoing apoptosis, assessed by TUNEL staining (Fig. 4.9a,b,e). In contrast,
RCAN1-over-expressing neurons exhibited a significant increase in neuronal apoptosis (46.4 ± 7.4% apoptotic neurons) (Fig. 4.9c,e). Notably, NGF added directly to neuronal cell bodies in compartmentalized cultures is known to promote survival by an endocytosis-independent mechanism (Ye, 2003), and in this condition, RCAN1 over-expression did not elicit increased apoptosis (17.6 ± 3.2% apoptotic neurons) (Fig. 4.9d,f). Thus, the over-expression of RCAN1 specifically compromises neuronal survival when NGF is present on distal axons, a scenario where internalization and long-distance trafficking of TrkA receptors is essential for retrograde NGF survival signaling.

We then sought to determine if RCAN1 over-expression would influence NGF-dependent axon growth, a process that is also dependent on calcineurin-mediated endocytosis of TrkA receptors (Bodmer, 2011). In these analyses, the broad-spectrum caspase inhibitor, boc-aspartyl(O-methyl)-fluoromethylketone (BAF, 50 mM), was added to cell bodies of compartmentalized cultures so that axon growth could be assessed independent of complications of RCAN1-mediated apoptosis. NGF (100 ng/ml) added only to distal axons of compartmentalized sympathetic neurons promoted robust axon growth in control neurons, with an average growth rate of 136 mm/day (Fig. 4.10a,b,e). In contrast, the growth-promoting effect of NGF was abolished in RCAN1 over-expressing neurons (Fig. 4.10c,d,e).

**RCAN1 triplication causes defects in sympathetic nervous system development**

Our findings in compartmentalized sympathetic cultures suggest a mechanism by which excess RCAN1 contributes to the sympathetic defects by impairing NGF-dependent TrkA endocytosis and retrograde trophic signaling. If
this is indeed the case, then trisomic expression of *RCAN1* should recapitulate the phenotypes in NGF and TrkA knockout mice. Thus, we employed *RCAN1* transgenic mice that express three copies of *RCAN1.4*, generated by targeting a myc-tagged third copy of *RCAN1.4* cDNA driven by its native promoter into the *Hprt* locus (Fig. 4.11) (Baek, 2009). These mice exhibit a \(\sim 2.5\)-3-fold increase in *RCAN1* mRNA levels, similar to Down syndrome human fetal tissues (Baek, 2009). We performed TH immunohistochemistry to visualize the formation of the SCGs, as well as Nissl staining to quantify neuronal numbers. Similar to the 45\% decrease in SCG neuronal numbers observed in *Dp(16)1Yey/+* mice, we found a significant loss of sympathetic neurons in *RCAN1* trisomic mice at birth, with 52.7\% fewer SCG neurons compared to litter-mate controls (13,002 \(\pm\) 1,112 neurons in P0.5 *RCAN1* mice *vs.* 27,463 \(\pm\) 1,774 in wild-type litter-mates) (Fig. 2.12a-c), as well as enhanced apoptosis in the SCGs (Fig. 4.12d-f) Additionally, the *RCAN1* transgenic mice had significantly reduced sympathetic fibers in the target tissues including the olfactory epithelium (Fig 2.13a-c) and salivary glands (Fig. 2.13d-f).

The onset of the developmental deficits in *RCAN1* transgenic mice was also remarkably similar to TrkA/Bax knockout mice. At early stages i.e., E16.5, there were no obvious differences between *RCAN1* and wild-type mice in the formation of the SCG and sympathetic neuronal numbers (Fig. 2.14a-c), indicating a selective effect on post-mitotic neurons that depend on target-derived NGF. Also, sympathetic innervation deficits preceded the neuronal loss in *RCAN1* trisomic embryos (Fig. 2.14d-i), suggesting that RCAN1 accumulation in the two different *RCAN1* trisomic mouse models may have an early effect on attenuating axon growth at sympathetic nerve terminals.
In light of our observations that reduced neuronal numbers is manifested at birth well after the deficits in sympathetic innervation of target tissues in both RCAN1 transgenic and Dp(16)1Yey/+ embryonic mice, we conclude that the neuronal loss may be a cumulative effect that reflects the failure of sympathetic axons to gain access to adequate levels of target-derived NGF due to the diminished innervation, as well as reduced retrograde trafficking of TrkA receptors from nerve terminals to neuronal cell bodies. Together, these findings support the notion that trisomy of RCAN1 alone is sufficient to perturb NGF-dependent trophic functions in the developing sympathetic nervous system.
Figure 4.1. RCAN1 isoform analysis in sympathetic neurons.

(a) Schematic representation of RCAN1.1 and RCAN1.4 isoforms generated by dual promoter usage. Mouse and human RCAN1 share the same genomic coding exon organization. Alternative selection of exons 1 and 4 as the first exon results in two different transcripts, with both sharing the last three coding exons. Arrows indicate the position of primers used for RT-PCR analyses. (b) Semi-quantitative RT-PCR analysis shows the relative expression of RCAN1.1 and RCAN1.4 isoforms in brain and SCG tissues. Arrows and arrowheads indicate the RCAN1 bands, as confirmed by sequencing.
Figure 4.2. RCAN1 is expressed in peripheral neurons during development.

(a) *In situ* hybridization shows endogenous expression of *RCAN1* mRNA in the developing mouse superior cervical ganglia at P0.5. Sense control is shown in (b). Scale bar: 100 mm. (c,d) *In situ* hybridization shows *RCAN1* expression in the developing mouse Dorsal Root Ganglia (DRG) at P0.5. Scale bar: 100 mm. (e-g) RCAN1 protein is localized to both cell bodies (f) and axons (g) of cultured sympathetic rat neurons as detected using a RCAN1 antibody. Staining with pre-immune serum control is shown in (e). Scale bar: 10 mm for (e,f) and 5 mm for (g).
Figure 4.3 Adenoviral overexpression of HA-tagged RCAN1 in sympathetic neurons

Adenovirus-mediated over-expression of HA-tagged human RCAN1.4 results in a 2.8-fold increase in RCAN1 protein expression. Mass cultures of sympathetic neurons were infected with either RCAN1.4 or control GFP adenovirus. 48 hr post-infection, neuronal lysates were prepared and immunoblotted for RCAN1. The blot was stripped and reprobed for HA and p85. (b) Densitometric quantification of RCAN1 protein levels normalized to p85. **p<0.01; n=6 independent experiments. Unpaired two-tailed Student’s t test for (b).
Figure 4.4. NGF-dependent TrkA internalization is attenuated by over-expression of RCAN1 in sympathetic neurons.

(a) A cell surface biotinylation assay shows that adenoviral over-expression of RCAN1.4 attenuates NGF-dependent TrkA internalization in cultured rat sympathetic neurons. Membrane proteins were subjected to cell-surface biotinylation. Internalized TrkA receptors were detected by surface stripping of biotin, neutravidin precipitation and TrkA immunoblotting. Supernatants were probed for p85 for normalization of protein amounts. (b) Densitometric quantification of internalized TrkA. Results are means ± SEM from 4 independent experiments. *p<0.05 significantly different from all other conditions. (c) RCAN1 over-expression attenuates NGF-dependent internalization of TrkA receptors in cultured DRG neurons as assessed by a cell surface biotinylation assay. (d) Densitometric quantification of internalized TrkA. n=3 independent experiments, *p<0.05 different from all other conditions.
Figure 4.5. RCAN1 overexpression does not disrupt all clathrin-dependent endocytic events.

(a) Uptake of biotin-labeled transferrin (biotin-Tfn) is unaffected by RCAN1 over-expression in rat sympathetic neuron cultures. After internalization at 37°C and acid washes to remove surface-bound transferrin, internalized biotin-Tfn was detected in neuronal lysates by neutravidin precipitation and immunoblotting using a transferrin antibody. Supernatants were probed for p85 for normalization of protein amounts. (b) Densitometric quantification of internalized biotin-Tfn. Results are means ± SEM from 5 independent experiments. *p<0.05 significantly different from corresponding controls at 4°C. (c-g) Uptake of Alexa-555-labeled Transferrin is unaffected by RCAN1 over-expression in sympathetic neurons. Images were acquired by confocal microscopy and 3-dimensional reconstructions from z-stacks using Image J. Internalized receptors were calculated as Alexa-555- fluorescent pixels per µm² of cell body. The values from conditions at 4°C in GFP-expressing neurons were set to 1, and all other conditions are represented relative to the 4°C GFP values. 40-50 neurons were analyzed per condition per experiment. *p<0.05 relative to 4°C conditions, n=3. Scale bar: 5 mm. (h-l) Similarly, internalization of Alex-555-EGF remains unchanged in sympathetic neurons over-expressing RCAN1. 3-dimensional reconstructions from confocal z-stacks were analyzed in Image J. Internalization was analyzed as Alexa-555-fluorescent pixels per µm² of cell body. All values are normalized to 4°C in GFP-expressing neurons which was set to 1. 20-30 neurons were analyzed per condition per experiment. *p<0.05 relative to
4°C conditions, n=3. Scale bar: 5 mm. Two way ANOVA followed by Bonferroni post-hoc test for (i,n).
Figure 4.6 NGF-dependent dynamin1 dephosphorylation is reduced by RCAN1 overexpression

(a) NGF stimulation results in dephosphorylation of dynamin1, which is abrogated by excess RCAN1. Neuronal lysates were immunoblotted using a phospho-Ser778 dynamin antibody. Immunoblots were stripped and reprobed for total dynamin1 for normalization. (b) Densitometric quantification of phospho-dynamin1 levels normalized to total dynamin1 levels. All values are expressed relative to the no neurotrophin treatment in GFP-expressing neurons. Results are means ± SEM from 7 independent experiments. *p<0.05 significantly different from all other conditions. Statistical analyses by two-way ANOVA and Bonferroni post-hoc test for (b).
Figure 4.7. RCAN1 over-expression blocks retrograde propagation of the NGF-TrkA signal in sympathetic neurons.

