RCAN1 in Mitochondrial Dysfunction and Down Syndrome

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RCAN1 IN MITOCHONDRIAL DYSFUNCTION AND DOWN SYNDROME

by

CAROLYN ANNE ARDIZZONE

B.A., University of California, Berkeley, 2013

A thesis submitted to the

Faculty of the Graduate School of the

University of Colorado in partial fulfillment

of the requirement for the degree of

Master of Science

Department of Integrative Physiology

2016
This thesis entitled:
RCAN1 in Mitochondrial Dysfunction and Down Syndrome
written by Carolyn Anne Ardizzone
has been approved for the Department of Integrative Physiology

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

IACUC protocol #1311.02
ABSTRACT

Ardizzone, Carolyn Anne (M.S., Integrative Physiology)

RCAN1 in Mitochondrial Dysfunction and Down Syndrome

Thesis directed by Assistant Professor Charles A. Hoeffer

The near-universal early age appearance of Alzheimer’s disease (AD)-related neuropathology in Down syndrome (DS) individuals suggests that factors related to the overexpression of genes on chromosome 21 may be related to the manifestation of AD in the population at large. The product of the gene *Regulator of calcineurin 1 (RCAN1)*, found on chromosome 21 is increased in the brains of DS individuals and is also found elevated in sporadic (non-DS) AD patients. Therefore, the goal of this proposal is to test the hypothesis that *RCAN1* overexpression promotes the development of AD-related neuropathology in DS. First, we examined activity of *RCAN1*’s main substrate, calcineurin (CaN), in DS model (Dp16(1)Yey/+ [Dp16]) neurons in culture using a novel FRET reporter for CaN activity. We found evidence for elevated CaN activity in DS neurons. Second, using DS cell culture models we explored the notion that RCAN1 is important in the manifestation of AD-related phenotypes. Specifically, we explored RCAN1-mediated mitochondrial dysfunction as a potential mechanism through which *RCAN1* overexpression leads to the development of AD-related pathology. We found that overexpression of *Rcan1* in DS mouse model cell culture promoted abnormal mitochondrial fission leading to reduced mitochondrial size and elevated ROS. Third, we addressed the important question of whether *Rcan1* knockdown in DS cell lines eliminates DS-linked AD neuropathology and mitochondrial dysfunction. We found that restoration of RCAN1 levels in *Dp16* neuronal culture rescued mitochondrial abnormalities found in *Dp16* (*Rcan1*...
trisomic) cells. Finally, using $Rcan1$ KO cell lines we also examined the idea that particular RCAN1 isoforms were specifically involved in mitochondrial dysfunction. Completion of this project will validate the generation of new tools, including brain specific $Rcan1$ expression mouse models, and may be of great value to the both the DS and AD research communities.
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CHAPTER I
INTRODUCTION

Down syndrome and Alzheimer’s disease

Down Syndrome (DS) is a congenital disorder characterized by low muscle tone, cognitive delays, slightly flattened facial features, and often a variety of other traits. The syndrome is caused by the partial or total trisomy of chromosome 21. The extra copy of chromosome 21 is derived in three different ways: nondisjunction results in an extra copy of the chromosome in every cell of the body and comprises 95% of all DS cases; mosaicism results in an extra chromosome in just a portion of the individual’s cells and occurs in only 1% of the DS population; translocation occurs in 4% of DS individuals when part or all of chromosome 21 attaches to another chromosome, usually chromosome 14, so the individual still has a total of 46 chromosomes (Korenberg, Chen et al. 1994). Maternal age at birth is the biggest risk factor for conceiving a child with DS, and since many people today are choosing to have children at older ages, the incidence of DS is expected to rise.

Recent advances in medical care have also increased the life expectancy of these individuals. However, by the age of 50, the majority of DS individuals exhibit symptoms consistent with early-onset Alzheimer’s disease (AD). AD is a devastating neurodegenerative disease affecting many people in the world today. It is projected that in the year 2050, over 88 million people in the United States will be over 65 years old, compared to less than 50 million today (Mattson 2004). Since the biggest risk factor for AD onset is age (Hebert, Weuve et al. 2013), the incidence of AD and DS-AD will increase, and with it, the importance of future treatments for this disorder.
AD is well known to be associated with abnormal synaptic plasticity and progressively severe cognitive deficits such as memory loss. It is characterized by amyloid-beta (Aβ) plaques, neurofibrillary tangles (NFTs), and gradual loss of neurons and synapses (Maslow 2010). It is generally accepted that the associated problems arise from deficits in synaptic function (Selkoe 2001, Neary, Snowden et al. 2005, Lemere and Masliah 2010) that can arise through numerous proposed cellular mechanisms. The relationship between DS and AD might help elucidate pathways that are critical in causing neurodegeneration since some factors causing DS might be the same factors causing AD. Because the trisomy of chromosome 21 leads to the overexpression of genes in that region and one gene also located on chromosome 21, the amyloid precursor protein (APP), is an obvious candidate for driving dysfunction stemming from the Aβ hypothesis. Indeed, synaptic plasticity impairments are observed both in APP model mice and in neuronal cultures following exposure to Aβ oligomers, derivatives of APP cleavage by enzymatic processes. Furthermore, neuritic Aβ plaques are found in essentially all DS adults and even some DS children (Vega, Rothermel et al. 2003). However, the fact that all Aβ-directed clearance therapies have failed to halt the progression of dementia (Oddo, Caccamo et al. 2003) casts some doubt on APP overexpression as a major causal mechanism for AD. Additionally, long-term potentiation (LTP) deficits and other cognitive impairments occur before significant Aβ plaque deposition, suggesting that Aβ is not the main driver of dysfunction in AD (Olson and Shaw 1969, Jacobsen, Wu et al. 2006, Ma, Hoeffner et al. 2010). Thus, other genes located on chromosome 21 may play critical roles in the expression of AD in both DS individuals and the general (sporadic) AD population.
Regulator of Calcineurin 1

