Investigating the Role of Neuronal Aging in Fragile X-Associated Tremor/Ataxia Syndrome

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INVESTIGATING THE ROLE OF NEURONAL AGING IN FRAGILE X-ASSOCIATED TREMOR/ATAXIA SYNDROME

by

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A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biology in the College of Sciences and in the Burnett Honors College at the University of Central Florida Orlando, Florida

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ABSTRACT

Fragile X-associated tremor/ataxia syndrome (FXTAS) is an X-linked late-onset neurodegenerative disorder caused by a noncoding trinucleotide repeat expansion in the *FMR1* gene. This gene produces fragile x mental retardation protein (FMRP), an RNA binding protein whose targets are involved in brain development and synaptic plasticity. One of the proposed mechanisms of FXTAS pathogenesis is an RNA gain-of-function in which the repeat expansion causes toxic mRNA that sequesters important proteins in the cell, interfering with their functions. Another suggested method of pathogenesis is through a mutant protein called FMRpolyG. This protein results from repeat-associated non-AUG (RAN) translation, in which the expanded repeats are translated where they otherwise would not be. This protein co-localizes with intranuclear inclusions and nuclear membrane proteins, causing disorganization of the nuclear lamina in FXTAS patient brain samples and neurons differentiated from FXTAS patient-derived induced pluripotent stem cells (iPSCs). iPSC technology involves reprogramming an adult somatic cell back to an embryonic-like state, allowing it to be differentiated into all cell types. A limit with iPSCs, though, is modeling late-onset disorders because the cells lose all age-related features during reprogramming. Progerin, a truncated form of the lamin A protein, has been used to age neurons differentiated from Parkinson Disease (PD) patient-derived iPSCs. Progerin-mediated aging was found to unmask PD-like phenotypes in those neurons, making it a promising technology for modeling late-onset disorders such as FXTAS. In this study, we investigated the link between the aging process and FXTAS pathogenesis in neurons differentiated from FXTAS patient-derived iPSCs with the use of progerin. Progerin transduction was successful in aging the FXTAS neurons. The presence of FMRpolyG was confirmed and an
interaction with Lap2β was observed. In some neurons, there was also an observed interaction between FMRpolyG and progerin. Overall, this data suggests that there is an interaction between the mutant FMRpolyG protein and the nuclear membrane during aging, which may contribute to the cell death that causes neurodegeneration in FXTAS patients.
DEDICATION

To my mother, for always believing in me
and
To my little brother, Trevor – you inspire me every day.
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# TABLE OF CONTENTS

LIST OF FIGURES ......................................................................................................................... ix

LIST OF ABBREVIATIONS ............................................................................................................... xi

CHAPTER I: INTRODUCTION ........................................................................................................ 1

CHAPTER II: RESULTS .................................................................................................................. 13

  PROGERIN TREATMENT CAUSES INCREASED NUCLEAR BLEBBING ........................................ 13

  SOME NEURONS REVEAL CO-LOCALIZATION BETWEEN FMRPOLYG AND LAP2β .................. 14

  PROGERIN MAY CO-LOCALIZE WITH FMRPOLYG IN SOME CELLS ....................................... 16

  SOME NEURONS MAY HAVE INCREASED NUCLEAR PERMEABILITY ..................................... 17

  CLEAVED CASPASE 3 WAS COMPARABLE IN PROGERIN AND CONTROL-TREATED NEURONS ... 18

  PROGERIN-TREATED NEURONS DISPLAY INCREASED DNA DAMAGE .................................... 20

  ABNORMAL MORPHOLOGY OBSERVED IN PROGERIN AND CONTROL-TREATED NEURONS ...... 21

  FXTAS NEURONS ARE DIFFICULT TO DISSOCIATE FOR SORTING ........................................ 22

CHAPTER III: DISCUSSION .......................................................................................................... 24

CHAPTER IV: METHODOLOGY ................................................................................................... 27

  EXPERIMENTAL CONDITIONS ................................................................................................. 27

  NEURAL STEM CELL CULTURE ............................................................................................... 27

  NEURONAL DIFFERENTIATION ............................................................................................... 28

  VIRAL TRANSDUCTION .......................................................................................................... 29

  IMMUNOCYTOCHEMISTRY ..................................................................................................... 29

  FLOW CYTOMETRY ............................................................................................................... 30
LIST OF FIGURES

Figure 1. Common physical features of Fragile X Syndrome. .................................................. 3

Figure 2. FMRpolyG and ubiquitin co-localization in FXTAS patient brain samples. ............ 6

Figure 3. Immunofluorescence of FXTAS patient and control brain samples reveals disruption of nuclear lamina. ........................................................................................................................................... 7

Figure 4. Immunocytochemistry of neurons differentiated from FXTAS patient-derived iPSCs reveals co-localization between FMRpolyG and Lap2β and a disruption of the nuclear membrane........................................................................................................................................... 9

Figure 5. Progerin treatment reveals phenotypes of aging in young and old donor midbrain dopaminergic (mDA) neurons differentiated from iPSCs. .......................................................... 11

Figure 6. A comparison of the amount of nuclear abnormality displayed in neurons treated with progerin and the control ........................................................................................................... 14

Figure 7. A comparison of the amount of co-localization between FMRpolyG and Lap2β in neurons treated with either AAV-progerin-GFP or AAV-nGFP .................................................. 15

Figure 8. Co-localization of FMRpolyG and Lap2β outside of the nucleus. .................... 16

Figure 9. Some FXTAS neurons display co-localization between FMRpolyG, Lap2β, and progerin. ........................................................................................................................................... 17

Figure 10. GFP signal outside of the nucleus was recorded in greater than 70% of the transduced nGFP neurons ........................................................................................................................................... 18

Figure 11. A comparison of the level of apoptosis in neurons treated with either AAV-progerin-GFP or AAV-nGFP ........................................................................................................... 19
Figure 12. A comparison between the presence of a DNA damage signal inside of the nucleus and outside of the nucleus.