(a) Schematic of the compartmentalized culture system used for biochemical analyses of NGF-dependent signaling locally in distal axons and retrogradely in cell bodies. (b) NGF stimulation of distal axons promotes phosphorylation of P-TrkA, P-Erk1/2 and P-Akt locally in axons and retrogradely in cell bodies of control GFP-expressing sympathetic neurons. RCAN1 over-expression disrupts the propagation of a retrograde NGF signal to cell bodies but does not affect local activation of these effectors in distal axons. Distal axons (da) of sympathetic neurons expressing GFP or RCAN1 were stimulated with NGF (100 ng/ml) for 8 hr. Cell body/proximal axon and distal axon lysates were prepared and subjected to immunoprecipitation with a P-Tyrosine (PY20) antibody followed by immunoblotting for TrkA to detect P-TrkA. Supernatants were immunoblotted for P-Erk1/2, P-Akt and p85. (c-e) Densitometric quantifications of levels of P-TrkA (c), P-Erk1/2 (d) and P-Akt (e). P-TrkA, P-Erk1/2 and P-Akt signals were all normalized to p85 levels. Results are means ± SEM from 5 independent experiments, and expressed relative to no neurotrophin conditions. n.s., not significant, *p<0.05 by two-way ANOVA and Bonferroni test.
a

IP: P-Tyr

Pellet

IB: TrkA

IB: P-Erk1/2

IB: P-Akt

IB: p85

Cell bodies

Distal axons

GFP RCAN1

- - + +

GFP RCAN1

- - + + NGF (da)

b

c

d

NGF (da) - - + +

Cell bodies

Distal axons

Relative P-TrkA

Relative P-Erk1/2

Relative P-Akt

n.s.

GFP

RCAN1

GFP

RCAN1

GFP

RCAN1

GFP

RCAN1

GFP

RCAN1

GFP

RCAN1

GFP

RCAN1

GFP

RCAN1

GFP

RCAN1

GFP

RCAN1

GFP

RCAN1
Figure 4.8. Calcineurin activity in distal axons is necessary to propagate a retrograde NGF signal.

(a) NGF-dependent retrograde accumulation of P-TrkA, P-Erk1/2 and P-Akt is attenuated by local inhibition of calcineurin activity in distal axons. Distal axons (da) of sympathetic neurons were stimulated with NGF (100 ng/ml) in the presence of the calcineurin inhibitors (CsA and FK506) for 8 hr. Lysates prepared from cell body and distal axon compartments were subjected to P-Tyr immunoprecipitation followed by immunoblotting for TrkA to detect P-TrkA. Supernatants were immunoblotted for P-Erk1/2, P-Akt and p85. (b-d) Densitometric quantifications of levels of P-TrkA (b), P-Erk1/2 (c), and P-Akt (d), normalized to p85 levels. Results are means ± SEM from 5 independent experiments and expressed relative to the NGF-stimulated condition. *p<0.05, **p<0.01 as determined by one-way ANOVA followed by Tukey’s post-hoc test.
Figure 4.9. Excess RCAN1 perturbs NGF-dependent neuronal survival.

(a) TUNEL labeling (green) indicates neuronal apoptosis in the presence of a neutralizing NGF antibody (anti-NGF, added to both cell body and axonal compartments). (b) Neuronal survival with NGF (100 ng/ml) present only on distal axons (da) in control LacZ-infected neurons. (c,d) RCAN1 over-expression compromises neuronal survival when NGF is present on distal axons (da) but not when NGF is added directly to cell bodies (cb). Neuronal apoptosis was assessed in neurons that had extended axons into the side chambers, visualized by retrograde accumulation of fluorescent microspheres (red). Neuronal nuclei are labeled with DAPI (blue). Scale bar: 10 mm. (e) Neuronal apoptosis was calculated by determining the percentage of projecting neurons that were TUNEL-positive. *p<0.05, **p<0.01, results are means ± SEM from 4 independent experiments. One-way ANOVA followed by Tukey’s post-hoc test for.
DAPI + NGF
LacZ
RCAN1

a DAPI TUNEL Beads

b NGF (da)

NGF (da) + NGF (da)

NGF (cb)

NGF (da)

NGF (cb)

LacZ

RCAN1

% Apoptosis

0 25 50 75 100

** *

NGF (cb) - - - +
NGF (da) - + + -

LacZ
RCAN1

0 25 50 75 100

** *

NGF (cb) - - - +
NGF (da) - + + -
Figure 4.10. NGF-dependent axon growth is abolished by RCAN1 over-expression.

Compartmentalized cultures of sympathetic neurons expressing GFP or RCAN1 were either deprived of NGF by including anti-NGF in media bathing cell body and axon compartments (a,c), or maintained with NGF (100 ng/ml) added solely to the axonal compartments (b,d). The caspase inhibitor, BAF (50 mM), was included in all experiments to prevent cell death. Panels in (a-d) are representative images of axons immunostained with anti-β-III tubulin 24 hr after addition of anti-NGF or NGF to distal axons. Scale bar: 50 mm. (e) Quantification of axon growth in compartmentalized cultures. Rate of axon extension (mm/day) was assessed in 24-hr intervals for a total of 72 hr. Results are means ± SEM from 5 independent experiments. **p<0.01 different from all other conditions. Two-way ANOVA and Bonferroni post-hoc test for (k).
METHODS

Animals

All procedures relating to animal care and treatment conformed to institutional and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. Mice were maintained on a C57BL/6 background and both sexes were used for analyses. Trisomic RCAN1 mice were a generous gift from Dr. Sandra Ryeom. RCAN1 trisomic mice were genotyped by PCR screening to ensure that only heterozygous mice were used for in vivo analyses of RCAN1 trisomy (Baek, 2009). Sprague Dawley rats were purchased from Charles River. Sympathetic neuron cultures were established from superior cervical ganglia dissected from P0.5 rat pups as previously described.

Constructs, reagents, antibodies

Human HA-tagged RCAN1.4, a gift from Dr. Kyle Cunningham, was sub-cloned into pShuttle-CMV vector (Stratagene) and a replication incompetent recombinant adenoviral construct was generated using the AdEasy™ adenoviral vector system (Stratagene). Recombinant viral backbone was transfected into HEK 293 cells (ATCC) using Lipofectamine™ (Invitrogen) and high-titer virus stocks purified using a CsCl gradient. The antibodies used in this study were previously validated for the following applications: P-Tyr (Sigma-Aldrich; P4110, immunoprecipitation), P-Erk1/2 (Cell signaling; 9106, western blotting), P-Akt (Cell Signaling; 9271, western blotting), TrkA (Millipore; AB1577, western blotting), p85 subunit of phosphatidylinositol-3-kinase (Upstate Biotechnology, 06-195, western blotting), dynamin1 (Abcam; ab3456, western blotting), dynamin phospho-Ser778 (Imgenex, IMG-670, western blotting), HA (Sigma-Aldrich; H9658, western blotting), transferrin (Santa Cruz Biotechnology; sc-52256,
western blotting), TH (Millipore; AB152, immunohistochemistry), and cleaved caspase-3 (Cell Signaling; 9661, immunohistochemistry). The RCAN1 antibody was generated by injecting rabbits with the peptide FLISPPASPPV, and blood serum was extracted (Pocono Rabbit Farm). RCAN1 anti-serum was used directly at a dilution of 1:1000 for immunoblotting and 1:200 for immunocytochemistry. FK-506 and Cyclosporin A were obtained from Sigma-Aldrich.

**In situ hybridization**

*In situ* hybridization was performed using a digoxigenin labeled probe spanning a 480 bp region within exons 5-7 of mouse RCAN1. P0.5 mouse pups were fresh frozen in OCT (Tissue-Tek) and serially sectioned (12µm) using a cryostat. Sections were post-fixed in 4% PFA, washed in PBS and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine with 0.9% NaCl. After hybridization with the labeled RNA probe (2 mg/ml) at 68°C overnight, sections were washed with 0.2X SSC buffer at 65°C, blocked with TBS containing 1% normal goat serum and then incubated with alkaline phosphatase-labeled anti-DIG antibody (1:5000; Roche) overnight at 4°C. The alkaline phosphatase reaction was visualized with NBT/BCIP, rinsed in PBS, fixed in 4% PFA and mounted in AquaMount (EMD Chemicals).

**RT-PCR analyses**

Total RNA was prepared from dissected SCGs using Trizol-chloroform extraction (Life Technologies). RNA was then reverse transcribed using a RETROscript kit (Ambion). RT-PCR analyses for *RCAN1.1* and *RCAN1.4* isoforms were performed using the following primers; (*RCAN1.1*-F 5'-ACTGGAGCTTCATCGACTGC-3') and (*RCAN1.4*-F: 5'-
AGCTCCCTGATTGCTTGTGT-3’) and a common reverse primer (5’-GTGTA\text{CTCGGTCTCGTGT-3’}).

**Neuronal cultures and adenovirus infection**

Sympathetic neurons were harvested from P0.5 Sprague-Dawley rats and grown in mass cultures or compartmentalized cultures as described previously (Kuruvilla, 2004). Dissociated DRG neurons were isolated from E15-16 rats and grown in mass cultures using culture conditions similar to that described for sympathetic neurons. Cells were maintained in culture with high-glucose DMEM media supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (1U/ml), and NGF (100 ng/ml) purified from male mouse submaxillary glands as described previously (Mobley, 1976). For immunocytochemistry, cells were plated on poly-D-lysine (1 µg/ml; Sigma-Aldrich) coated coverslips. To withdraw NGF before any stimulation experiments, neurons were placed in high-glucose DMEM containing 0.5% FBS with 1:1000 anti-NGF (Sigma-Aldrich) and boc-aspartyl(O-methyl)-fluoromethylketone (BAF) (50 µM; MP Biomedical) for 48 hr. For adenoviral infections, neuronal cultures were infected with high-titer CsCl-purified adenoviruses for 48 hr as described previously (Kuruvilla, 2000).

**Neuronal survival and axon growth assays**

Sympathetic neurons grown in compartmentalized cultures were infected with adenoviral constructs expressing LacZ or RCAN1. To ensure survival scoring of only the neurons that had projected axons into the side chambers, fluorescent microspheres (Invitrogen) were added to the distal axon compartments 24 hr prior to the experiments. Neurons were either completely deprived of NGF (by
adding anti-NGF to both cell body and distal axon compartments) or supported by NGF (100 ng/ml) added only to distal axons or cell bodies. After 72 hours, neurons were fixed and dying cells were visualized using TUNEL staining (Roche) according to the manufacturer’s protocol. Neuronal apoptosis was calculated by determining the percentage of neurons that had extended axons into the side chambers (visualized by fluorescent microsphere uptake) that were also positive for TUNEL label.

For assessing axon growth, compartmentalized neuronal cultures were infected with GFP or RCAN1. Neurons were either completely deprived of NGF or NGF (100 ng/ml) was added only to distal axons as for the survival assays. The broad-spectrum caspase inhibitor, BAF (50 mM) was also included to allow assessment of axon growth without the complications of cell death. Axon growth was quantified by capturing phase contrast images of the distal axon compartments over consecutive 24-hr intervals for 3 days, using a Zeiss Axiovert 200 microscope with a Retiga EXi camera. Rate of axonal growth (µm/day) was measured using Openlab 4.04. Measurements from 30-50 neurons were averaged for each condition for a single experiment. For representative images, neurons were immunostained with β-III-tubulin (1:200; Sigma-Aldrich) 24 hr after the NGF treatment.