An alternative to the overexpression of APP is overexpression of the gene *Regulator of calcineurin 1 (RCAN1)*, which has also been shown to play a role in AD pathogenesis. RCAN1 has been found overexpressed in brains of DS individuals as well as in those of sporadic AD patients (Sun, Wu et al. 2011, Wu and Song 2013, Wong, Levenga et al. 2015). The gene *RCAN1* encodes seven exons that can be differentially spliced to produce mRNA for the translation of at least three different protein isoforms, RCAN1.1L, RCAN1.1S and RCAN1.4. The isoforms differ by their promoters or the length of transcript (Figure 1A). The glucocorticoid-responsive promoter preceding exon 1 is responsible for the transcription of both RCAN1.1L and RCAN1.1S mRNA, while RCAN1.4 mRNA is transcribed from a nuclear factor of activated T-cells (NFAT) – responsive promoter preceding exon 4. The functions of these distinct isoforms have not yet been comprehensively explored, although RCAN1.1L has been the most intensively studied. RCAN1.1L has been shown to initiate mitophagy, a process that targets dysfunctional mitochondria for degradation (Sun, Hao et al. 2014), and even more recent research suggests that RCAN1.1S plays a role mitochondrial dysfunction as well. The overexpression of RCAN1.1S in transgenic mice produces age-dependent mitochondrial abnormalities (Wong, Levenga et al. 2015). These mice also exhibited age-specific hippocampus-dependent memory deficits and pathological tau protein, which present similarly to the cognitive decline and histopathology observed in AD patients during middle age. The mitochondria in these mice are irregular in that there are increased numbers of small mitochondria compared to wild-type (WT) controls. This finding could account for the increased levels of reactive oxygen species (ROS) found in AD (Maccioni, Munoz et al. 2001), DS (Busciglio and Yankner 1995), and normal aging (Bishop, Lu et al. 2010) in which RCAN1 levels are also increased. Indeed, the aged transgenic mice
Figure 0: Expression of Rcan1 (A) Schematic adapted from Harris et al. 2007, representing the RCAN1 gene, which consists of seven exons that can be differentially spliced to produce specific isoforms. Two of the three identified isoforms are transcribed from a glucocorticoid-responsive promoter preceding exon 1 – RCAN1.1L (long) and RCAN1.1S (short). RCAN1.4 is transcribed beginning at an NFAT-responsive promoter preceding exon 4. (B) Western blot showing a clear increase in RCAN1 in Dp16 mice. The removal of one copy of Rcan1 as in Dp16/Rcan1 heterozygous mice restores RCAN1 to almost wild-type levels. This is preliminary data and needs to be repeated in order to quantify RCAN1 levels and ensure significance. (C&D) Immunostaining of dentate gyrus (C) and CA1 region (D) of the hippocampus in WT, Dp16, and Rcan1 KO mice showing increased levels of RCAN1 in the Dp16 brain and only background fluorescence in Rcan1 KO mice, indicating antibody specificity.
showed increased levels of ROS compared to age-matched WT controls (Wong, Levenga et al. 2015). These results suggest that \textit{Rcan1.1S} overexpression promotes dysregulated mitochondrial fission, as abnormal numbers of small mitochondria appear in middle age. One mechanism by which this may occur is through altered activity of mitochondrial fission control protein dynamin-related protein-1 (DRP1). DRP1 localization is altered in neurons overexpressing \textit{Rcan1.1S}, suggesting a connection between RCAN1 levels and DRP1 localization, although they have not yet been shown to directly interact (Wong, Levenga et al. 2015).