Figure 13. Both neurons treated with AAV-progerin-GFP and AAV-nGFP showed abnormal morphology.

Figure 14. Flow cytometry analysis to test cell dissociation protocol.
LIST OF ABBREVIATIONS

AAV – Adeno-associated Virus
AUG – Start Codon
BSA – Bovine Serum Albumin
cAMP – Cyclic Adenosine Monophosphate
CGG – Cytosine Guanine Guanine (Codon)
CO₂ – Carbon Dioxide
DAPI – 4’,6-diamino-2-phenylindone
DGCR8 – DiGeorge Syndrome Critical Region 8
DNA – Deoxyribonucleic Acid
FACS – Fluorescence Activated Cell Sorting
FMRP – Fragile X Mental Retardation Protein
FMR1 – Fragile X Mental Retardation 1
FXS – Fragile X Syndrome
FXPOI – Fragile X-associated primary ovarian insufficiency
FXTAS – Fragile X-associated tremor/ataxia syndrome
GFP – Green Fluorescent Protein
HGPS – Hutchinson-Gilford Progeria Syndrome
hnRNP A2/B1 – Heterogeneous Nuclear Ribonucleoprotein A2/B1
ICC - Immunocytochemistry
iPSC – Induced Pluripotent Stem Cell
kb – Kilobases
LAP2β - Lamin-associated Polypeptide 2-Beta
LAP2 – Lamin-associated polypeptide 2
MAP2 – Microtubule Associated Protein 2
MCP – Middle Cerebral Peduncle
miRNA – Micro-Ribonucleic Acid
mRNA – Messenger Ribonucleic Acid
n-GFP – Nuclear Green Fluorescent Protein
NGS – Normal Goat Serum
NSC – Neural Stem Cell
ORF – Open Reading Frame
PBS – Phosphate Buffered Solution
Purα – Pur-alpha
RAN – Repeat-associated Non-AUG
rhBDNF – Human Recombinant Brain-Derived Neurotrophic Factor
rhGDNF – Human Recombinant Glial-Derived Neurotrophic Factor
RNA – Ribonucleic acid
RPM – Revolutions per Minute
uORF – Upstream Open Reading Frame
UTR – Untranslated Region
CHAPTER I: INTRODUCTION

The fragile X mental retardation 1 (FMR1) gene is located on the long arm of the X-chromosome (Xq23.3) and is responsible for producing FMR protein (FMRP). The FMR1 gene carries a CGG trinucleotide repeat in the 5’ untranslated region (UTR). In the normal population, the repeat size ranges from 5 to 40 and is most commonly found around 30. Expansion mutations of this CGG repeat may lead to different disorders. Fragile X Syndrome (FXS), one of the leading genetic causes of intellectual disability, results from an expansion to over 200 CGG repeats, classified as a full mutation. A repeat expansion between 55 and 200 is classified as a premutation and puts carriers at risk for fragile x-associated primary ovarian insufficiency (FXPOI) and fragile X-associated tremor/ataxia syndrome (FXTAS). Currently, the only treatments for these disorders are aimed at controlling symptoms, with no disease-modifying treatments available at this time.

The FMR1 gene has 17 exons, with a messenger RNA (mRNA) transcript of about 3.9 kilobases (kb). The FMR1 transcript undergoes alternative splicing, which results in six isoforms of FMRP throughout different body tissues. Although FMRP is found throughout the body, the brain is one of the organs with the highest amount. The protein is present not only in the cell bodies of neurons but in the dendrites and synaptic clefts as well. FMRP has RNA binding domains and it is estimated to bind with ~4% of mRNAs in the brain, whose functions include neuronal development and regulation of synaptic plasticity. It has been shown in sedimentation assays that FMRP co-localizes with active polyribosomes, the machinery responsible for the translation of mRNA into protein, which indicates a role in mRNA metabolism. Deletion of FMRP results in upregulation of expression of FMRP binding
partners, suggesting that FMRP has an inhibitory role in mRNA processing\(^9,10\). Furthermore, FMRP has both a nuclear localization signal and a nuclear export signal, which indicates a potential function of shuttling mRNA from the nucleus to the cytoplasm\(^9,10\).

FXS is a neurodevelopmental disorder that results from complete silencing of \(FMR1\)^11. The large expansion to over 200 CGG causes the gene to become hypermethylated, turning off the promoter, which in turn stops transcription of \(FMR1\) mRNA\(^11\). While the full mutation can affect both males and females, it affects males to a higher degree due to X-inactivation in females\(^11\). This occurs because females have two X-chromosomes, whereas males have one X and one Y-chromosome. Cells only need the genetic information from one X-chromosome, so in females, one of the two is randomly inactivated in each cell during early embryonic development, which is then conserved through subsequent cell divisions\(^12\). The inactivated chromosome is permanently packaged in a way that prevents gene transcription\(^12\). Because the selection of which X-chromosome is inactivated is random, some cells will inactivate the X-chromosome with the \(FMR1\) mutation, attenuating its deleterious effects. Conversely, males only have one X-chromosome, so all cells will contain the mutant \(FMR1\) gene, resulting in a more severe disorder than is seen in females. Clinical features of FXS may include large ears, an elongated face, hypotonia, and hyperflexible joints (figure 1)^11,13,14. Symptomatic presentation may range from mild to severe intellectual disability, autistic-like behavior, mood disorders, hyperactivity, and fine motor and learning deficits^11,13. The prevalence of FXS is about 1 in 4,000 males and 1 in 7,000 females\(^6\).
The CGG tract of the *FMR1* gene is unstable, which can result in intergenerational expansion from the normal range of 5-40 to between 55 and 200 during gametogenesis\(^\text{15}\). This range is classified as the premutation, which can subsequently expand to greater repeat lengths within the premutation range or to the full mutation (over 200 repeats) in future generations\(^\text{15}\). The premutation range is associated with lower penetrance disorders distinct from FXS.