TrkA receptor internalization assays

Cell-surface biotinylation assays were performed in cultured rat sympathetic neurons as previously described (Kuruvilla, 2004). Briefly, neurons were biotinylated with a reversible membrane-impermeable form of biotin (EZ-Link NHS-S-S-biotin, 1.5mg/ml in 1X PBS; Pierce Chemical) at 4°C for 25 min.
Neurons were washed briefly with 1X PBS containing 50mM glycine (Sigma-Aldrich) to remove remaining unconjugated biotin. Neurons were moved to 37°C to promote internalization under the appropriate conditions for 30 min. Neurons were returned to 4°C and the remaining biotinylated surface receptors were stripped of their biotin tag with 50mM glutathione (Sigma-Aldrich). After this stripping process, cells were washed twice with 50mM iodoacetamide (Sigma-Aldrich) to quench excess glutathione. Neurons were lysed with 500 µl of RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.25% deoxycholate), and supernatants were subjected to precipitation with 40 µl-immobilized neutravidin agarose beads (Pierce Chemical) and immunoblotting with a TrkA antibody.

**Transferrin and EGF internalization assays**

Sympathetic neurons, infected with RCAN1 or GFP adenoviruses, were serum-starved and incubated with anti-NGF in the presence of BAF (50 mM) for two days prior to the internalization assay. For the biochemical transferrin uptake assay, neurons were incubated with biotin-labeled transferrin in PBS (25 mg/ml) at 4°C for 30 min. Cells were then washed two times with PBS and either left at 4°C or moved to 37°C for 5 min to allow transferrin internalization. To measure internalized transferrin, surface-bound transferrin was stripped by adding 2 ml of ice-cold acidic buffer to the cells for 2 min, followed by a wash with 10 ml of ice-cold PBS. The stripping and washing steps were performed twice. After the stripping process, neurons were lysed with 500 ml of RIPA buffer, and supernatants were subjected to precipitation with 40 ml-immobilized
neutravidin agarose beads (Pierce Chemical) and immunoblotting with a transferrin antibody (1:1000; Santa Cruz Biotechnology).

For the fluorescent-based uptake assays, neurons were incubated with Alexa-555-labeled transferrin (25 mg/ml) or Alexa-555-labeled EGF in PBS (2 mg/ml) at 4°C for 30 min. Neurons were then either left at 4°C or moved to 37°C for 5 min or 15 min respectively, followed by washes with ice-cold PBS, fixation with 4% paraformaldehyde and then mounted on slides with Fluoromount (Vectashield) and DAPI. Levels of labeled transferrin that accumulated intracellularly were assessed within 5 minutes to ensure that we were monitoring transferrin internalization in the absence of significant recycling back to the plasma membrane. Images were acquired using confocal microscopy and creating a 3-dimensional model from z-stacks. Internalized receptors were calculated as Alexa-555 fluorescent pixels per µm² of cell body. 40-50 cells were analyzed per condition per experiment.

**Immunoblotting and Immunoprecipitation assays**

For biochemical analyses of local and retrograde NGF signaling, sympathetic neurons were grown in compartmentalized chambers for 7-10 DIV until robust axonal projections were evident in the side compartments. Neurons were then infected with control GFP or RCAN1 adenoviruses and deprived of NGF for 48 hr by adding anti-NGF in the presence of BAF (50 mM). Neurons were then either stimulated with NGF (100 ng/ml) added exclusively to distal axons for 8 hr or treated with control medium alone, after which lysates were prepared separately from the cell body and distal axon compartments using RIPA buffer. To detect P-TrkA, lysates were subjected to immunoprecipitation with anti-phosphotyrosine (PY-20; Sigma-Aldrich) and incubated with Protein-A agarose
beads (Santa Cruz Biotechnology) and immunoprecipitates were then immunoblotted for TrkA. The supernatants from the immunoprecipitation were subjected to immunoblotting for P-Akt, and P-Erk1/2. Normalization for protein amounts was done by immunoblotting for p85 (Upstate Biotechnology). In experiments to assess the role of local calcineurin activity on NGF signaling, distal axons of compartmentalized neurons were pre-treated with the calcineurin inhibitors, Cyclosporin A (CsA) (2 mg/ml) and FK506 (0.2 mg/ml) for 30 minutes prior to the NGF stimulation.

To examine the phosphorylation level of dynamin1 in vitro, sympathetic neurons grown in mass cultures were stimulated with NGF for 30 min or treated with control medium. Lysates were prepared by laemmli sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% Glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) and subjected to immunoblotting with the phospho-dynamin1 (P-Ser 778, 1:5000; Imgenex) and dynamin1 (1:1000; Abcam) antibodies. All immunoblots were visualized with ECL Plus Detection Reagent (GE Healthcare) and scanned with a Typhoon 9410 Variable Mode Imager (GE Healthcare).

**Immunohistochemical analyses of mouse tissues**

Mice at various developmental ages were fixed in 4% PFA at 4°C for 3-4 hr, cryoprotected in 30% sucrose in PBS, frozen in OCT and serially sectioned (12 mm). For immunofluorescence, sections were washed in PBS, permeabilized in PBS containing 1% Triton X-100, and blocked using 5% goat serum in PBS + 0.1% Triton X-100. Sections were incubated in the following primary antibodies overnight: rabbit anti-TH (1:200; Millipore) and rabbit anti-cleaved caspase 3 (1:200; Cell Signaling). Following PBS washes, sections were incubated with anti-rabbit Alexa-488 secondary antibodies (1:200; Life Technologies). Sections were
then washed in PBS and mounted in VectaShield (Vector Laboratories) containing 100 mg/ml DAPI.

Quantification of sympathetic innervation density in the salivary glands and nasal epithelium was done by calculating integrated TH fluorescence density per unit area (ImageJ) from multiple random images.

**Neuronal cell counts**

E16.5 and P0.5 mice for neuronal counts were prepared as described (Bodmer, 2009). Briefly, mouse torsos were fixed in PBS containing 4% PFA, and then cryoprotected overnight in 30% sucrose-PBS. SCG sections (12 mm) were stained with a solution containing 0.5% cresyl violet (Nissl). Cells with characteristic neuronal morphology and visible nucleoli were counted in every fifth Nissl stained section.

**Statistical Analyses**

Samples sizes were similar to those reported in previous publications (Kuruvilla, 2004; Ascano, 2009; Bodmer, 2011). Data were collected randomly and the assessment of human tissues was done in a manner blinded to the group allocation. For practical reasons, analyses of innervation in mouse tissues were done in a semi-blinded manner such that the investigator was aware of the genotypes prior to the experiment, but conducted the immunostaining and data analyses without knowing the genotypes of each sample. InStat software was used for statistical analyses. All Student’s *t* tests were performed assuming Gaussian distribution, two-tailed, unpaired, and a confidence interval of 95%. For peripheral innervation of human tissue analyses, we used a one-tailed *t* test, based on the prediction of directionality obtained from analyses in mice. One-way or two-way ANOVA analyses were performed when more than two groups
were compared. Statistical analyses were based on at least 3 independent experiments, and described in the figure legends. All error bars represent the standard error of the mean (S.E.M.).
DISCUSSION

Here, we show that overexpression of a single Down syndrome gene, RCAN1 is sufficient to disrupt sympathetic nervous system development. Adenoviral overexpression of RCAN1 in vitro is sufficient to alter dynamin1 phosphorylation state, block NGF-dependent TrkA internalization, perturb retrograde trafficking of the NGF-TrkA signal, and disrupt trophic function. The RCAN1 transgenic mice show a marked reduction in sympathetic target innervation as early as E16.5. Interestingly, the axon growth defects precedes the cell death defect, which only presents at later stages of development, P0.5. One possibility is that RCAN1 overexpression specifically disrupts axon growth, reducing access to the NGF survival signal. This is supported by our in vitro data where RCAN1 overexpression blocks NGF-dependent axon growth in the presence of an apoptotic inhibitor.

During endocytic trafficking in neurons, neurotrophin receptors are not passive passengers being carried along by a constitutively operating endocytic machinery. Rather, Trk receptor tyrosine kinase-initiated signals actively modulate the endocytic machinery to drive their own trafficking (Ascano, 2012). We previously demonstrated that TrkA signaling drives its own internalization by recruiting and activating the effector enzyme, PLC-g, which then stimulates calcineurin-dependent dephosphorylation of neuron-specific spliced isoforms of dynamin1 (Bodmer, 2011). Here, we show that excess RCAN1 interferes with this TrkA-initiated endocytic signaling pathway, but does not impede clathrin-mediated endocytosis of EGF and transferrin receptors. Furthermore, we found that RCAN1 over-expression uniquely compromises neuronal survival when NGF is present on distal axons, but that RCAN1 over-expressing neurons are
healthy and viable with NGF added directly to cell bodies. Together, these findings support the view that excess RCAN1 does not elicit non-specific endocytic deficits in sympathetic neurons, but interferes with the propagation of long-distance NGF endosomal signaling.

While calcineurin has several downstream targets (Li, 2011), NGF signaling may selectively regulate a subset of calcineurin-substrate interactions. Previously, increased gene dosage of RCAN1 was proposed to contribute to embryonic development and tumor suppression phenotypes in Down syndrome by disrupting calcineurin-dependent regulation of NFAT transcription factors (Arron, 2006; Baek, 2009). In the PC12 cell line, RCAN1 over-expression was found to attenuate NGF-induced activation of NFAT promoter activity (Stefos, 2013). However, NGF does not elicit NFAT nuclear localization or transcriptional activity in sympathetic neurons (Bodmer, 2011). In contrast, NGF stimulation does lead to dephosphorylation of dynamin1, which was blocked by either pharmacological inhibition of calcineurin (Bodmer, 2011) or RCAN1 over-expression (this study). Additionally, expression of phosphomimetic or nonphosphorylatable forms of calcineurin-interacting dynamin1 isoforms in sympathetic neurons perturbed NGF-dependent TrkA internalization and axon growth (Bodmer, 2011), similar to the effects of excess RCAN1. Together our findings suggest, that although excess RCAN1 in sympathetic neurons could inhibit other calcineurin-dependent signaling pathways, the disruption of calcineurin-dynamin1 interactions is the most pertinent mechanism that underlies impaired TrkA endocytosis and NGF-dependent neuronal survival and growth with RCAN1 over-expression.
Chapter 5: RCAN1 overexpression is required for full sympathetic defects in Down syndrome

A portion of the work in this chapter has been submitted for publication at Nature Neuroscience:

Ami Patel, Naoya Yamashita, Maria Ascaño, Daniel Bodmer, Erica Boehm, Chantal Bodkin-Clarke, Yun Kyoung Ryu, and Rejji Kuruvilla. RCAN1 links impaired neurotrophin trafficking to aberrant development of the sympathetic nervous system in Down syndrome.
INTRODUCTION