A pathway through which RCAN1 might indirectly regulate DRP1 activity is through calcineurin (CaN), one of RCAN1’s main targets. CaN is a calcium- and calmodulin (CaM)-dependent serine/threonine phosphatase (Figure 2B) comprising 1\% of all protein in the brain (Harris, 2007), and which has many substrates in the brain. CaN regulates many cellular processes, and its dysregulation is implicated in many human diseases. It is comprised of two subunits, a catalytic calcineurin A (CaNA) subunit and a regulatory calcineurin B (CaNB) subunit. CaNA contains a N-terminal catalytic domain typical of phosphoprotein phosphatases and a C-terminal CaNB-binding domain, CaM-binding domain, and a site of autoinhibition. During an influx of calcium, such as in response to membrane depolarization, calcium binds to both CaNA and CaNB, and CaM binds to CaNB, causing conformational changes that release autoinhibition. This allows CaN to exert its phosphatase activity on a variety of proteins.

DRP1 is a CaN substrate and, therefore, its activity is likely to be affected by RCAN1 levels (Cribbs, 2007; Cereghetti, 2008). Thus, CaN might provide the missing link between RCAN1 and altered levels of mitochondrial-specific ROS through DRP1 activity (proposed interaction in Figure 2A). The main goal of this project was to characterize the influence of
RCAN1 on CaN activity in DS model mice to help elucidate the mechanism by which mitochondrial dysfunction occurs in DS and AD.
Figure 1: Role of calcineurin (CaN) in ROS production (A) Proposed mechanisms of RCAN1 regulation in neurodegeneration. Upregulation of RCAN1 causes increased CaN activity resulting in excessive mitochondrial fission via DRP1 leading to increased reactive oxygen species (ROS) (B) Ribbon diagram of CaN structure interacting with FK506, a specific CaN inhibitor. The two subunits calcineurin A (CaNA) and calcineurin B (CaNB) are shown in red and purple, respectively, while FK506 is shown in yellow.
CHAPTER II
MATERIALS AND METHODS

Mouse models

In order to investigate the role of RCAN1 in calcineurin phosphorylation and mitochondrial dysfunction, we used several mouse models with varying numbers of Rcan1 alleles. B6D2F-1/J mice served as our wild type controls with two Rcan1 alleles, while Rcan1\(^{(-/-)}\) (Rcan1 KO), Rcan1\(^{(+/-)}\) (Rcan1 Het) and Dp(16)1Yey/+ (Dp16) (Li, Yu et al. 2007) mice served as experimental mice (Hoeffer, 2007). Since the mouse’s chromosome 16 is orthologous to human chromosome 21 (Hsa21), these mice have been genetically manipulated at chromosome 16 (Mmu16) to simulate DS chromosomal abnormalities. The Dp16 mouse, a DS model mouse, has an extended chromosome 16 containing several genes that are triplicated in human trisomy 21. The Dp16 model was chosen over other Down syndrome mouse models for several reasons. The most extensively studied model mouse, the Ts65Dn, contains a partial trisomy with many, but not all, of the homologous Hsa21 genes, but it also contains triplicated genes that are not found on the human chromosome. Therefore, it has high face validity, but mediocre construct validity (Rueda, Florez et al. 2012). The Dp(16)1Yey/+ mouse created by Li et al. contains 110 orthologous genes, which covers the whole Hsa syntenic region on Mmu16 without the addition of genes not triplicated in Hsa21 in Down syndrome. This mouse exhibits cognitive, cardiovascular and gastrointestinal phenotypes resembling those observed in humans with DS (Li, Yu et al. 2007, Yu, Li et al. 2010, Das and Reeves 2011). The Rcan1 KO mice have both alleles for the Rcan1 knocked out on Mmu16 and this mouse model shows pronounced spatial learning and memory deficits (Hoeffer, Dey et al. 2007). The Rcan1 Het mice have one allele
coding for \textit{Rcan1}, so \textit{Rcan1} levels are between levels of WT and \textit{Dp16} (Figure 1B). The \textit{Rcan1} KO mice have both alleles for the \textit{Rcan1} knocked out in Mmu16, but are otherwise normal. They show pronounced spatial learning and memory deficits (Hoeffer, Dey et al. 2007). The \textit{Rcan1} Het mice have one allele coding for \textit{Rcan1}, so RCAN1 levels fall in between levels of WT and \textit{Dp16} RCAN1 (Figure 1B).

The \textit{Dp16} mouse, a DS model mouse, has an extended chromosome 16 containing several genes that are triplicated in human trisomy 21. The \textit{Dp16} model was chosen over other Down syndrome mouse models for several reasons. The most extensively studied model mouse, the Ts65Dn, contains a partial trisomy with many, but not all, of the homologous Hsa21 genes, but it also contains triplicated genes that are not found on the human chromosome. Therefore, it has high face validity, but mediocre construct validity (Rueda, Florez et al. 2012). The Dp(16)1Yey/+ mouse created by Li et al. contains 110 orthologous genes, which covers the whole Hsa syntenic region on Mmu16 without the addition of genes not triplicated in Hsa21 in Down syndrome. This mouse exhibits cognitive, cardiovascular and gastrointestinal phenotypes resembling those observed in humans with DS (Li, Yu et al. 2007, Yu, Li et al. 2010, Das and Reeves 2011), and is the mouse we used in our experiments.