Fragile X-associated tremor/ataxia syndrome is a late onset neurodegenerative disorder caused by the premutation expansion of the CGG tract. Symptoms of FXTAS include cerebellar ataxic gait, intention tremor, Parkinsonism, decline in cognitive function, and peripheral neuropathy\(^\text{6}\). While approximately 1 in 130-250 females and 1 in 250-810 males carry the *FMR1* premutation, only about 16% of female and around 40% of male carriers will develop FXTAS in their lifetime\(^\text{6,13}\). Individuals with premutation repeat lengths above 70 are at greater risk for developing FXTAS, and severity is correlated with increasing repeat length\(^\text{16}\). Symptom onset

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*Figure 1. Common physical features of Fragile X Syndrome.* These features can include large ears and an elongated face. Adapted from\(^\text{14}\).
generally begins around the age of 50, but increasing repeat length and age of onset are inversely correlated\textsuperscript{16}.

FXTAS neuropathology may present with atrophy throughout the cerebrum and cerebellum\textsuperscript{1,6}. Loss of Purkinje cells and astrocytosis predominantly in the subcortical cerebral white matter are observed\textsuperscript{1}. Ubiquitin-positive intranuclear inclusions are observed in neurons and astrocytes throughout the brain and brainstem and are prominent in the hippocampus\textsuperscript{1}. Interestingly, these inclusions are rarely present in Purkinje cells despite their observed drop out, which may indicate that the inclusions are not necessary for cellular degeneration and may even play a protective role\textsuperscript{1}. One predominant neuropathological feature of FXTAS is an MCP sign, where the middle cerebral peduncle atrophies. The middle cerebral peduncle is the connection point from the body to the cerebellum and is useful in planning and refining movement and motor function\textsuperscript{17}.

Compared to normal individuals, carriers of the \textit{FMR1} premutation have about a 5 to 10-fold increase in \textit{FMR1} mRNA levels, in contrast to FXS where mRNA is virtually absent\textsuperscript{1}. Counterintuitively, although mRNA levels are increased in FXTAS, levels of FMRP are normal or reduced. It is also important to note that individuals with FXS do not develop the neurodegeneration seen in FXTAS. From this, it is hypothesized a potential mechanism of FXTAS pathology is RNA gain-of-function. Expanded \textit{FMR1} mRNAs form folded hairpins that cause accumulation and intranuclear inclusions that sequester RNA-binding proteins essential for cell viability\textsuperscript{1}. One of particular importance is DiGeorge syndrome critical region 8 (DGCR8), which binds preferentially to the repeat expansion\textsuperscript{1,5}. This protein is a binding partner to DROSHA, a double-stranded RNA-specific endonuclease that is involved in the processing of primary microRNA (miRNA) to precursor miRNA\textsuperscript{1,5}. Further cleavage of precursor miRNA by
the endonuclease DICER produces mature miRNA that is then able to perform posttranscriptional gene suppression. This posttranscriptional regulation is crucial for the growth and survival of neurons, which suggests that sequestration of regulatory proteins may cause FXTAS-related cellular dysfunction and death. Other RNA binding/processing proteins, hnRNP A2/B1, Purα, and Sam68, are known to interact with FMR1 mRNA inclusions in vitro, but unlike DGCR8, these interactions are not modified by CGG expansion. Thus, it is unclear if their sequestration contributes to disease pathogenesis.

A second potential mechanism of FXTAS pathogenesis is repeat-associated non-AUG (RAN) translation. RAN translation occurs when repetitive mRNA sequences are translated without an AUG initiation site. RAN translation can occur in all possible reading frames, which can result in aberrant production of multiple repeat-containing proteins. In FXTAS, this process involves a near-cognate ACG codon upstream of the CGG repeats in a short upstream open reading frame (uORF) in the 5’ UTR of the FMR1 mRNA. This codon causes leaky ribosomal scanning that produces a polyglycine-containing protein called FMRpolyG (GGC) and a polyalanine-containing protein, FMRpolyA (GCG). However, no RAN translation product is detected in the CGG polyarginine frame. Furthermore, the translation of the FMRpolyA product is less efficient than the FMRpolyG product, leading researchers to postulate that FMRpolyG is the main pathogenic driver through RAN translation in FXTAS. Leaky ribosomal scanning is a mechanism that occurs in the presence of an uORF in the 5’ UTR of mRNA to ensure that translation is re-initiated at the main open reading frame (ORF), for FMRP in this case. If this did not occur, the FMRP ORF would not be initiated, and FMRP production would not occur. With that said, the ORFs of FMRpolyG and FMRP overlap by about 20 amino acids, which could cause an interruption in translation re-initiation. This may explain why the
levels of FMRP can be decreased in pre-mutation carriers despite increased amounts of mRNA\textsuperscript{4}. It is also important to note that FMRpolyG has only been detected with CGG repeats above 60 and it co-localizes with ubiquitin-positive nuclear inclusions in FXTAS patient brain samples (figure 2\textsuperscript{4}).

\textbf{Figure 2. FMRpolyG and ubiquitin co-localization in FXTAS patient brain samples.} Immunofluorescence of post-mortem brain samples shows the presence of ubiquitin-positive inclusions (green) containing FMRpolyG (red) co-localized in the nucleus of FXTAS neurons. This shows that the mutant protein FMRpolyG forms inclusions inside of the nucleus and that they can co-localize with the inclusions previously noted in FXTAS neuropathology. Adapted from\textsuperscript{4}.

Association studies with FMRpolyG revealed that it interacts with lamina-associated polypeptide 2-beta (LAP2\textbeta), an isoform of the LAP2 protein that is anchored to the nuclear membrane\textsuperscript{4}. Lap2\textbeta assists in maintaining the inner nuclear membrane organization through interactions with lamin B1 and lamin B2 proteins. In FXTAS patient brain, Lap2\textbeta and
FMRpolyG are co-localized and lamin B1 is mis-localized, demonstrating that the organization of the nuclear membrane is perturbed (figure 3). Disruption of the nuclear membrane often leads to cell death, and overexpression of Lap2β, which has no effect on control neurons, is sufficient to rescue neurons expressing FMRpolyG. Further study of these protein interactions could reveal clues about FXTAS pathogenesis and could lead to potential therapeutic targets.