One of the major goals of Down syndrome research is identifying genotype-phenotype correlations. Understanding which genes contribute developmentally to the pathogenesis of DS not only gives insight into normal development, but can also direct research for treatment options. In the past 20 years, it has been proposed that a relatively small number of genes on Hsa21 actually contribute to, or play a dominant role in DS disorders (Korenberg, 1994; Sinet, 1994; Luke, 1995; Shapiro, 1999; Nikolaienko, 2005; Olson, 2007). This idea was supported by the observation that some genes on Hsa21 have expression levels in DS that were within the normal range (Lyle, 2004; Kahlem, 2004; Spielman, 2007; Sultan, 2007). Further, the use of partial trisomic mouse models, such as one containing the “Down syndrome critical region” (DSCR) showed that many DS phenotypes were conferred by trisomy of a small number of genes. However, these mice did not have all the phenotypes associated with DS (Olson, 2007). More recently, the complexity of this disorder has led researchers to focus efforts on determining which specific genes are responsible for specific phenotypes. Of the originally designated “DSCR” genes, the endogenous calcineurin regulator RCAN1 remains one of the most “dominant” genes, being implicated in cardiovascular, immune, and cognitive development (Bhoiwala, 2011; Dierssen, 2011; Bhoiwala, 2013; Martin, 2013; Xing, 2013; Torac, 2014). Our previous work agrees with the idea that RCAN1 overexpression is sufficient for sympathetic defects, it remains unknown if it is the “dominant” gene causing reduced NGF-TrkA trafficking, innervation and survival defects. To address this, we restored RCAN1 to disomic levels in the context of DS by crossing a RCAN1
heterozygous knockout mouse with the \textit{Dp(16)1Yey/+} mouse model and analyzed sympathetic development.

Our findings support the idea that RCAN1 overexpression is the dominant gene that contributes to NGF-TrkA trafficking defects in sympathetic neurons in Down syndrome. When RCAN1 is restored to wild type levels, calcineurin activity is increased, and dynamin1 phosphorylation and Trk receptor trafficking are similar to wild type. Returning RCAN1 to wild type levels also ameliorates sympathetic innervation and survival defects observed in the \textit{Dp(16)1Yey/+} mouse. Together, this data supports the hypothesis that sympathetic nervous system development defects are a result of impaired NGF-TrkA endocytosis due to RCAN1 overexpression.
RESULTS

Reducing RCAN1 gene dosage improves calcineurin activity and corrects endocytic defects in Dp(16)1Yey/+ mice

To further test the causal role of RCAN1 trisomy in defects in TrkA trafficking and NGF-dependent development of sympathetic neurons, we eliminated one copy of RCAN1 in Dp(16)1Yey/+ mice by crossing these mice with RCAN1+/- mice (Ryeom, 2008) to generate Dp(16)1Yey/+;RCAN+/- mice, which will have two copies of RCAN1 but maintain trisomy of the other 112 genes. Quantitative real-time PCR analysis of SCGs confirmed that RCAN1 levels were restored to physiological levels in Dp(16)1Yey/+;RCAN+/- mice (Fig. 5.1).

To assess the effects of an extra copy of RCAN1 on restraining calcineurin signaling, we compared calcineurin phosphatase activity in SCG lysates from P0.5 wild-type mice with that in Dp(16)1Yey/+ mice containing two versus three copies of RCAN1. Calcineurin activity was substantially decreased in SCG lysates from Dp(16)1Yey/+ mice with three RCAN1 copies (42% of wild-type activity), but reducing RCAN1 gene dosage significantly improved calcineurin activity (64% of wild-type activity) in Dp(16)1Yey/+;RCAN+/- mice (Fig. 5.2a). To test whether the improvement in calcineurin activity by removing one copy of RCAN1 is reflected by alterations in dynamin phosphorylation, we assessed the levels of phospho-dynamin1 in the salivary glands, a sympathetic target field, in vivo. The salivary glands are richly innervated by axonal projections from the SCG, and immunoblotting of salivary gland lysates with the phospho-dynamin1 antibody should reveal the phosphorylation status of dynamin1, a neuron-specific protein, locally in nerve terminals. We found a significant increase (2.5 ± 0.4 fold increase) in phosphorylated dynamin1 (P-Ser-778) in sympathetic axons...
innervating the salivary glands in Dp(16)1Yey/+ mice compared to wild-type controls. Notably, this increase in phosphorylated dynamin1 was corrected by reducing the RCAN1 dosage in Dp(16)1Yey/+:RCAN+/− mice (Fig. 5.2b,c). There were no significant differences in the levels of phosphorylated dynamin1 between Dp(16)1Yey/+:RCAN+/− mice and wild-type litter-mates (p=0.46, one way ANOVA, Tukey’s post-hoc test). Together, these findings support a key contribution of the extra copy of RCAN1 in suppressing calcineurin activity and increasing dynamin1 phosphorylation in Dp(16)1Yey/+ sympathetic neurons.

Given that reducing RCAN1 gene dosage ameliorated deficits in calcineurin activity and dynamin1 phosphorylation in Dp(16)1Yey/+ mice, we then asked whether this genetic rescue approach would also alleviate defects in TrkA trafficking in Dp(16)1Yey/+ neurons. Thus, we monitored the internalization of Trk receptors in compartmentalized cultures of sympathetic neurons isolated from P0.5 SCGs of Dp(16)1Yey/+ and litter-mate Dp(16)1Yey/+:RCAN+/− and wild-type mice. Sympathetic neurons expressing FLAG-tagged chimeric TrkB:TrkA receptors were live-labeled with a FLAG antibody. After 1 hour of exposure to the ligand, BDNF, to allow for internalization of labeled receptors, the surface-bound antibodies were stripped, leaving antibodies bound only to the internalized pool of receptors. FLAG antibodies bound to internalized receptors were then visualized with Alexa-546-labeled secondary antibodies. We found that, in contrast to the internalization defect in Dp(16)1Yey/+ neurons, neurotrophin stimulation resulted in significant Trk receptor internalization in Dp(16)1Yey/+:RCAN+/− rescue neurons similar to that in wild-type neurons (Fig. 5.3a-f). The normalization of receptor endocytosis with reduced RCAN1 gene dosage was evident in both cell bodies (Fig. 5.3g) and axons (Fig. 5.3h) from
Dp(16)1Yey/+:RCAN<sup>+/−</sup> sympathetic neurons. Together, these results establish a causal link between excess RCAN1 and defects in neurotrophin receptor trafficking in Dp(16)1Yey/+ neurons.

**Reducing RCAN1 gene dosage alleviates developmental defects in Dp(16)1Yey/+ mice**

We then addressed whether reducing RCAN1 levels would rescue the phenotypes in sympathetic nervous system development in Dp(16)1Yey/+ mice. We employed TH immunohistochemistry to visualize the developing SCGs and axonal innervation of targets, as well as Nissl staining to quantify SCG cell numbers in P0.5 Dp(16)1Yey/+, and litter-mate Dp(16)1Yey/+:RCAN<sup>+/−</sup> and wild-type mice. As expected, we observed a substantial decrease in SCG cell numbers in Dp(16)1Yey/+ mice as compared to wild-type pups (7,601 ± 334 neurons in Dp(16)1Yey/+ mice vs. 22,680 ± 1,251 in wild-type litter-mates) (Fig. 5.4a,b,d). Genetically reducing RCAN1 levels ameliorated the loss of sympathetic neurons since we found a significant increase (32.4%) in SCG cell numbers in Dp(16)1Yey/+:RCAN<sup>+/−</sup> mice compared to their Dp(16)1Yey/+ litter-mates (11,259 ± 556 neurons in Dp(16)1Yey/+:RCAN<sup>+/−</sup> mice vs. 7,601 ± 334 neurons in Dp(16)1Yey/+ litter-mates) (Fig. 5.4c,d). Notably, the reduction in RCAN1 dosage did not fully rescue the neuronal number deficit. Examination of sympathetic innervation also revealed a beneficial effect of reducing RCAN1 levels since more TH-positive sympathetic fibers were found within the nasal epithelium (Fig. 5.5a-d) and salivary glands (Fig. 5.5e-h) in Dp(16)1Yey/+:RCAN<sup>+/−</sup> mice compared to their Dp(16)1Yey/+ litter-mates.
Together, these findings indicate that increased gene dosage of RCAN1 significantly contributes to the disruptions in NGF-dependent development of sympathetic neurons in the $Dp(16)1Yey/+\$ mouse model of Down syndrome.
Figure 5.1 Restoration of RCAN1 to wild type levels in \textit{Dp}(16)1Yey/+ mice.

Quantitative RT-PCR shows a 2.9-fold increase in \textit{RCAN1} mRNA in SCGs from \textit{Dp}(16)1Yey/+ mice compared to wild-type litter-mates. \textit{RCAN1} levels are reduced in \textit{Dp}(16)1Yey/+:\textit{RCAN1}−/+ mice that are diploid for \textit{RCAN1} and trisomic for the rest of human chromosome 21 syntenic region on mouse chromosome 16. Results are mean ± SEM, n=7 mice for each genotype. *p<0.05 different from all other conditions.
Figure 5.2 Reducing RCAN1 to WT in a DS mouse model restores calcineurin activity and phospho-dynamin1 levels

(a) Calcineurin activity is significantly reduced in SCG lysates from P0.5 $Dp(16)1Yey/+\) mice. Genetic reduction of $RCAN1$ significantly improves calcineurin activity in $Dp(16)1Yey/+:RCAN^{+/}\) mice relative to $Dp(16)1Yey/+\) littermates. Calcineurin phosphatase activity was measured using a colorimetric assay that detects free phosphate released from the calcineurin-specific RII phosphopeptide. Results are mean ± SEM from n=6 mice per genotype. **p<0.01, ***p<0.001. (b,c) Reducing $RCAN1$ gene dosage restores the dynamin1 phosphorylation status in $Dp(16)1Yey/+\) mice. $Dp(16)1Yey/+\) mice have increased levels of phospho-dynamin1 in sympathetic axons in vivo, that is corrected by removing one copy of $RCAN1$ in $Dp(16)1Yey/+:RCAN^{+/}\) mice. Salivary gland lysates from P0.5 wild-type, $Dp(16)1Yey/+\) and $Dp(16)1Yey/+:RCAN^{+/}\) mice were immunoblotted using phospho-dynamin1 (Ser778) antibody. Immunoblots were stripped and reprobed for total dynamin1 for normalization. (c) Densitometric quantification of phospho-dynamin1 (Ser778) after treatments as described in (c). Values are expressed relative to wild-type. Results are means ± SEM from n=7 mice per genotype. *p<0.05, **p<0.01. Statistical analyses by one-way ANOVA and Tukey’s post-hoc test for (a,c).
Calcineurin activity

WT
Dp(16)1Yey/+ Dp(16)1Yey/+:RCAN1

Phosphorylated dynamin1

WT Dp(16)1Yey/+ Dp(16)1Yey/+:RCAN1

Phosphorylated dynamin1

WT Dp(16)1Yey/+ Dp(16)1Yey/+:RCAN1
Figure 5.3. Reducing RCAN1 gene dosage rescues the defect in Trk receptor endocytosis in Dp(16)1Yey/+ mice.