We performed several combinations of crosses between \textit{Dp16} and \textit{Rcan1} KO, \textit{Rcan1} Het, or WT mice to achieve some combination of four genotypes (Figure 3). When a \textit{Dp16} mouse was bred with an \textit{Rcan1} KO, the resulting progeny would be either \textit{Rcan1} Het or \textit{Dp16/Rcan1} Het with two \textit{Rcan1} copies. When a \textit{Dp16} mouse was bred with an \textit{Rcan1} Het the resulting progeny would be one of four genotypes: WT with two copies of \textit{Rcan1}, \textit{Dp16} with three copies of \textit{Rcan1}, or either of the two previously described genotypes. With varying numbers of copies
of *Rcan1* in conjunction with the extended chromosome 16, we are able to observe whether or not RCAN1 levels alone can modulate mitochondrial dysfunction and/or CaN activity.
Figure 2: Use of Dp16(1)Yey/+ (Dp16) and Rcan1 heterozygous (Rcan1 Het) mice to produce genetic variability. Although WT and Dp16/Rcan1 Het mice both have two copies of Rcan1, the Dp16/Rcan1 Het mouse contains additional genes triplicated on chromosome 16, which distinguishes it from the wild-type (WT). The sole manipulation of the Rcan1 Het mouse is the deletion of a copy of Rcan1, while the Dp16 mouse has many triplicated genes including Rcan1.
Animals

All procedures relating to animal care and treatment conformed to Institutional Animal Care and Use Committee and Office of Animal Resources guidelines. *Rcan1* KO mice were generated as previously described (Vega, Rothermel et al. 2003). *Dp16* mice were purchased from The Jackson Laboratory. These mice were generated as described in (Li, Yu et al. 2007). Mice were maintained on a 12:12 hour light:dark schedule with food and water available *ad libitum*. Both males and females were used in these experiments.

Tissue isolation

Pregnant dams carrying embryos between E16 and E18 were sacrificed via cervical dislocation and embryos removed. At this time, individual tails were taken for genotyping and hippocampi were isolated and stored individually on ice until PCR results were complete. Hippocampi were then combined according to genotype. Hippocampi were washed 2x with 10mL 1X Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Waltham, MA) in a laminar flow hood. DMEM was aspirated and 1mL of 1X Trypsin (Gibco, Waltham, MA) was added to each tube and placed in a 37°C water bath for 15 minutes to break up protein adhesions between cells. Trypsin was then aspirated and cells were washed with 5mL DMEM, with 2mL DMEM then added to each tube. Cells were dissociated with fire-polished Pasteur pipettes of two different diameters. After that, 3mL DMEM was added to each tube to bring total volume to 5mL/tube and cells were centrifuged at 1000 rpm for 5 min. Supernatant was then aspirated and 1mL of pre-warmed Neurobasal Medium+++ ((NBM+++)) Neurobasal Medium (Gibco, Waltham, MA), 2% B27 (Gibco, Waltham, MA), 1% penicillin streptomycin (Thermo Fisher Scientific), and 1% GlutaMAX (Gibco, Waltham, MA)) was added to each tube. Cells were counted using a
hematocytometer, and 25,000 cells were plated per coverslip in a 24-well plate. The glass coverslips were treated with 100µg/mL poly-D-lysine (Sigma-Aldrich, St. Louis, MO) in 0.1M borate buffer overnight hidden from light, and then washed 2x with autoclaved water and left to dry. Next, 50µg/mL laminin (Sigma-Aldrich, St. Louis, MO) was added to the dry coverslips for at least 30 minutes at 37°C and then aspirated and replaced with 40uL DMEM and stored at 37°C until use. At 21 days in vitro (DIV) the cells were fixed with 4% paraformaldehyde (PFA) and washed with 1X phosphate-buffered saline (PBS).

**PCR for genotype determination**

*Rcan1* KO and Dp16(1)Yey/+ mouse genotyping was performed by PCR using mutant and wild-type-specific primer sets (Vega, Rothermel et al. 2003, Yu, Li et al. 2010). PCR products were visualized by 1% agarose gel electrophoresis.