Figure 3. Immunofluorescence of FXTAS patient and control brain samples reveals disruption of nuclear lamina. (Left) Co-localization of Lap2β (red) and FMRpolyG (green) in FXTAS patient brain samples. (Right) lamin B1 (red) mis-localization to intranuclear inclusions containing FMRpolyG (green) in FXTAS patient brain samples. In control brain samples, Lap2β and lamin B1 are localized to the nuclear membrane as they should be. In the presence of FMRpolyG in FXTAS patient samples, Lap2β and lamin B1 co-localize with FMRpolyG-positive inclusions. Adapted from 4.

Induced pluripotent stem cells (iPSCs) can be differentiated into any cell type in the body, making them a unique tool to model regional and tissue-specific effects of various
disorders and provide a valuable method to study patient neurons\textsuperscript{19}. However, reprogramming adult cells, such as from a skin biopsy or blood sample, to iPSCs removes all markers of aging, thus, returning cells to an embryonic-like state\textsuperscript{19,20}. This \textit{in vitro} method is appealing because it is not subject to the same ethical considerations that are associated with embryonic stem cell research, and allows for patient-specific research\textsuperscript{20}. However, they are limited in the study of adult onset diseases. In neurons differentiated from FXTAS patient-derived iPSCs, \textit{FMR1} mRNA levels are elevated up to three fold, which is consistent with the increased levels of the \textit{FMR1} mRNA noted in FMR1 pre-mutation carriers\textsuperscript{4}. Consistent with patient brain samples, FMRpolyG inclusions and Lap2\textbeta are co-localized in FXTAS patient-derived neurons (figure 4), which disrupts the normal localization of Lap2\textbeta\textsuperscript{4}. In the presence of FMRpolyG inclusions, lamin B1 is also mis-localized (figure 4)\textsuperscript{4}. 
Figure 4. Immunocytochemistry of neurons differentiated from FXTAS patient-derived iPSCs reveals co-localization between FMRpolyG and Lap2β and a disruption of the nuclear membrane. (Left) Representative images (top) and quantification (bottom) of co-localization of Lap2β (red) and FMRpolyG (green) in neurons differentiated from FXTAS patient-derived iPSCs. (Right) Representative images (top) and quantification (bottom) of mis-localization of lamin B1 (red) in the presence of FMRpolyG (green) intranuclear inclusions in FXTAS iPSC-derived neurons. This shows that in the presence of FMRpolyG, the localization and organization of Lap2β and lamin B1 are disrupted. Adapted from Ref. 4.

One limitation in using iPSCs to model late-onset disorders, such as FXTAS, is that the cells lose all age-related features during reprogramming, reverting back to an embryonic-like state19. This is evidenced by longer telomeres, increased mitochondrial fitness, healthier nuclear organization, decreased DNA damage, and decreased reactive oxygen species compared to adult
donor cells prior to reprogramming\textsuperscript{19}. This reversion to a developmental state confounds the study of late onset phenotypes. Acceleration of aging via progerin treatment has been used to overcome this\textsuperscript{19}. Progerin is a truncated form of lamin A that causes Hutchinson Gilford Progeria Syndrome (HGPS), a disease of premature aging\textsuperscript{19}. Progerin is not only produced in HGPS, but is produced at low levels in healthy individuals, as well\textsuperscript{19,21}. Progerin production increases over time in the absence of a mutation, suggesting a role in normal aging\textsuperscript{21}. The Lamin A protein is involved in organization of the nuclear membrane and progerin disrupts the normal organization\textsuperscript{19}. This disorganization perturbs nuclear processes, including mRNA production, the DNA damage response, and the cell cycle\textsuperscript{19}. Progerin treatment is able to induce cellular aging in neurons differentiated from iPSCs, restoring markers of natural aging\textsuperscript{19}. Progerin ‘aged’ neurons display increased nuclear blebbing, DNA damage, protein aggregation, and reactive oxygen species, decreased dendrite length, and changes in the cell cycle and chromatin modification processes, demonstrating advanced biological age (figure 5)\textsuperscript{19}.
Figure 5. Progerin treatment reveals phenotypes of aging in young and old donor midbrain dopaminergic (mDA) neurons differentiated from iPSCs. (Top Left) Compared to nuclear GFP control, progerin treatment increases nuclear blebbing (Lamin B2) and DNA damage (γH2AX) in iPSC-derived neurons, both age-like phenotypes. (Top Right) Progerin treatment induces reactive oxygen species in iPSC-derived neurons from young and old donors, another age-like phenotype. (Bottom) Progerin treatment reduces dendrite length (MAP2) in iPSC-derived mDA neurons from both young and old donors. Adapted from 19.
Using progerin, we are investigating whether there is a link between neuronal aging and FXTAS pathogenesis. If progerin-induced aging of FXTAS patient iPSC-derived neurons reveals greater disease-specific pathology, it would suggest that aging is directly involved in FXTAS pathogenesis and provide rationale for the study of anti-aging therapies on FXTAS onset and progression.
CHAPTER II: RESULTS

Progerin Treatment Causes Increased Nuclear Blebbing

Neurons transduced with AAV-progerin-GFP displayed increased nuclear blebbing, a sign of dysfunction in the nuclear scaffolding. Compared to neurons transduced with AAV-nuclear-GFP (nGFP), neurons treated with progerin showed a 19% increase in nuclear blebbing (figure 6B). This nuclear abnormality is seen in cells during aging. Since the cells treated with progerin showed this increased abnormal nuclear morphology, we can conclude that the progerin was successful in aging the neurons. While some cells treated with the nGFP displayed nuclear abnormalities, it was not as dramatic as those treated with progerin. Figure 6A shows the differences between a cell with nuclear blebbing compared to one without.
Figure 6. A comparison of the amount of nuclear abnormality displayed in neurons treated with progerin and the control. A) Immunocytochemistry revealing the differences between a normal and an abnormal nucleus (nuclear blebbing/folding). Cells were labeled using DAPI (nucleus) and GFP (AAV-progerin-GFP or AAV-nGFP). B) Quantification of the amount of nuclear blebbing between neurons treated with progerin and neurons treated with the control.