(a-f) Ligand-dependent Trk internalization in Dp(16)1Yey/+:RCAN+/− sympathetic neurons was comparable to that in wild-type neurons. Trk receptor internalization was monitored by a modified FLAG antibody feeding assay where surface-bound FLAG antibodies were stripped after internalization. FLAG immunoreactivity (red) representing internalized Trk receptors was assessed in cell bodies and axons in neurons treated with or without BDNF. GFP is co-expressed with FLAG-TrkB:TrkA in infected neurons. Scale bar: 5 mm and 10 mm for axons and cell bodies respectively. (g,h) Quantification of internalized Trk in cell bodies and axons after treatments described in (a-f), by estimating FLAG immunofluorescence (pixels) per µm² of cell body or axon, (for axons, measurements were taken from a stretch of axon equal to 100-250 µm length). 40-50 cells were analyzed per condition per experiment. Quantification is represented as fold-change relative to wild-type neurons with no ligand. Results are the mean ± SEM from at least 5 independent experiments. n.s., not significant, *p<0.05, **p<0.01, ***p<0.001. Statistical analyses done by two-way ANOVA and Bonferroni post-hoc test for (g,h).
WT

Dp(16)1Yey/+

Dp(16)1Yey/+;RCAN1

Internalized Trk receptors

Cell bodies

Distal axons

neurotrophin - - - + + +

*** n.s. **

Internalized Trk receptors in cell bodies

Internalized Trk receptors in axons

WT Dp(16)1Yey/+ Dp(16)1Yey/+;RCAN1

- - - + + +

*** n.s. **

neurotrophin

- - - + + +

*** n.s. **

- - - + + +

*** n.s. **
Figure 5.4. Sympathetic cell numbers are rescued by reduction of RCAN1 in Dp(16)1Yey/+ mice

(a-d) Genetic reduction of RCAN1 ameliorates the loss of sympathetic neurons in Dp(16)1Yey/+ mice. P0.5 SCGs from Dp(16)1Yey/+:RCAN1+/− mice show increased size and neuronal numbers compared to Dp(16)1Yey/+ litter-mates. SCGs were visualized by TH immunohistochemistry and cell counts were performed on Nissl stained tissue sections. Results are the mean ± SEM from n=5 mice per genotype. *p<0.05, ***p<0.001. Scale bars: 100 µm. Statistical analysis by one way ANOVA and Tukey’s post-hoc test.
Figure 5.5. TH immunohistochemistry reveals a marked improvement of sympathetic innervation in target tissues in P0.5 Dp(16)1Yey/+ :RCAN<sup>+/−</sup> mice compared to Dp(16)1Yey/+ litter-mates.

(a-h) Innervation of nasal epithelium is shown in (a-c) and that of salivary glands in (e-g). Quantification of innervation density (d, nasal epithelium; h, salivary glands) was estimated from n=6 mice per genotype. Values are the mean ± SEM. The results are represented as a percentage of the mean for wild-type mice. *p<0.05, **p<0.01, ***p<0.001. Scale bar: 50 µm for (a,b,c) and 100 µm for (e,f,g).

Statistical analyses by one-way ANOVA and Tukey's post-hoc test for (d,h).
Nasal Epithelium

Innervation density of SG (% WT)

0 25 50 75 100

+/-

******

Salivary Glands

Innervation density of NE (% WT)

0 25 50 75 100

* ***

****

TH+DAPI

WT Dp(16)1Yey/+ Dp(16)1Yey:

RCAN1+/−

P0.5

WT Dp(16)1Yey/+ Dp(16)1Yey:

RCAN1+/−

P0.5
METHODS

Animals

All procedures relating to animal care and treatment conformed to institutional and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. Mice were maintained on a mixed C57BL/6 and Balb/C background. RCAN1+/− mice were a generous gift from Dr. Sandra Ryeom. Dp(16)1Yey/+;RCAN+/− mice that were diploid for RCAN1 and trisomic for the rest of human chromosome 21 syntenic region on mouse chromosome 16 were generated by mating RCAN1+/− mice with Dp(16)1Yey/+ mice, and litters were genotyped as previously described (Li, 2007 #3138; Baek, 2009).

Constructs and antibodies

The generation of the FLAG-TrkB/A adenovirus has been previously described (Ascano, 2009). The antibodies used in this study were previously validated for the following applications: dynamin1 (Abcam; ab3456, western blotting), dynamin phospho-Ser778 (Imgenex, IMG-670, western blotting), FLAG M2 (Sigma-Aldrich; H9658, antibody feeding assays), TH (Millipore; AB152, immunohistochemistry), and cleaved caspase-3 (Cell Signaling; 9661, immunohistochemistry). Calcineurin activity was assessed using the Enzo cellular activity assay kit (AK816, Enzo Life Sciences) and substrate reaction was measured using a plate reader.

Immunohistochemical analyses of mouse tissues

Mice at various developmental ages were fixed in 4% PFA at 4°C for 3-4 hr, cryoprotected in 30% sucrose in PBS, frozen in OCT and serially sectioned (12 mm). For immunofluorescence, sections were washed in PBS, permeabilized in PBS containing 1% Triton X-100, and blocked using 5% goat serum in PBS + 0.1%
Triton X-100. Sections were incubated in the following primary antibodies overnight: rabbit anti-TH (1:200; Millipore) and rabbit anti-cleaved caspase 3 (1:200; Cell Signaling). Following PBS washes, sections were incubated with anti-rabbit Alexa-488 secondary antibodies (1:200; Life Technologies). Sections were then washed in PBS and mounted in VectaShield (Vector Laboratories) containing 100 mg/ml DAPI.

Quantification of sympathetic innervation density in the salivary glands and nasal epithelium was done by calculating integrated TH fluorescence density per unit area (ImageJ) from multiple random images.

**RT-PCR analyses**

Total RNA was prepared from dissected SCGs using Trizol-chloroform extraction (Life Technologies). RNA was then reverse transcribed using a RETROscript kit (Ambion). Real-time qPCR was performed using a Maxima SYBR Green/Rox Q-PCR Master Mix (Thermo Scientific), in a 7300 Real time PCR System (Applied Biosystems). *RCAN1* mRNA levels were measured by using primers targeting exons 5-7 (*RCAN1*-F: 5’-TTCCTGGGGAAGGAAATGAA-3’ and *RCAN1*-R: 5’-GGTGGTGTCCCTTGTCATATG-3’). GAPDH was used as a control (*GAPDH*-F: 5’-CCTGCACCACCAACTGCTTA-3’ and *GAPDH*-R: 5’-CCACGATGCCAAAGTTGTCA-3’). Each sample was analyzed in triplicate reactions. Fold change in *RCAN1* transcript levels was calculated using the $2^{ΔΔCt}$ method, normalizing to GAPDH transcript.

**Calcineurin activity assay**
SCGs, salivary glands, and heart tissues were dissected from P0.5 mouse pups and homogenized in lysis buffer containing protease inhibitors. Supernatants were incubated with the calcineurin-specific substrate, the RII phosphopeptide, at 37°C for 30 minutes. Phosphatase activity was measured by colorimetric detection of free phosphate using a plate reader. Total phosphatase activity was assessed under conditions where no exogenous substrate was included, in a calcineurin-permissive buffer (inclusion of calmodulin) and a calcineurin-inhibiting buffer (inclusion of the calcium chelator, EGTA). Calcineurin phosphatase activity was calculated by subtracting calcineurin-independent phosphatase activity (in the EGTA condition) from total phosphatase activity. Phosphatase activity was normalized to total protein using a BCA assay.

**Neuronal cell counts**

E16.5 and P0.5 mice for neuronal counts were prepared as described (Bodmer, 2009). Briefly, mouse torsos were fixed in PBS containing 4% PFA, and then cryoprotected overnight in 30% sucrose-PBS. SCG sections (12 mm) were stained with a solution containing 0.5% cresyl violet (Nissl). Cells with characteristic neuronal morphology and visible nucleoli were counted in every fifth Nissl stained section.

**TrkA receptor internalization assay**

A modified version of the antibody feeding assay (Ascano, 2009) was employed in experiments in Fig. 7e-j, where neurons, labeled with the FLAG antibody under non-permeabilizing conditions and stimulated with BDNF to allow ligand-mediated internalization, were then quickly stripped of surface-bound FLAG antibodies that had not internalized by three quick washes in an ice-cold acidic buffer (0.2N acetic acid, 0.5M NaCl, pH 3.0). Internalized receptors
were then visualized by fixing, permeabilizing and incubation with fluorescently conjugated anti-mouse secondary antibody. Images were acquired using confocal microscopy and creating a 3-dimensional model from z-stacks. Internalized receptors were calculated as Alexa-546 fluorescent pixels per \( \mu \text{m}^2 \) of cell body or axon (for axons, measurements were taken from a stretch of axon equal to 100-250\( \mu \text{m} \) length). For all imaging, 40-50 cells were analyzed per condition per experiment.

**Immunoblotting assays**

To detect phosphorylated dynamin1 *in vivo*, salivary glands were dissected from P1 mouse pups. To examine the phosphorylation level of dynamin1 *in vitro*, sympathetic neurons grown in mass cultures were stimulated with NGF for 30 min or treated with control medium. Lysates were prepared by laemmli sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% Glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) and subjected to immunoblotting with the phospho-dynamin1 (P-Ser 778, 1:5000; Imgenex) and dynamin1 (1:1000; Abcam) antibodies. All immunoblots were visualized with ECL Plus Detection Reagent (GE Healthcare) and scanned with a Typhoon 9410 Variable Mode Imager (GE Healthcare).