**Fluorescence resonance energy transfer (FRET)**

Fluorescence resonance energy transfer (FRET) works by means of a distance-dependent transfer of energy between two fluorescent proteins flanking a molecular switch or incorporated into two separate proteins. When a donor fluorophore protein is excited, it can transfer energy in a non-radiative manner to an acceptor fluorophore if the acceptor is close enough, and that acceptor can then emit energy at a particular wavelength, which we can detect. If the acceptor fluorophore is not close enough to the donor fluorophore, the donor emits the energy at its own wavelength without transfer. The excitation and emission spectra of the two fluorophores must be taken into consideration in order to ensure efficient energy transfer. Cyan fluorescent protein
Figure 4: Fluorescence resonance energy transfer (FRET) (A) Representation of the CaN activity reporter (CaNAR) as adapted from Mehta et al. 2014 (Mehta and Zhang 2014). Two fluorescent proteins whose interactions allow detection of nuclear factor of activated T-cells (NFAT) phosphorylation flank 297 N-terminal amino acids of NFAT. Increased dephosphorylation of CaNAR by CaN shifts excitation-induced photon emission from CFP emission to YFP emission. FRET ratio = (YFP emission/CFP emission), so an increased FRET ratio indicates higher CaN activity (B) Representative images of CaNAR visualization in neuronal cell culture. NeuN marks neurons while CFP and YFP detect different states of NFAT phosphorylation. (C) Preliminary evidence of CaNAR as a functional reporter. Specific inhibition of CaN by FK506 led to a small decrease in FRET ratio while CaN activation by ionomycin increased the FRET ratio. (D) FRET ratios from WT, Dp16, Dp16/Rcan1 Het, and Rcan1 Het mice. Trends are as follows: Dp16 mice showed increased FRET ratios while removal of one Rcan1 allele in Dp16/Rcan1 Het mice rescued the phenotype. Further removal of Rcan1 in Rcan1 Het mice led to increased FRET ratios similar to those of Dp16 mice.
(CFP) and yellow fluorescent protein (YFP) are often used together since there is spectral overlap between CFP emission and YFP excitation, and minimal spectral overlap between the excitation of the two. That ensures that only CFP is being excited when targeted with a 440nm laser and that the two fluorophores’ emissions can be distinguished. CFP absorbs at a wavelength of 440nm and emits at 477nm while YFP absorbs at 514nm and emits at 530nm.

To examine the dephosphorylation activity of calcineurin as a potential mechanism of RCAN1-regulated dysfunction, we used a CaN activity reporter (CaNAR) in neuronal cell culture (Figure 4A). This tool is a genetically-encoded fluorescent protein biosensor that allows quantification of the phosphorylation states of NFAT. NFAT is one of CaN’s main target and is a transcription factor involved in immune responses and ubiquitously co-expressed along with calcineurin in humans. The CaNAR sequence consists of 297 N-terminal amino acids in between a CFP and YFP. This N-terminal sequence serves as a regulatory domain that is constitutively phosphorylated by cytoplasmic kinases at several serine-rich sites. This reporter works by utilizing a molecular switch – an NFAT conformational change in response to dephosphorylation by CaN – that changes the relative positioning of CFP and YFP intramolecularly. This difference in conformation changes the energy-transfer dynamics of CaNAR in response to CFP excitation. This means that when the NFAT-regulatory site is phosphorylated, excitation at 440nm results in CFP emission, while when the NFAT-regulatory site is dephosphorylated by CaN, excitation at 440nm results in a CFP-to-YFP energy transfer and YFP emission. Detection of the YFP to CFP emission ratio allows for quantification of the relative NFAT phosphorylation states, and thus, relative CaN activity in different conditions or geneotypes (Mehta and Zhang 2014).
**CaNAR infection**

At DIV 12, neuronal cultures were treated with an adeno-associated virus (AAV8) as follows: 30 minutes before virus was added to the wells, conditioned media was removed from each well and stored at 37°C leaving only 250 µl for cells to habituate to the low media volume. After 30 minutes in the incubator, enough AAV8 CANAR virus in fresh NBM+++ for a final concentration of $10^4$ GC/cell was added to each well and left at 37°C for 1.5 hours. At that time, the warm conditioned media was added back into the wells.

**CaNAR efficacy controls**

*Ionomycin*: At DIV 20 media was removed from wells and replaced with Hank’s Balanced Salt Solution with HEPES (HBSS + HEPES) for 2 minutes prior to a 1 µM ionomycin treatment with 10mM CaCl$_2$ for either 5 or 10 minutes, or a 5 µM ionomycin treatment without CaCl$_2$ for 10 minutes at 37°C prior to fixing. *FK506*: Cells were treated with 5µM FK506 (tacrolimus) in NBM+++ for 12 hours prior to fixing.

**RCAN isoform transfection**

To observe effects of specific RCAN1 isoforms on CaN activity, *Rcan1* KO neurons were transfected with *RCAN1.IL*, *Rcan1.1S*, *RCAN1.4*, or an empty red fluorescent protein-labeled plasmid. Plasmids at a concentration of 1µg/well were introduced using Lipofectamine 2000 (Thermo Fisher Scientific) at DIV 19. A cell transfected with a particular isoform then expressed no other RCAN1 isoforms besides that which was transfected. Each plasmid was tagged with either hemagglutinin (HA) or c-Myc for subsequent fluorescent immunocytochemical identification after fixation at DIV 21.
CellLight treatment

To compare mitochondrial sizes between cells of different genotypic origin, we treated cells with a fluorescent protein that tags the first subunit (E1) of pyruvate dehydrogenase complex (PDH). PDH is a heterotetrameric enzyme complex found in the mitochondrial matrix that catalyzes pyruvate, the end product of glycolysis, into acetyl-CoA that is then used in the citric acid cycle. As such, tagging this protein that is prevalent in mitochondria with fluorescent protein allows for mitochondrial localization and approximate size-detection. Cells were treated with CellLight-Mitochondria-GFP fusion construct (ThermoFisher, Waltham, MA) in NBM++ at 30 particles per cell for 16-24 hours before the cells were fixed.