Some Neurons Reveal Co-Localization Between FMRpolyG and Lap2β

Following a study that showed the co-localization of Lap2β with FMRpolyG in telencephalic FXTAS neurons, we expected our FXTAS neurons to display this co-localization. Our lab wanted to investigate whether aging neurons with progerin would increase the amount of FMRpolyG inclusions, and if those inclusions would co-localize with Lap2β. Comparing neurons treated with AAV-progerin-GFP and AAV-nGFP, there was no significant difference noted in the number of cells with FMRpolyG and Lap2β co-localization (Figure 7). It was also expected that the inclusions would form in the nucleus, as established in previous studies. Of the neurons in our study that showed the protein co-localization, a majority of the proteins were in
the cytoplasm of the cell (Figure 7). It is possible that the nuclei of these cells had increased permeability from transducing them with a nuclear protein, which could cause the protein aggregates to leave the nucleus. Nonetheless, the FMRpolyG found in these cells did mis-localize the Lap2β from the nuclear membrane, which can lead to cellular dysfunction.

It was expected that the neurons treated with the AAV-nGFP would display less co-localization between FMRpolyG and Lap2β, as we hypothesized that this process would increase in aged cells. Our results only include one cell treated with the control virus and stained for FMRpolyG and Lap2β, of which co-localization was noted. Because of the small sample size, we cannot conclude that there was a difference between the two viral conditions in this set of immunocytochemistry (ICC). Further analysis of the co-localization between FMRpolyG and Lap2β in greater numbers of neurons treated with either progerin or the control is needed before a conclusion can be formed.

Figure 7. A comparison of the amount of co-localization between FMRpolyG and Lap2β in neurons treated with either AAV-progerin-GFP or AAV-nGFP. Immunocytochemistry reveals co-localization between
FMRpolyG (red) and Lap2β (magenta) in neurons treated with AAV-progerin-GFP, both inside and outside of the nucleus (top). Of the one neuron gathered for analysis that was treated with AAV-nGFP, FMRpolyG and Lap2β display co-localization outside of the nucleus (bottom).

Figure 8 shows an example of the presence of FMRpolyG and Lap2β co-localizing outside of the nucleus. This was not expected because Lap2β is a nuclear membrane scaffolding protein, therefore it is expected that inclusions would form inside the nucleus. While this was not expected, the cells are still showing that the presence of FMRpolyG can possibly disrupt the nuclear membrane by interfering with the localization of Lap2β.

**Figure 8. Co-localization of FMRpolyG and Lap2β outside of the nucleus.** The presence of FMRpolyG aggregates (red) in the cytoplasm of the cell co-localize with Lap2β (magenta) in a large proportion of our FXTAS neurons.

Progerin May Co-localize with FMRpolyG in Some Cells

Some neurons from each of the FXTAS cell lines displayed co-localization between FMRpolyG, Lap2β, and progerin (figure 9). This co-localization was not present in cells treated with the nGFP control. This is not entirely surprising, as progerin is a truncated form of the lamin A protein, which is another scaffolding protein of the nuclear membrane. Progerin is
known to localize to the nuclear membrane, whereas the nuclear-GFP control should be diffuse throughout the nucleus. Since Lap2β mis-localizes from the nuclear membrane in the presence of FMRpolyG in some cases, it is plausible that the progerin protein could also mis-localize. This co-localization was noted in about one-third of the neurons showing a co-localization between FMRpolyG and Lap2β.

![Figure 9: Some FXTAS neurons display co-localization between FMRpolyG, Lap2β, and progerin.](image)

In neurons treated with AAV-progerin-GFP, some cells showed a co-localization between FMRpolyG (red), Lap2β (magenta), and progerin (green); this co-localization disrupts the nuclear membrane structure as seen in the photos above.

Some Neurons May Have Increased Nuclear Permeability

Both the AAV-progerin-GFP and the AAV-nGFP should produce protein that specifically labels the nucleus. The progerin-GFP typically localizes to the nuclear membrane, whereas the nuclear-GFP is typically diffuse within the nucleus. About 70% of cells transduced with nGFP had GFP staining throughout the cell, compared to about 20% of progerin-treated cells that had this filling outside of the nucleus (figure 10B). It could be possible that this signal was produced from background fluorescence or non-specific binding of the antibody. It is also possible that the cells transduced with nGFP have greater nuclear permeability from using a diffuse nuclear protein. This could explain why the GFP signal was diffuse throughout many of the cells. Figure 10A shows a representative image of the GFP cell filling.
Figure 10. GFP signal outside of the nucleus was recorded in greater than 70% of the transduced nGFP neurons. In neurons treated with AAV-nGFP, many of the cells had GFP (green) diffuse throughout the cell when the GFP protein should have been in the nucleus. Some of the cells transduced with AAV-progerin-GFP had this cell filling, but the percentage was much lower.

Cleaved Caspase 3 Was Comparable in Progerin and Control-Treated Neurons

Cleaved caspase 3 is a marker of apoptosis. This marker is present in dying cells, so it is expected that cells treated with progerin will have a greater amount of cleaved caspase 3 in the cytoplasm compared to cells treated with the control. In comparing FXTAS neurons treated with AAV-progerin-GFP and AAV-nGFP, the levels of cleaved caspase 3 were similar (figure 11B).
The presence of this apoptotic marker was seen in less than half of the cells in both conditions, indicating that the levels of cell death were not significantly high. This was not expected, as the aging caused by progerin was hypothesized to cause greater dysfunction in FXTAS cells compared to “young” cells. Interestingly, the number of cells containing cleaved caspase 3 was greater in neurons treated with the nGFP control. Figure 11A is a representative image of the cleaved caspase 3 signal in FXTAS neurons.