**Statistical Analyses**

Samples sizes were similar to those reported in previous publications (Kuruvilla, 2004; Ascano, 2009; Bodmer, 2011). For practical reasons, analyses of innervation in mouse tissues were done in a semi-blinded manner such that the investigator was aware of the genotypes prior to the experiment, but conducted the immunostaining and data analyses without knowing the genotypes of each
sample. InStat software was used for statistical analyses. One-way ANOVA analyses were performed when more than two groups were compared. Statistical analyses were based on at least 3 independent experiments, and described in the figure legends. All error bars represent the standard error of the mean (s.e.m).
DISCUSSION

A key question is whether, in the context of trisomy of 113 genes in the Dp(16)1Yey/+ mouse model of Down syndrome, increased expression of RCAN1 is primarily responsible for the sympathetic nervous system phenotypes that we observed? We found that triplication of RCAN1 alone was sufficient to mimic the loss of neurons and diminished sympathetic innervation in Dp(16)1Yey/+ mice (Chapter 4). We also showed that deleting a single copy of RCAN1 in the Dp(16)1Yey/+ mouse model led to marked improvements in the sympathetic nervous system phenotypes, demonstrating the key contribution of RCAN1 trisomy to the disruptions, although reducing RCAN1 dosage did not fully restore neuronal numbers and innervation in Dp(16)1Yey/+ mice. Interestingly, although calcineurin activity was significantly improved by reducing RCAN1 to two copies in Dp(16)1Yey/+ mice, this rescue strategy did not fully restore calcineurin activity. Thus, in Dp(16)1Yey/+ mice, the decreased calcineurin activity may not simply be attributable to an increase in RCAN1 expression, but may also result from a complex imbalance of RCAN1 activity, perhaps mediated by interactions with other trisomic gene products. RCAN1 phosphorylation by dual specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1a), a trisomic gene product also known to regulate calcineurin signaling and implicated in Down syndrome phenotypes, has been reported to augment RCAN1’s inhibitory activity toward calcineurin (Jung, 2011). Therefore, increased gene dosage of Dyrk1a could act together with RCAN1 over-expression to suppress calcineurin activity and contribute to the observed sympathetic nervous system phenotypes in Dp(16)1Yey/+ mice. Our results, therefore, do not preclude the contribution of other trisomic genes to the disruptions in
sympathetic nervous system development in Dp(16)1Yey/+ mice, either by impinging on RCAN1 regulation as exemplified above for Dyrk1a, or via RCAN1-independent mechanisms. Nevertheless, based on our findings that RCAN1 gene triplication alone is sufficient to elicit substantial defects in neuronal survival and target innervation of NGF-dependent sympathetic neurons, and importantly, that these defects in Dp(16)1Yey/+ mice are significantly ameliorated by restoring RCAN1 to disomic levels, we conclude that an imbalance in RCAN1 gene dosage is a key contributing mechanism to the sympathetic phenotypes in the Down syndrome mice.

While several cellular processes could potentially contribute to the enhanced apoptosis and diminished innervation of sympathetic neurons in Dp(16)1Yey/+ mice, our findings provide evidence in support of excess RCAN1-mediated disruption of TrkA endocytosis as a key mechanism. Indeed, we found that ligand-dependent TrkA endocytosis was abolished in isolated Dp(16)1Yey/+ and RCAN1 over-expressing sympathetic neurons, whereas deleting a copy of RCAN1 in Dp(16)1Yey/+ sympathetic neurons normalized receptor internalization, which correlated with improved neuronal survival and target innervation. Additionally, the ability of axon-applied NGF to support retrograde signaling, neuronal survival and axon growth in compartmentalized sympathetic neurons was pronouncedly compromised by RCAN1 over-expression. Together, these findings provide evidence in support of a relationship between excess RCAN1, impaired TrkA endocytosis and decreased neurotrophic support of NGF-responsive sympathetic neurons in Dp(16)1Yey/+ mice.
Chapter 6: Calcineurin is required for basal forebrain cholinergic neuron development

A portion of the work in this chapter has been submitted for publication at Nature Neuroscience:

Ami Patel, Naoya Yamashita, Maria Ascaño, Daniel Bodmer, Erica Boehm, Chantal Bodkin-Clarke, Yun Kyoung Ryu, and Rejji Kuruvilla. RCAN1 links impaired neurotrophin trafficking to aberrant development of the sympathetic nervous system in Down syndrome.
INTRODUCTION

RCAN1 overexpression has previously been linked to disease, and specifically to Down syndrome neurodegeneration. Intriguingly, similar to Down syndrome individuals, RCAN1 mRNA levels are elevated 2-3-fold in the brains of Alzheimer’s disease patients (Ermak, 2001). Almost all individuals with Down syndrome exhibit early-onset neurodegeneration with pathological features similar to Alzheimer’s disease and many familial cases of Alzheimer’s are linked to human chromosome 21 genes, indicative of common pathogenetic mechanisms (Wisniewski, 1985; Mann, 1989; Schupf, 1998). These findings raise a question of whether RCAN1 accumulation could underlie decreased neurotrophic support and ultimate degeneration of NGF-responsive adult CNS neurons. Basal forebrain cholinergic neurons are a TrkA-expressing CNS population that undergo age-dependent atrophy in humans with Down’s syndrome and Alzheimer's disease (Whitehouse, 1981; Mann, 1985; Auld, 2002). Since mouse models of Down syndrome and Alzheimer’s disease exhibit decreased retrograde transport of NGF derived from hippocampal and cortical target regions of basal forebrain cholinergic neurons, disturbed trophic support has been suggested to underlie neuronal atrophy (Cooper, 2001; Salehi, 2003; Salehi, 2006). In contrast to the well-established trophic dependence of developing peripheral neurons on NGF:TrkA signaling and trafficking, genetic deletion of NGF or TrkA in mice does not compromise the survival of the majority of basal forebrain cholinergic neurons (Crowley, 1994; Smeyne, 1994; Fagan, 1997; Sanchez-Ortiz, 2012; Muller, 2012), but results in defects in cholinergic input to the cortex and hippocampus and the down-regulation of cholinergic neurotransmitter synthesizing enzymes (Smeyne, 1994; Fagan, 1997;
Sanchez-Ortiz, 2012). When tested in behavioral tasks, conditional mutant mice lacking NGF:TrkA signaling showed either no cognitive impairment (Muller, 2012) or mild cognitive decline (Sanchez-Ortiz, 2012). Unlike sympathetic neurons that rely on a single neurotrophin, NGF, for neuronal survival and target innervation during development, basal forebrain cholinergic neurons are responsive to multiple neurotrophins (Wu, 2004). Additionally, while the requirement of TrkA trafficking in promoting sympathetic neuron development is well-documented (Ascano, 2012; Harrington, 2013), there is less evidence to support a role for TrkA trafficking in basal forebrain cholinergic neuron development and maturation. Thus, further studies are warranted to clarify the relationship between TrkA signaling/trafficking and the trophic support of basal forebrain cholinergic neurons, and to define a possible contribution of excess RCAN1 to neurodegeneration in pathological situations.

As a first step to addressing this question, we first asked if calcineurin is required for NGF-TrkA dependent trophic functions in the basal forebrain cholinergic neurons. Preliminarily, this study found that mice that have either TrkA or calcineurin knocked out of cholinergic neurons have fewer cholinergic projections to the hippocampus. These mice also have atrophy of BFCN somas reminiscent of NGF and TrkA knock down experiments, supporting that a similar calcineurin-dependent mechanism is used for retrograde trafficking of NGF-TrkA in both sympathetic neurons and BFCNs. Both of these processes are likely disrupted in DS through RCAN1 overexpression.
RESULTS

The TrkA-expressing basal forebrain cholinergic neurons (BFCNs) play an important role in learning and memory. This function is mediated by projections from the BFCNs to the hippocampus, which secretes NGF. Though it has been accepted that NGF and TrkA are required for normal innervation, whether NGF signaling is required during development or only later for maintenance is still debated. To characterize the role of TrkA in the development of BFCNs, a conditional knockout mouse that lacks TrkA expression in neurons was generated. BFCN projections to the hippocampus were assessed using acetylcholine esterase (AChE) activity for histochemistry. At 6 weeks of age, TrkA conditional knockout mice have a reduction in cholinergic projections to the hippocampus (Figure 6.1a,b). Innervation is reduced in all regions including the CA1, CA3, and dentate gyrus, all of which receive innervation from BFCNs. NGF-TrkA retrograde trafficking in BFCNs is required for trophic function. Our work has shown that calcineurin is required for retrograde trafficking of NGF-TrkA in sympathetic neurons. As a first step to determine if calcineurin is also required for trafficking of NGF is BFCNs, the regulatory subunit of calcineurin CNB was specifically knocked out in cholinergic neurons using a floxed CNB mouse crossed to a choline-acetyl transferase (ChAT)-Cre mouse. Knocking out the regulatory subunit results in degradation of the catalytic subunit as well. Mice lacking calcineurin in BFCNs have decreased AChE activity in the hippocampus compared to control mice at 6 weeks (Fig. 6.1c,d). Previously, it has been suggested that decreased NGF-TrkA signaling reduces the cholinergic identity of BFCNs through downregulation of cholinergic markers. To determine if the reduced AChE activity in the hippocampi of CNB knockout mice is a result
of downregulation of cholinergic markers or overall degeneration of BFCN axons, cholinergic axons were genetically labeled with alkaline phosphatase (AP). Using enzyme histochemistry, BFCN innervation of the hippocampus was assessed in a cholinergic-independent manner. CNB knockout mice have reduced innervation from cholinergic neurons in the hippocampus when compared to control mice (Fig. 6.1e,f).

NGF-TrkA signaling in BFCN cell bodies has been shown to regulate gene expression, soma size, and neuronal survival. To determine if NGF-TrkA trophic functions are disrupted in CNB knockout mice, cholinergic neurons genetically labeled with AP were used to assess cell number. No change in BFCN cell number is observed in CNB conditional knockout mouse compared to controls at 6 weeks (Fig. 6.2a-c). Using the same marker, soma size was measured to determine if BFCNs undergo atrophy in the absence of CN. Mice with CNB conditionally deleted from BFCNs have a smaller soma size compared to control mice (Fig. 6.2a-b,d). This preliminary study indicates a role for CN in innervation and soma size of BFCNs, possibly through mediating NGF-dependent TrkA trafficking and trophic signaling.
Figure 6.1. Reduced hippocampal innervation in TrkA and calcineurin knockout animals

(a-d) Cholinergic innervation to the hippocampus is revealed using AChE histochemistry. (a,b) 6 week old mice that have TrkA knocked out of all neurons have reduced cholinergic labeling in the CA1, CA3 and dentate gyrus regions of the hippocampus. (c,d) Knockdown of calcineurin specifically in cholinergic neurons reduces AChE activity throughout the hippocampus. (e,f) Genetic labeling of BFCN axons reveals reduction of overall innervation to the hippocampus. Arrow: CA1, black arrowhead: CA3, white arrow: dentate gyrus. Scale bar: 150μm.
Figure 6.2. Mice lacking calcineurin have normal BFCN number with reduced cell size

(a-b) Immunohistochemistry revealing BFCN cell number and cell size in the medial septum of 6 week old mice. (c) No changes were seen in BFCN cell number in the medial septum (MS) and ventral diagonal band (VDB) when calcineurin is knocked out. (d) BFCNs in calcineurin knockout mice have reduced size compared to controls. N=2. Values are the mean ± SEM. Scale bar: 50 μm.
METHODS

Animals

All procedures relating to animal care and treatment conformed to institutional and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. Calcineurin knockout and ChAT-Cre mice were both purchased from Jackson Labs (Stock # 017692 and 006410 respectively). Conditional alkaline phosphatase expressing mice were a gift from the Hattar Lab. CNB\(^{+/+}\) or ChAT-Cre:AP:CNB\(^{+/+}\) were used as controls.