MitoSox treatment

In order to compare levels of mitochondria-specific reactive oxygen species (ROS), we used MitoSox Red mitochondrial superoxide indicator (ThermoFisher, Waltham, MA). When added to cell culture this reagent is readily targeted to mitochondria, a major site of ROS production. The fluorogenic dye interacts with superoxide, a byproduct of oxidative phosphorylation, to produce red fluorescence. MitoSox is specific to mitochondrial ROS and will not interact with reactive nitrogen species (Thermofisher, Waltham, MA). Cells were incubated with 5µM MitoSOX at DIV 21 for 10 minutes at 37°C in the dark prior to fixing.

Western blotting

Cortical tissue from Dp16 and WT mice were prepared as whole lysates for western blotting. Soluble protein extracts were prepared by homogenizing the tissue samples in ice-cold buffer [in mM: 50 Tris-HCl, pH 7.5, 150 KCl, 1 DTT, 1 EDTA, 1% complete protease inhibitor
cocktail III (Sigma-Aldrich, St. Louis, MO), 1% phosphatase inhibitor cocktail I (Sigma-Aldrich); 1% phosphatase inhibitor cocktail II (Sigma-Aldrich). Proteins were resolved on SDS-polyacrylamide gels, and immunoblotting was performed using standard techniques. A rabbit polyclonal antiserum raised against RCAN1 was diluted 1:1000 in 1% 0.2% I-Block (Tropix, Foster City, CA). All blots were developed using enhanced chemiluminescence detection (GE Healthcare, Fairfield, CT).

Confocal microscopy

Immunocytochemistry of embryonic hippocampal cell culture was used in conjunction with CaNAR, CellLight, and MitoSox treatments. Regardless of treatment, all cells were washed in 1X PBS for 3× 10 min. To block and permeabilize, we used “blocking buffer” containing “staining buffer” (0.05 M Tris, 0.9% NaCl, 0.25% gelatin, and 0.5% Triton X-100, pH 7.4) plus 2% donkey serum (Millipore, Billerica, MA). Coverslips containing CaNAR-treated cells were incubated overnight at 4°C with mouse anti-NeuN primary antibody (1:300; Novus Biologicals) diluted in blocking buffer. The next day, the cells were washed in PBS and incubated with donkey anti-mouse Cy3 antibody (1:200; Jackson Immunoresearch) diluted in staining buffer in addition to Hoechst (1:2000 ….) for one hour at room temperature. Finally, the cells were washed in 1X PBS and mounted in Mowiol mounting solution (Mowiol 4–88). Cells treated with CellLight or MitoSox were washed in 1X PBS and incubated in staining buffer with Hoechst for 5 minutes and then mounted in Mowiol. FRET ratios were determined by emission wavelength detection after CFP excitation. MitoSox fluorescence data was collected as pixel intensity with the same laser power and excitation time used for each image. Laser power used to visualize CellLight staining was determined on a cell-by-cell basis, as transduction varied greatly from
cell-to-cell and between cultures. This did not alter results as pixel intensity was not measured in the mitochondrial size analysis.

Data analysis

All data are presented as mean values ± standard error of the mean (SEM). Data were statistically evaluated using R software. A student’s t test or one-way ANOVA was applied as appropriate. All statistical tests were two-tailed with $\alpha = 0.05$. 
CHAPTER III
RESULTS

*Rcan1* overexpression in *Dp16* mice

We first measured *Rcan1* expression in *Dp16* mice to ensure that RCAN1 is more abundant in *Dp16* mice than WT mice. Western blot analysis confirmed higher levels of RCAN1 in the cortex of *Dp16* mice as compared to WT littermates (Figure 1B). Moreover, RCAN1 levels are decreased following the removal of one copy of *Rcan1* in *Dp16/Rcan1* Het mice. Although we only tested a few animals, the assay suggests that RCAN1 levels depend on the number of *Rcan1* alleles present in a mouse, but we plan to confirm these results with additional testing.

CaNAR as a functional reporter

In order to confirm the efficacy of the CaN activity reporter (CaNAR) several control conditions were tested. FK506 (tacrolimus) acts to inhibit CaN activity by binding directly to CaN, and more specifically, to the CaNB-binding site of CaNA. Binding of FK506 inhibits the ability of CaNB to release autoinhibition and, thus, the ability of CaN to function (Victor, Thomas et al. 1995). The addition of this inhibitory drug should block CaN directly, which should cause a decrease in the CaNAR FRET ratio if the reporter is functional and enough FK506 is used. Ionomycin is an antibiotic isolated from *Streptomyces conglobatus* that acts to increase intracellular calcium (Liu and Hermann 1978). It both mobilizes intracellular Ca$^{2+}$ stores and acts as an ionophore, bypassing ion channels and moving Ca$^{2+}$ from extracellular fluid into the cell across the plasma membrane (Morgan and Jacob 1994). Since CaN is Ca$^{2+}$-
dependent, a large influx of Ca\(^{2+}\) should induce higher CaN activity through release of autoinhibition on CaNA, which should result in a higher FRET ratio if the CaNAR is functional. As indicated by preliminary data, neurons treated with ionomycin showed increased CaNAR activity as increased FRET ratios compared to the vehicle conditions (Figure 4C), while FK506 treatment seemed to decrease CaNAR activity. Although this graph represents data from only one culture, the trends indicate that the CaNAR is fully functional.