Figure 11. A comparison of the level of apoptosis in neurons treated with either AAV-progerin-GFP or AAV-nGFP. A) Immunocytochemistry showing a representative image of the presence of cleaved caspase 3 (magenta) in
FXTAS neurons treated with either progerin or control. B) Quantification of the percentage of cells with a cleaved caspase 3 signal between FXTAS neurons treated with progerin or control. The levels of cleaved caspase 3 were similar between treatments, indicating that apoptosis was not increased in either condition.

Progerin-Treated Neurons Display Increased DNA Damage

DNA damage is another marker of aging in cells. DNA is the backbone of all cellular processes; therefore, it is necessary for cell viability. In neurons treated with AAV-progerin-GFP, the presence of DNA damage was increased compared to neurons treated with AAV-nGFP (figure 12). Interestingly, a large majority of the neurons treated with AAV-nGFP showed the signal for DNA damage staining throughout the cytoplasm (figure 12). This signal could be due to bleed through from other color channels or may represent a leaky nucleus. The latter is unlikely, though, as DNA is a large charged biomolecule unlikely to pass through even a very disrupted membrane, and if this were to happen, DAPI would also be expected to stain the whole cell. This is because DAPI binds to specific bases in the DNA, all of which would be present in the cytoplasm of these cells. Because of the strange signal in cells treated with AAV-nGFP, it is difficult to conclude a definitive difference between the levels of DNA damage in each condition.
Figure 12. A comparison between the presence of a DNA damage signal inside of the nucleus and outside of the nucleus. A small percentage of neurons treated with AAV-progerin-GFP (green) showed DNA damage (15A3, red), characterized by foci inside of the nucleus. A large percentage of neurons treated with AAV-nGFP (green) showed DNA (15A3, red) damage signal outside of the nucleus, often diffuse throughout the cell.

Abnormal Morphology Observed in Progerin and Control-Treated Neurons

FXTAS neurons were stained for MAP2 and Beta-III Tubulin to assess general health and complexity. Figure 13 shows representative images of the neurons. The shapes of the neurons from each treatment are similar, and the number of dead cells per condition were insignificant. Overall, the neurons of both treatment groups displayed complex and abnormal morphology. The cells did not have normal neuronal shapes, but the MAP2 staining confirms that they are neurons, as MAP2 is a neuron-specific protein. The abnormal morphology is most likely due to the low density of cells, allowing them to grow unhindered by other cells. We were unable to conclude anything about general neuronal health and complexity due to the abnormal morphologies.
Both neurons treated with AAV-progerin-GFP and AAV-nGFP showed abnormal morphology. Dendritic complexity was visualized using MAP2 (red) and general neuronal health was visualized using Beta III Tubulin (magenta). While these cells do not resemble normal neuronal morphology, staining with MAP2 confirms that they are neurons.

**FXTAS Neurons Are Difficult to Dissociate for Sorting**

Cell sorting is useful in studies such as this, as analysis of only GFP-positive cells is desired. The ability to sort cells into different populations based on size, complexity, and fluorescence would be useful for Western blot analysis of FMRpolyG in only progerin and control-treated cells. In an attempt to separate individual neurons in this study, a dissociation protocol was used. The cells were then run through a flow cytometer to analyze each sample for different population. The size and complexity of the components of the sample were measured along with the fluorescent intensity of GFP. Unfortunately, no single populations were recorded in the analysis. Figure 14 shows a graph comparing size and complexity, as well as complexity and fluorescence intensity. It is not possible to discern any different components from the mass...
of signal that was recorded. The fluorescent signal was high, indicating that viral transduction could have been successful in the neurons. The components of the sample with low size and complexity are likely various cellular debris. The larger components with greater complexity could be clumps of neurons present in the sample, which would indicate that the dissociation protocol was not able to break the neuronal connections. In the future, our lab is going to determine where GFP alone falls on the graph to determine if any of the “debris” is GFP. This could give us insight into whether the dissociation protocol was simply too harsh on the cells and caused them to lyse and spill their contents. Further investigation is required to troubleshoot this protocol for the use in future cell sorting procedures.

Figure 14. Flow cytometry analysis to test cell dissociation protocol. A) This graph is comparing the size to the complexity of the components of the sample of neurons. No single population is detected, indicating that a single cell suspension was not achieved B) This graph is comparing the fluorescence intensity to the complexity of the components of the sample. The signal of GFP is high in this case.
CHAPTER III: DISCUSSION

The viral transduction of AAV-progerin-GFP and AAV-nGFP was successful in that about a 40% transduction rate was achieved. On the contrary, the number of total cells used in this study was insufficient to answer many of the questions that were originally proposed. In FXTAS neurons treated with AAV-progerin-GFP, there was a significant increase in nuclear morphological changes and an increase in DNA damage compared to neurons treated with AAV-nGFP. There was no significant increase in the number of apoptotic cells, and it was not able to be determined if cells treated with progerin displayed increased co-localization between FMRpolyG and Lap2β.

One conclusion of this study is that the cells treated with progerin were aged to some degree compared to the cells treated with the control. The presence of nuclear blebbing is a sign of nuclear dysfunction, which often leads to cell dysfunction and death. The number of cells with nuclear blebbing was increased by about 20% in cells treated with progerin, which confirms that the nuclear integrity of these cells was compromised more than those treated with the control. While this isn’t a definitive quantification of aging itself, it is one sign of aging in neurons.