Reagents

The antibodies used in this study were previously validated for the following applications: p75 (Promega G3231).

AChE enzyme histochemistry

Mice were sacrificed with an overdose of avertin, perfused with PBS followed by 4% PFA. Brains were dissected out and incubated in 4% PFA overnight at 4°C, 30% sucrose overnight at 4°C and embedded. Serial coronal cryostat sections (40 µm) were collected in 0.1M phosphate buffer (pH 7.4), washed in 65 mM sodium maleate (pH 6.0) and incubated as floating sections for staining for 1 hr at room temperature in 0.05 mg/mL acetylthiocholine iodide, 0.1 mM tetra-isopropyl-pyrophosphatamide, 0.05 mM potassium ferricyanide, 0.3 mM CuSO4, 0.5 mM sodium citrate and 65 mM sodium maleate (pH 6.0). Following PB washes, sections were mounted on slides with Fluoromount (Thermo F4680).

Alkaline phosphatase enzyme histochemistry

Mice were sacrificed, perfused, fixed and embedded in OCT. 40µm sections were collected in 1XPB and incubated at 65°C for 30 min in PBS to inactivate other
phosphatases. They were subsequently incubated in the dark for overnight in NBT/BCIP tablets diluted in PBS (Roche 11697471001). After rinsing with 0.1% Tween-20 in PBS and post-fixing for 3 hours, Sections were dehydrated through an ethanol series. Sections were then cleared and mounted in benzyle benzoate:benzyl alcohol.

**Neuronal cell counts**

Neurons were counted using mice that had cholinergic neurons labeled with AP. Following AP staining protocol, septal and diagonal band cholinergic neurons were defined by using anatomical landmarks according to the Allen Brain Atlas. Four 40-µm thick coronal sections per mouse were taken through the complete MS/VDB with a 160-µm interval between each to avoid counting the same cells twice.

**Cell size**

Cell size was determined using AP-labeled BFCN mice. Four sections through the MS/VDB were collected and all labeled neurons within the defined region were traced using ImageJ. Cell size is presented as total area of cell in µm².
DISCUSSION

This work provides a framework for the hypothesis that calcineurin mediated NGF-TrkA trafficking is disrupted in the basal forebrain cholinergic neurons of DS patients as a result of RCAN1 overexpression. Preliminary analyses of TrkA and calcineurin knockout mice show similar defects in hippocampal innervation. Knockout of calcineurin shows soma atrophy similar to what has been reported in the literature. The role of NGF-TrkA signaling in BFCNs is still highly debated. Some studies indicate that the major function of NGF-TrkA is in maintenance, with reduced signaling resulting in degeneration of cholinergic projections in older animals. Other studies suggest a developmental role for NGF-TrkA signaling, where knockdown of TrkA in the basal forebrain results in reduced innervation during early stages of adulthood. Our data are consistent with the latter idea, implicating an important developmental role of TrkA signaling in establishing cholinergic circuitry. Our finding that knocking down calcineurin in these neurons leads to similar innervation defects suggests that calcineurin may be in the same pathway as NGF-TrkA. Similarly, the fate of BFCNs that lack trophic support has been debated. Some studies suggest that without NGF-TrkA signaling, BFCNs undergo cell death. However, others found that there is a downregulation of cholinergic markers and soma atrophy, with no associated cell death. Knocking down calcineurin in BFCNs leads to soma atrophy, not cell death, suggesting that calcineurin may indeed be regulating NGF-TrkA trafficking. However, the fate of these neurons after 6 weeks remains unknown. Though preliminary results suggest that calcineurin knockout has phenotypes similar to that of TrkA knockout, whether calcineurin directly regulates TrkA internalization is unclear.
The mechanism by which TrkA is trafficked in BFCNs has not been studied in detail. Though it is widely accepted that NGF trafficking is disrupted in diseases such as Alzheimer’s and Down syndrome, the molecular mechanisms underlying these defects have been minimally studied. Our findings in the sympathetic nervous system that calcineurin is required for TrkA internalization may provide insight into the BFCNs. To address this, further studies on how NGF-TrkA trafficking is disrupted in BFCNs by inhibition or knockdown of calcineurin are necessary. If calcineurin does play a role in trafficking, it would be of interest to determine the role of RCAN1. RCAN1 is highly expressed throughout the brain and excess RCAN1 has been associated with Alzheimer’s and Down syndrome. RCAN1 overexpression in Down syndrome BFCNs may contribute to this degeneration by impairing NGF-TrkA trafficking.
Chapter 7: Concluding Remarks
CONCLUDING REMARKS

This study is the first to implicate altered sympathetic nervous system development in autonomic dysfunction reported in Down syndrome. It also provides a molecular basis for these developmental defects: the disrupted trafficking of the neurotrophin NGF and its receptor TrkA. Through cell culture studies and genetic manipulation in mice, this study implicates the overexpression of a single gene, RCAN1, as the main contributor to this defect (Figure 7.1). Further, this study provides a basis for the role of calcineurin activity in NGF-TrkA trafficking in neuronal defects of the basal forebrain cholinergic neurons.

Sympathetic nervous system development and pathogenesis of Down syndrome

The finding that there are defects in the sympathetic nervous system in Down syndrome mice and human tissue samples provides a mechanism for peripheral abnormalities observed in DS individuals. The sympathetic nervous system acutely regulates tissue function to maintain homeostasis and allows an organism to respond quickly and fully to stressors. Many of these responses are disrupted in Down syndrome individuals. For example, DS children have dampened cardiovascular responses to stress and exercise, functions that are regulated by sympathetic innervation to the heart and blood vessels. Sympathetic signaling is also required for the innate immune response, regulating cytokine, natural killer cell, and macrophage activity. Down syndrome individuals are not able to properly respond to immune challenges, with infants 62 times more likely to contract pneumonia during their first year,
and an increased frequency in infection throughout their lifetime (NIH). Reduced sympathetic innervation to immune organs such as the thymus and spleen, similar to that reported in this study, may contribute to these immune deficiencies. Cardiac dysfunction and immune deficiencies are major causes of morbidity and mortality in DS individuals, and determining how sympathetic defects contribute to them would provide a platform for possible treatment options.

Sympathetic innervation may also contribute to DS pathogenesis by affecting the morphogenesis of target tissues during development. Recent studies point to peripheral innervation playing an important role in development of targets. For example, acetylcholine and VIP released from parasympathetic neurons are required for tubulogenesis and progenitor maintenance in salivary glands (Nedvetsky et al., 2014). A handful of studies have reported decreased salivation in Down syndrome children that may result in to poor oral hygiene and oral infections (Chaushu et al., 2002). Furthermore, mice that are genetically sympathectomized have aberrant development of islets in the pancreas, leaving them in a pre-diabetic state. The islet architecture defects can be rescued by administration of a β-adrenergic receptor agonist, again implicating the neurotransmitter norepinephrine as a developmental signal (Borden et al., 2013). Improper islet development, as a result of reduced innervation, may underlie the prevalence of diabetes in Down syndrome children. Together, these studies indicate a vital role of peripheral innervation and neurotransmitter release for normal tissue development that may be disrupted in Down syndrome. Understanding the role of sympathetic innervation in development of organs
such as the thymus and heart, both of which often have abnormal structures in Down syndrome individuals, will give us insight into the pathogenesis in disorders associated with DS.

Altered sympathetic innervation may also provide a basis to one of the most interesting disparities of Down syndrome individuals: the marked decrease in solid cancers with the prevalence of leukemia. Down syndrome individuals have reduced tumorogenesis and a cancer mortality rate that is <10\% than expected. One possible explanation is that reduced innervation provides protection against cancer progression. Recently, a study showed that autonomic axon growth into a prostate cancer cell mass enhances tumor growth. This affect is ameliorated by knockout of norepinephrine or acetylcholine receptors (Magnon et. al 2013). The reduction of innervation in DS individuals may therefore prevent tumorogenesis, providing protection against many cancers. Alternately, Down syndrome children are 10 times more likely to develop leukemia than individuals without DS (NIH). Impaired sympathetic innervation of the bone marrow may increase the risk of leukemia, as sympathetic signaling has been proposed to regulate the hematopoietic stem cell niche (Aguila 2006). Changes in the bone marrow microenvironment are suggested to be an important step in the progression of leukemia and other blood disorders (Hanoun et al, 2015). Further investigation of the interactions between sympathetic innervation and their targets would provide insight into the progression of cancer in Down syndrome individuals, as well as an overall understanding of the role of innervation in cancer.

In this study we propose the overexpression of the endogenous calcineurin regulator RCAN1 as the primary mechanism by which NGF-TrkA
trafficking is inhibited and sympathetic development is disrupted. However, in the Down syndrome mouse model with only two copies of RCAN1, neurotrophin trafficking is rescued, but sympathetic innervation and cell number is not returned to wild type levels. This suggests that there are potentially other mechanisms by which sympathetic development is disrupted in Down syndrome. Many studies have implicated DS genes such as DYRK1A, SIM2, and KCNJ6 in various aspects of brain development (Lana-Elola et al., 2011). Many of these genes have been shown to be expressed in sympathetic neurons in screens, including Olig2, DSCAM, and APP (biogps). DYRK1A has even been shown to interact with NGF signaling in PC12 cells (Kelly et al., 2005). Further studies would allow us to gain a better understanding of which of these genes is involved in sympathetic nervous system development and how its overexpression in Down syndrome may contribute to sympathetic defects.

**RCAN1 regulation of vesicle dynamics**

RCAN1 has been proposed to play an important role in vesicle dynamics through regulation of its primary target, the serine-threonine phosphatase calcineurin. Whether RCAN1 is required in calcineurin-mediated endocytic events other than NGF-TrkA internalization remains unclear. Calcineurin-mediated endocytosis is especially important in the development and maintenance of synapses. Calcineurin regulates clathrin-dependent synaptic vesicle recycling in the pre-synapse via dephosphorylation of dephosphins, and synaptic scaling in the post-synapse via maintenance of surface AMPA receptor number (Cousin et al., 2001, Kim et al., 2014). In the Down syndrome brain, developmental defects have been attributed to an imbalance of excitatory and
inhibitory synapses, which require proper vesicle recycling and scaling. Further studies are required to understand how RCAN1 overexpression may affect the excitatory-inhibitory synapse balance in the DS brain. Calcineurin-mediated endocytosis is also important in non-neuronal secretory cells such as chromaffin cells, and RCAN1 may play a role in endocytosis similar to that in synaptic vesicle recycling in neurons (Wu et. al, 2014). Interestingly, calcineurin and RCAN1 both play roles in exocytosis as well.