**Higher CaN activity in Dp16 mice**

To test whether CaN activity differs between Dp16, Dp16/Rcan1 Het, Rcan1 Het and WT mice, neurons in culture were infected with CaNAR. The results show that NFAT, the regulatory unit of CaNAR, has a higher ratio of dephosphorylation to phosphorylation ([YFP emission/CFP emission] aka FRET ratio) in Dp16 mice as compared to WT (Figure 4D). This suggests that expression of the triplicated genes on Mmu16 in Dp16 mice causes an increase in CaN activity in neurons in cell culture. Deletion of a copy of Rcan1, as in the Dp16/Rcan1 Het mice, rescues this increase in CaN activity. This result strongly suggests that Rcan1 is responsible for the increased CaN phosphatase activity in Dp16 mice. The other genes besides Rcan1 that are triplicated in Dp16 mice on Mmu16 are still present in Dp16/Rcan1 Het mice, so they likely are not responsible for the rescue. Interestingly, though, Rcan1 Het cells show the same increased CaN activity as the Dp16 cells, which suggests that altering RCAN1 levels in neurons causes some level of system dysregulation.
Mitochondrial dysfunction in *Dp16* mice

Mitochondrial fission is a crucial cellular process that depends on DRP1 activity (Cribbs and Strack 2007, Cereghetti, Stangherlin et al. 2008). Since DRP1 is a substrate of CaN, aberrant CaN activity could result in mitochondrial fission dysregulation. CaN dephosphorylates DRP1 at serine 637, causing cytosolic DRP1 to translocate to mitochondria where it initiates fission (Wong, Levenga et al. 2015). We found significantly more small mitochondria in *Dp16* cells compared to WT cells using CellLight transduction (Figure 5A, 5B). Additionally, the total density of mitochondria per cell did not differ between the genotypes. This suggests that the mitochondria fission rate is elevated suggesting that activity of DRP1 is increased in these cells, which could be caused by increased CaN activity. It is important to note here that the mitochondria were analyzed in glial cells only. However, the smaller mitochondria in glial cells could indicate *Rcan1* overexpression also affect this cell type in the brain.

Higher ROS levels in *Dp16* mice

Abnormally small mitochondrial size is indicative of dysregulated mitochondrial fission and fusion dynamics (Wang, Su et al. 2009). As such, we predicted that cells with smaller mitochondria would contain more ROS. MitoSox data show a correlation between ROS levels in both soma and processes and number of *Rcan1* alleles in various genotypes (Figure 6A and 6B). *Dp16* mice have the highest levels of ROS, and levels in neuronal processes are significantly higher than WT mice as might be expected in the context of DS and neurodegeneration. High levels of ROS indicate cellular stress (Bishop, Lu et al. 2010), and the smaller mitochondria
Figure 3: Mitochondrial size in \textit{Dp16} and WT mice. (A) Representative images of CellLight fluorescence in cell culture. There are significantly more small mitochondria in \textit{Dp16} cells than WT cells as visualized in (A) and quantified in (B). Notably, the DAPI nuclear stains did not colocalize with the neuronal marker NeuN (not shown here), so these cells are most likely glial cells. * indicates a significant difference between WT and \textit{Dp16} mitochondria less than 50\(\mu\text{m}^2\) in size as detected by a student’s t-test with a \(p<0.05\).
present in *Dp16* cells suggest dysregulation of mitochondrial fission and fusion homeostasis. Removal of one copy of *Rcan1* in the *Dp16/Rcan1* Het mice reduces ROS levels back toward WT ROS levels, which correlates with the CaNAR results.
Figure 4: ROS levels in WT and Dp16 mice. (A) Representative images of ROS fluorescence in WT and Dp16 neurons in culture. (B) Quantification of MitoSox pixel intensity in WT, Dp16, Dp16/Rcan1 Het, and Rcan1 Het mice showing a positive correlation between number of Rcan1 alleles in mice and intensity of MitoSox fluorescence.
CHAPTER IV
DISCUSSION AND FUTURE DIRECTIONS