FMRpolyG and Lap2β were co-localized in FXTAS neurons treated with progerin, but the localization of these proteins in the cytoplasm was not expected. It is unclear whether this was from over permeabilization of the cells, but further investigation into the relationship between FMRpolyG and Lap2β is still required. The neuron treated with the nuclear-GFP control also showed co-localization between these two proteins. A conclusion cannot be made regarding whether progerin and control-treated neurons display differing levels of co-localization between these two proteins because the sample size of both conditions are too low. One interesting note is
of the co-localization between FMRpolyG inclusions and progerin in this study. Since FMRpolyG seems to disrupt proteins of the nuclear membrane, it is possible that it can also co-localize with progerin, a shortened form of the lamin A protein. Again, the sample size is too small to draw any conclusions, but the progerin co-localization question is a future aspect to study further.

The diffuse signal of the DNA damage antibody and GFP in some cells is another interesting question that needs further analysis. It is possible that the nuclei of these cells are highly permeable, leaking their contents into the cytoplasm. It is also possible that the antibody signals are from bleed through from other colors in the sample. Another trial with a larger sample is needed to study this more.

For future protein analysis using a Western blot, it is important to optimize the dissociation protocol for neuronal cultures. Neurons are tricky to dissociate because they form complex connections between one another. In the next dissociation trial, our lab is planning to perform extra mechanical steps to aid the dissociation of the clumps of cells to a single cell suspension. If the protocol is optimized for sorting cells into a population of single cells, the samples could be sorted by GFP fluorescence. This would ensure that only GFP-positive cells are used in a Western blot, giving a more accurate comparison of the FMRpolyG levels in progerin and control-treated cells.

The biggest limitation of this study was that a control cell line was not available for use in the analysis. This study will be repeated and will include two cell lines that do not have expanded CGG tract lengths to compare to the two FXTAS lines. Adding data from AAV-progerin-GFP and AAV-nGFP treated control lines will hopefully give us a greater
understanding of the co-localization speculations and of the effect of aging on FXTAS pathogenesis.
CHAPTER IV: METHODOLOGY

Experimental Conditions

There are a total of six conditions for this study. Two FXTAS and one control NSC lines were treated with adeno-associated virus (AAV)-progerin-GFP or AAV-nuclear GFP (nGFP) control. Immunocytochemistry (ICC) was performed.

Neural Stem Cell Culture

FXTAS neural stem cells (NSCs) (Generous gift, Dr. Nicolas Charlet-Berguerand) and control NSCs (Generous gift, Dr. Virginia Mattis) were expanded in suspension as neurospheres. Tissue culture flasks were used with no cell matrix treatment. The cells were expanded in NSC Maintenance Media: DMEM/F-12 (STEMCELL Technologies) supplemented with 1X SM1 (STEMCELL Technologies), 0.2 ng/mL Human Recombinant Epidermal Growth Factor (rhEGF) (Fisher Scientific), 2.0 ng/mL Human Recombinant Fibroblast Growth Factor (rhFGF) (STEMCELL Technologies), 0.5 U/mL Heparin (Millipore Sigma), 1X Penicillin/Streptomycin. The media was changed when a color change close to yellow was noted. Once the spheres reached about 1mm in size, they were manually passaged by placing the neurospheres in a petri dish with little media and using a sterile blade to chop them. They were incubated at 37°C and 5% CO₂ for the duration of expansion.
Neuronal Differentiation

The neurospheres in suspension were collected and passed through an 18-gauge needle attached to a syringe four times to break up the spheres. The cells were centrifuged at 1,000 RPM for 4 minutes. Following centrifugation, the supernatant was removed, and the pellet was resuspended in 12mL NSC maintenance media for plating a total of 24 wells of a 24 well plate. The media and pellet were pipetted 15 times using a 10mL serological pipette in order to homogenize the mixture. The cells were seeded onto coverslips (for ICC) and regular wells (for Western blot) coated in poly-L-lysine and matrigel\textsuperscript{21}. The media and cell mixture were evenly distributed between all of the wells for each line, 0.5 mL per well. The plates were left in an incubator at 37°C and 5% CO\textsubscript{2} overnight and checked the next day for survival. Another 0.5 mL of NSC maintenance media was added to the wells the next day. On day 5, half of the media was removed and replaced with BrainPhys Neuronal Differentiation Media (STEMCELL Technologies): BrainPhys basal media, 20 μL/mL SM1 (STEMCELL Technologies), 10 μL/mL N2 Supplement-A (STEMCELL Technologies), 20 ng/mL Human Recombinant Brain-Derived Neurotrophic Factor (rhBDNF) (STEMCELL Technologies), 20 ng/mL Human Recombinant Glial-Derived Neurotrophic Factor (rhGDNF) (STEMCELL Technologies), 1mM Dibutyryl-cAMP (STEMCELL Technologies), and 200 nM ascorbic acid (Fisher Scientific). A half-media change was performed every 2-3 days for 21 days for the neurons to reached maturity.
**Viral Transduction**

The progerin-GFP and nuclear GFP control were delivered to the cells using an adeno-associated virus. The virus was delivered on day 21 of neuronal differentiation and the cells were collected 9 days later. Preliminary experiments determined that the virus takes 4 days to reach full expression. Consistent with a study utilizing progerin-induced aging in Parkinson Disease neurons, the progerin was expressed for an additional 5 days.