Calcineurin and, more recently, RCAN1 have been shown to be required for exocytosis. One important role for calcineurin-mediated exocytosis is in the maturation of sperm. In order for sperm to fertilize an egg, it must exocytose an organelle called an acrosome containing digestive enzymes required for breakdown of the zona pellucida (Bennett et. al, 2010). The overexpression of RCAN1, and the inability to form a proper acrosome may contribute to the infertility of Down syndrome males. Calcineurin and RCAN1 are also required for exocytosis of vesicles in chromaffin cells. Chromaffin cells release large amounts of catecholamines into the bloodstream in response to stress, a process that is dampened when RCAN1 is either knocked out or overexpressed. Alterations of fusion pore kinetics resulting in a reduction of catecholamine release per vesicle is also seen when RCAN1 is overexpressed. These effects are mediated by RCAN1 regulation of calcineurin activity (Keating et. al, 2007 and Zanin et. al 2013). Interestingly, DS patients have reduced catecholamine circulation, a defect that may be due to a combination of reduced sympathetic innervation and defects in chromaffin cell exocytosis. However, the role of calcineurin and RCAN1 in vesicle exocytosis in neurons remains to be seen. While the role of RCAN1 in vesicle dynamics has been studied mainly in the
context of disease, it would be of interest to examine the role of RCAN1 of endocytosis during normal development.

In this study, we show that the overexpression of RCAN1 via trisomy disrupts trafficking, but we do not have an understanding of the role of RCAN1 in TrkA trafficking during normal development. The precise coordination of environmental cues and intrinsic responses is required to form proper connections between sympathetic neurons and their targets. One of the most important target-derived cues is NGF, which requires the retrograde trafficking of its receptor TrkA to have full trophic function. This study finds that RCAN1 regulates trafficking, and that altered protein levels disrupts this process, leading to sympathetic defects. To better understand when during RCAN1 activity is required, a detailed developmental time course, isoform expression analysis, and determination of interacting partners in the context of sympathetic development is required. It is likely that NGF regulates RCAN1 expression and function, and vice versa. RCAN1 and NGF signaling intersect at many points, including NFAT-mediated transcription of RCAN1 (Yang et. al, 2000), GSK3β dependent phosphorylation of RCAN1, (Vega et. al, 2002) and possible interactions between RCAN1 and APP (Olah et. al, 2011). These two seemingly opposing signals, with NGF promoting endocytosis, and excess RCAN1 inhibiting it, maintain a homeostasis for the normal growth and development of the sympathetic nervous system. A better understanding of how these signals interact and what other players are involved in this process will give us a better understanding of sympathetic nervous system development and how it is disrupted in disease.
Neurotrophin trafficking in disease

Neurotrophin trafficking defects have been implicated in many diseases including Charcot-Marie-Tooth, ALS, Huntington’s, Alzheimer’s disease, and Down syndrome. However, the mechanisms that underlie these defects are often general defects and not specific to neurotrophins. Misregulation of proteins such as Rabs (Bucci et. al, 2014), microtubule-associated proteins and motors (Millecamps and Julien, 2013), and huntingtin (El-Daher et. al, 2015) cause disease by disrupting all axonal transport. In this study, we found a mechanism by which the trafficking of NGF-TrkA is specifically disrupted, leaving trafficking of other receptor tyrosine kinases intact. While RCAN1 may play a role in other endocytic events important for sympathetic development, this study gives insight into the specific mechanisms that underlies neurotrophin trafficking and how disruption contributes to disease. Whether this mechanism is required for trafficking of other neurotrophin or in other cell types, and how it may be impaired in disease remains unclear. One of the most likely roles, however, is in the trafficking of NGF-TrkA in basal forebrain cholinergic neurons.

In Alzheimer’s disease and Down syndrome, basal forebrain cholinergic neuron (BFCN) degeneration has been attributed to disrupted NGF-TrkA trafficking (Mufson et. al, 1995, Crowley et. al, 2001). Overexpression of APP has been suggested to contribute to by directly interacting with TrkA trafficking. However, restoration of APP in DS mice does not fully rescue NGF trafficking defects, suggesting that there are other mechanisms at play here (Salehi et. al, 2006). One possible mechanism is the overexpression of RCAN1, which may inhibit NGF-dependent TrkA endocytosis as this study found in sympathetic neurons. However, there are also intrinsic differences between NGF-TrkA
trophic affects BFCNs and sympathetic neurons. There may also be differences in trafficking machinery in these neurons. To begin to understand the role of calcineurin activity in TrkA trafficking I showed knocked calcineurin out of BFCNs. Innervation defects similar to those found in the TrkA knockout mouse were seen. In the future, the role of TrkA in BFCN development and maintenance, as well as the role of calcineurin on TrkA endocytosis would be an interesting avenue to pursue.
Figure 7.1 RCAN1 trisomy links impaired neurotrophin receptor trafficking to neurodevelopmental deficits in Down syndrome

Post-ganglionic sympathetic neurons project to diverse peripheral organs and tissues to regulate tissue homeostasis, in a manner dependent on the target-derived neurotrophic factor, NGF, during development. NGF promotes endocytosis of TrkA receptors in nerve terminals by activating calcineurin-mediated dephosphorylation of neuron-specific splicing isoforms of dynamin1 that harbor a calcineurin interaction PxIxIT motif. NGF:TrkA-containing signaling endosomes are then retrogradely transported back to neuronal cell bodies to initiate transcriptional programs necessary for neuronal survival and growth. In Down Syndrome, over-expression of RCAN1, an endogenous calcineurin inhibitor, interferes with NGF-mediated phosphoregulation of dynamin1, internalization of TrkA receptors and retrograde trophic signaling. The loss of target-derived neurotrophic support contributes to diminished sympathetic innervation of target tissues and enhanced apoptosis of NGF-responsive sympathetic neurons in Down syndrome.
Normal state
- TrkA endocytosis
- Retrograde signaling
- Target innervation
- Neuronal survival

Disease state
- Inhibition of endocytosis
- Decreased retrograde signaling
- Decreased innervation
- Apoptosis
REFERENCES


Muller, M. et al. Loss of NGF-TrkA signaling from the CNS is not sufficient to induce cognitive impairments in young adult or intermediate-aged mice.


Stefos, G. C., Soppa, U., Dierssen, M. & Becker, W. NGF upregulates the plasminogen activation inhibitor-1 in neurons via the calcineurin/NFAT


RESEARCH INTERESTS

Understand how neuronal circuits influence perception and behavior. Determining how systems integrate information for decision-making. Understanding how circuits develop the correct connections to create networks.

EDUCATION

Johns Hopkins University, Baltimore, MD
PhD, Biology Expected 08/2015

Syracuse University – Renee Crown Honors Program, Syracuse, NY
BS, Biochemistry and Psychology, Magna Cum Laude 05/2009
Capstone project: “Metal-Citrate Transport and Metabolism in Streptomyces coelicolor“

RESEARCH & PROFESSIONAL EXPERIENCE

PhD student in the lab of Dr. Rejji Kuruvilla
Johns Hopkins University, Baltimore, MD 06/2010 – Current

▪ Research experience: Developed study and collaborated with lab members to identify the role of RCAN1 in impaired neurotrophin trafficking in Down syndrome. Identified previously unstudied innervation defects in Down syndrome mouse model and human tissue. Initiated new techniques and protocols in the lab including culturing BFCNs, the use of microfluidic chambers, and human tissues analysis.

▪ Supervisory experience: Mentored 1 undergraduate and 5 graduate students, developing short-term projects that resulted in departmental presentations.

▪ Teaching experience: Led basic biology lab course consisting of 12 TAs and 230 students. Instructed and evaluated classes of 20-30 students on aspects of biology and experimental design. Conducted lectures on behalf of instructor.

Undergraduate Researcher in the lab of Dr. Robert Doyle
Syracuse University, Syracuse, NY 08/2006 – 12/2008

▪ Established the function of a novel metal transporter in Streptomyces coelicolor through structure and function analysis. Performed capstone project on specificity of metal-citrate complexes transported by Cit, a bacterial membrane transporter.

Laboratory Assistant
International Flavors & Fragrances, Union Beach, NJ 01/2009 – 08/2009
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- Organized fragrance trials; analyzed, interpreted and presented trial results for senior research scientists.

**Laboratory Intern**

- Analyzed metal samples on GDMS, evaluated results, and compiled data into reports for customers.

**PUBLICATIONS**

- **Patel, A., Yamashita, N., Ascaño, M., Bodmer, D., Boehm, E., Bodkin-Clarke, C., Ryu, Y.K., Kuruvilla, R.** Impaired neurotrophin-dependent trafficking and trophic functions by increased expression of RCAN1, a Down syndrome-associated protein. *In review at Nature Neuroscience.*


**PRESENTATIONS**


- **Patel, A., Ascaño, M., Bodmer, D., Greil, M., Kuruvilla, R.** (2011) Negative regulator of Calcineurin, RCAN1, blocks NGF-dependent retrograde signaling and neuronal survival. Johns Hopkins Department of Biology Colloquium, Baltimore, MD.

**TECHNICAL SKILLS**

- *Molecular genetics/Biochemistry:* RNA/DNA/Protein isolation; generation of plasmids and riboprobes; gel electrophoresis; immunoprecipitation; western blot; ELISA; enzyme activity assays

- *Tissue culture:* mammalian cell lines; rodent primary culture of PNS and CNS neurons; generation of adenovirus and lentivirus; use of compartmentalized culture system

- *Microscopy:* Fixed and live cell imaging on confocal microscopy

- *Animal work:* handling and maintaining mouse colony; stereotaxic injection into hippocampus; intracameral injection of anterior chamber

**VOLUNTEER & LEADERSHIP EXPERIENCE**
AMI PATEL
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Mentor, Thread 06/2012 – Present

- Guiding an inner city Baltimore high school student to meet personal and educational goals individually and as part of a team of 4-8 mentors.

Resident Advisor, Syracuse University 08/2006 – 05/2008

- Facilitated the establishment of a residential community with 30-35 residents through monthly floor meetings and social activities.
- Along with 10 colleagues, developed and organized hall-wide educational and social programs to create a hall community.

Tutor, Literacy Corps 09/2005 – 05/2006

- Assisted in teaching classrooms of 20-30 middle school students as group and individually.
- Developed and oversaw educational and recreational afterschool activities for 15-20 students aged 7-13.