Understanding which genes play a role in Down syndrome (DS) will help us develop targets for therapies while elucidating relevant pathways in sporadic Alzheimer’s disease (AD). The growing aging population and the increased life expectancy of DS individuals who almost universally develop AD have made progress in the field an even more urgent matter. The relationship also provides a unique opportunity to observe aspects of AD pathology development. In the non-DS population, we are unable to identify those who will develop sporadic AD later in life, so we miss a potentially critical window to examine causal factors in the disease. With the universal development of early-onset AD-like symptoms in DS individuals, it may be assumed that genetic factors are causal in the two disorders. More specifically, overexpression of genes on human chromosome 21 is the likely cause for both sporadic and DS-linked AD. For many years, the Amyloid Hypothesis dominated the field of AD research (Hardy and Selkoe 2002) in which the amyloid precursor protein (APP) was thought to cause most dysfunction. This theory has now lost traction because of the failure of amyloid-beta (Aβ) clearance therapies to rescue cognitive impairment and evidence that AD-like symptoms still develop in Ts1Cje DS model mice that have APP deleted (Shukkur, Shimohata et al. 2006). Although Aβ is still being actively researched, many have shifted their attention to other candidate genes on chromosome 21.

Regulator of calcineurin 1 (RCAN1) is one of the genes proven to play a role in neuronal dysfunction. RCAN1 has been found to be overexpressed in brains of AD patients, DS individuals, and in those of the normally aging population (Cook, Hejna et al. 2005, Wu and Song 2013, Perluigi, Di Domenico et al. 2014). RCAN1 levels increasing with age suggests that
the protein could contribute to the age-dependent aspect of AD. We were able to examine specifically RCAN1 contribution in the DS mouse model because of the ability to manipulate Rcan1 independently of other triplicated Mmu16 genes. The results of this study suggest that altered RCAN1 levels lead to elevated CaN activity as assessed by a CaN activity reporter (CaNAR). This increase in CaN activity seems to lead to excessive mitochondrial fission and possibly either more reactive oxygen species (ROS) production in the brain or an inability to clear ROS. Although more cultures are needed to more confidently assess the effect of RCAN1 upregulation on CaN activity, the trends appear strong and consistent in these experiments. One reason the CaNAR results have not yet reached significance is that there is high between-culture variability. Thus, despite the fairly consistent FRET ratio differences between genotypes within each culture, we must overcome the variability introduced by the differences in FRET ratios between separate cultures by increasing the number of cultures included in our analysis. If the trends we observe at this point are maintained after further investigation, we might next look at what specifically drives the increased ROS levels we see in the DS model mice.

Our results showing increased numbers of small mitochondria in Dp16 cells compared to WT littermates correlate with both ROS and CaNAR data. Despite these promising results, it is important to note that the mitochondria were examined in glia while CaN activity and ROS levels were examined in neurons, as CellLight preferentially tags glia. This might be the due to the fact that CellLight works best with low plating densities (ThermoFisher Scientific, Waltham, MA). Glia tend to be present along the edges of cell culture cover slips where cell density is the lowest, which could contribute to this phenomenon. However, the complete lack of CellLight in neurons suggests that this is not the only factor influencing uptake since neurons are also present on cover slip periphery. To get around this issue, we will use the abundant inner mitochondrial
membrane protein Adenine nuclear transporter 1 (ANT1) staining to look at mitochondrial size, as this has proven effective in neurons (Wong, Levenga et al. 2015).

The decreased levels of ROS we observed in Rcan1Het with the removal of a second Rcan1 allele are somewhat surprising considering the heightened FRET ratios we observed in these mice, although the standard error for these results is fairly high. It is important to keep in mind, too, that however close to significance the FRET ratio results might be, they are not yet definitive, and so with the addition of analysis from future cultures we might see the FRET ratio shift to align more readily with the ROS results or vice versa.

RCAN1 has been shown to be both an activator and an inhibitor of CaN depending on context (Rothermel, Vega et al. 2000, Liu, Busby et al. 2009). For example, overexpression of the protein decreases CaN dephosphorylation of the microtubule-binding protein tau, resulting in hyperphosphorylated tau, which is found in multiple neurodegenerative diseases, while upregulation of RCAN1 leads to decreased DRP1 phosphorylation, implicating increased activity of CaN (Wong, Levenga et al. 2015). To reveal how these seemingly opposing roles of RCAN1 are regulated, we can look to see if the different isoforms of RCAN1 have distinct roles in both normal cellular processes and neuropathology. Determination of their native functions might help elucidate how altered function of these isoforms can contribute to disease. We have begun in vitro studies of Rcan1KO mice to look at how the different isoforms regulate CaN activity (via CaNAR), mitochondrial fission, and mitochondria-specific ROS (Figure 7). We hope that these experiments will help us better understand how RCAN1 regulates CaN, which will help to eventually identify potential therapeutic targets in Down syndrome and Alzheimer’s disease.
**Figure 5:** Identification of specific RCAN1 isoform contributions to mitochondrial dysfunction in Rcan1 KO cell culture. (A) Method for transfecting cell culture with bacterial plasmids containing RCAN1 isoform mRNA transcripts tagged with identifying epitopes. (B) Representative image of an RCAN1.1L-containing neuron alongside a non-transfected Rcan1 KO neuron in the context of FRET.
BIBLIOGRAPHY