**Immunocytochemistry**

The cells were fixed in 4% paraformaldehyde at room temperature for 30 minutes. After 3 5 min washes in PBS, the coverslips were incubated in blocking solution (1X PBS, 3% Bovine Serum Albumin (BSA), 5% Normal Goat Serum (NGS), and 0.3% Triton-X100) at room temperature for 30 minutes. The coverslips were incubated in the primary antibody solution at 4°C overnight. The primary antibody solution is the blocking solution containing the primary antibodies (see antibodies and dilutions below) Three more wash steps were performed using 1X PBS, each for 5 minutes. The coverslips were incubated in the secondary antibody solution (blocking solution with the secondary antibodies, see below) at room temperature for 2 hours, ensuring the samples were in complete darkness to protect the light-sensitive fluorophores. Three wash steps were performed using 1X PBS, each for 5 minutes. The coverslips were removed from the plates and transferred to a slide in groups of 3. The slide contained drops of ProLong® Gold Antifade mounting media containing DAPI (Thermo Fisher P36931) and coverslips were placed face-down. Visualization was done using a Zeiss LSM 710 confocal microscope and the ZEN imaging software. Analysis was done using ImageJ software.
Three sets of ICC were performed. The first set included antibodies for MAP2 (1:50, Invitrogen PA5-17646) and Beta III tubulin (1:300, Abcam ab131205) to investigate dendritic complexity and neuronal health. The second set was stained for DNA damage (15A3, 1:500, Abcam ab62623) and cleaved caspase 3 (1:400, Cell Signaling 9664) to investigate DNA damage and apoptosis. Greater than 3 foci were used as a benchmark to determine the presence of DNA damage. The final set was stained for FMRpolyG (1:100, Generous gift, Nicolas Charlet-Berguerand) and Lap2β (1:200, Millipore Sigma 06-1002) to investigate FXTAS pathology and nuclear lamina integrity. All three sets were also stained for GFP (1:2000, Abcam ab13970) to reveal progerin-GFP or nuclear-GFP control.

**Flow Cytometry**

One well of each of the two FXTAS lines treated with either progerin or the control were dissociated and run on a flow cytometer (BD Biosciences Accuri C6 Plus) to determine if separation of the neuronal network was possible. The dissociation solution (Dulbecco’s Phosphate Buffered Solution, 2% BSA, 25 mM HEPES, 5 mM MgCl₂, 0.2 mg/mL DNase 1) was added to each well. A large pipette tip was used to scrape the wells into 400µL of the solution and were collected into a 5mL snap cap tube. A 200µL wash was performed to collect any remaining cells. The cells in each tube were then passed through a 22-gauge needle 10 times to aid the dissociation. Each sample was passed through the flow cytometer to determine if a population of single cells was present using GFP fluorescent signal. This trial was performed to determine if this protocol could be used in Fluorescence Activated Cell Sorting (FACS) to separate GFP-positive cells for use in a Western blot.
APPENDIX: COPYRIGHT APPROVAL LETTERS
Dear Dr. Nicolas Charlet-Berguerand,

I am completing an Honors undergraduate thesis for a bachelor’s degree at the University of Central Florida entitled, “Investigating the role of the aging brain to Fragile X-associated tremor/ataxia syndrome.” I would like your permission to reprint in my thesis excerpts from the following:


This work involves studying a mutant protein called FMRpolyG that results from repeat-associated non-AUG translation in patients with an FMR1 pre-mutation CGG expansion. FMRpolyG is pathogenic in various cell types and in mice, and this study showed its interaction with nuclear membrane scaffolding proteins.

The excerpts to be reproduced are:

Figure 2. A Minimum of 60 Expanded CGG Repeats Is Required to Detect FMRpolyG, page 334.

(D) Immunofluorescence against the FMRpolyG N terminus and ubiquitin on brain sections (hippocampal area) of FXTAS or control individuals.

Figure 6. FMRpolyG Interacts with LAP2β and Alters Its Nuclear Localization, page 341.

(H) Immunofluorescence against FMRpolyG N terminus and LAP2β on brain sections (hippocampal area) of FXTAS patients of age-matched controls.

Figure S6. FMRpolyG alters LAP2β and lamin B1 nuclear organization, supplemental.

(E). Immunofluorescence against the N-terminal part of FMRpolyG (green, 8FM antibody) and lamin B1 (LMNB1, red) on brain sections (hippocampal area) of FXTAS patients or age-matched control individual.

Figure 7. LAP2β Rescues Neuronal Cell Death Induced by FMRpolyG, page 342.
(A). Upper, immunofluorescence against FMRpolyG N terminus and LAP2β on neuronal cultures differentiated 40 days from iPSCs of FXTAS patients or control individuals. Lower, quantification of LAP2β co-localization with FMRpolyG in neurons from iPSCs of FXTAS and control individuals.

(B). Upper, immunofluorescence against FMRpolyG N terminus and LMNB1 on neuronal cultures differentiated 40 days from iPSCs of FXTAS patients or control individuals. Lower, quantification of lamin B1 alteration in FMRpolyG-positive cells in neurons from iPSCs of FXTAS and control individuals.

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If these arrangements meet with your approval, please sign this letter where indicated below and return it to me. Thank you for your attention to this matter.

Sincerely,

Katlin Hencak
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PERMISSION GRANTED FOR THE USE REQUESTED ABOVE:

By: Nicolas Charlet

Date: 21 of June 2019
Dear Dr. Lorenz Studer,

I am completing an Honors undergraduate thesis for a bachelor’s degree at the University of Central Florida entitled, “Investigating the role of the aging brain to Fragile X-associated tremor/ataxia syndrome.” I would like your permission to reprint in my thesis excerpts from the following:


This work involves modeling Parkinson’s disease using induced pluripotent stem cells and aging them using progerin. The use of progerin to age various iPSC-derived cells reveals age-related features and suggests that progerin may be used to study late-onset diseases.

The excerpts to be reproduced are:

Figure 4. Progerin Overexpression Induces a Subset of the Fibroblast Age-Associated Signature in iPSC-mDA Neurons Derived from Both Young and Old Donors, page 698

(C). Progerin overexpression enhances nuclear folding and blebbing (as seen by lamin B2, pink) and increases DNA-damage accumulation (γH2AX) in both young and old donor-derived iPSC-mDA neurons.

(D). Flow Cytometry analysis of mitochondrial superoxide levels (MitoSOX) demonstrates increased mitochondrial dysfunction with progerin overexpression (n=3 independent RNA transfections of iPSC-DA neurons derived from independent iPSC clones).

Figure 5. Progerin Overexpression Elicits Features Consistent with Neuronal Aging in iPSC-mDA Neurons, page 699

(B). MAP2 immunocytochemistry reveals reduced intact dendrite lengths following overexpression of progerin in most but not all (insets) iPSC-mDA neurons derived from both young and old donors.

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Sincerely,

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Date: _____________June 18, 2019_____________
REFERENCES


