TITLE: **Repeat RNA toxicity drives ribosomal RNA processing defects in SCA2.**

Authors – Geena Skariah[1] and Roger L. Albin[1,2]
Affiliations – [1]Department of Neurology, University of Michigan, Ann Arbor, Michigan, USA; [2]Neurology Service and GRECC, VAAAHS, Ann Arbor, Michigan, USA.

Address correspondence to: geskaria@med.umich.edu

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/mds.28795

Spinocerebellar Ataxia type 2 (SCA2) is an autosomal dominant ataxia with no effective treatments. It is the second most prevalent form of dominant ataxia, accounting for approximately 15% of cases worldwide [1, 2]. Clinical features of SCA2 include gait ataxia, tremor, abnormal eye movements, peripheral neuropathy, and other multisystem features. SCA2 neuropathology is characterized by olivopontocerebellar atrophy with marked loss of Purkinje cells, and inferior olivary, pontocerebellar, and substantia nigra neuron loss [3-6].

SCA2 belongs to a growing class of nucleotide repeat expansion disorders arising from unstable repeats in the genome [7, 8]. In this issue of Movement Disorders, Pan P. Li et al. add to an expanding body of literature indicating that repeat expansion disorders are often characterized by plural molecular pathogenic mechanisms [9].

SCA2 is caused by expansion of CAG trinucleotide repeats in the first exon of the *ATXN2* gene, encoding a polyglutamine (polyQ) repeat containing protein. In individuals with SCA2, repeat numbers are in the range of 37-39 repeats, as opposed to a normal range of 13-23 repeats [10]. Intermediate repeat lengths of 27-33 CAG repeats are associated with increased risk for Amyotrophic Lateral Sclerosis (ALS) [11]. The exact function of ATXN2 protein is not yet known, but it is implicated in RNA processing and translation, stress granule assembly, endoplasmic reticular (ER) calcium response, and cytoskeletal reorganization [12]. The expanded CAG repeat in ATXN2 encodes an abnormal stretch of polyglutamine (polyQ) in the N-terminal region. In multiple polyQ disorders, these canonically translated repeat harboring proteins show gain of function toxicities separate from their normal cellular functions [13]. These pathogenic protein species may exert their effects via multiple proximate mechanisms [14]. In the case of the polyQ disorder Huntington disease (HD), haploinsufficiency may also contribute to neurodegeneration [7].

A second general molecular mechanism in nucleotide expansion disorders is the phenomenon of repeat-associated Non-AUG (RAN) translation, which gives rise to toxic repeat peptides due to aberrant translation from expanded repeat regions. RAN translation, however, from the expanded SCA2 transcript appears to be minimal and may not be a significant pathogenic process in SCA2 [15].

Pan P. Li et al. evaluated a third potential pathogenic molecular mechanism involving repeat containing RNA transcripts. In some nucleotide expansion disorders, such as Type 1 Myotonic Dystrophy, expanded repeats exert neurotoxicity at the RNA level by disrupting mRNA processing. One of the mechanisms of RNA mediated toxicity is sequestration of key RNA binding proteins (RBPs), making them unavailable for their normal cellular functions [16]. RNA toxicity is often accompanied by formation of RNA foci. These authors find that expanded sense *ATXN2* RNA transcripts cause neurotoxicity and disrupt ribosomal RNA processing. They present evidence that overexpression of an expanded *ATXN2* transcript, not undergoing RAN translation, shows increased Caspase 3/7 activity in human neuroblastoma cells, and shows increased toxicity in nuclear condensation assays performed with primary mouse cortical neurons. The authors go on to show that the mutant transcripts bearing either 58 or 104 CAG triplets form more RNA foci when transfected into cells. RNA foci were also detected in cerebellar Purkinje cells of SCA2 transgenic mice and in one out of five postmortem human patient brains.

To examine the possibility of RBP sequestration, the authors performed *in vitro* pull down of the mutant *ATXN2* transcript followed by Mass Spectrometry. They identified a number of RBPs with a predominant nuclear and nucleolar localization pattern, suggesting the nucleus as the possible site for aberrant interactions. Several of the sequestered RBPs are critical for maturation of the small rRNA subunit. The authors selected TBL3 (transducing β-like protein 3) for further analysis due to its known interaction with the expanded Huntingtin transcript, implicating a possible common disease mechanism in HD and SCA2. With a series of elegant biochemical studies, the authors demonstrate that TBL3 binds to the aberrant hairpin structure formed by continuous CAG repeats in the expanded repeat *ATXN2* transcript.

The yeast homolog of TBL3 plays a role in 35S rRNA processing and 18S rRNA biogenesis [17]. Based on these findings, Pan P. Li et al tested the levels of 45S pre-rRNA (human equivalent of yeast 35S pre-rRNA) and the ratios of mature 18S and 28S rRNA after TBL3 knockdown in HEK293T cells. They observed an increase in 45S pre-rRNA levels and a decrease in the ratio of mature 18S and 28S rRNA, suggesting defective rRNA processing and maturation, respectively, in the absence of TBL3. Similar trends, albeit without statistical significance, were observed in postmortem HD and SCA2 brains. Defects in ribosome biogenesis are reported in HD, accompanied by nucleolar aggregates [18, 19]. This study opens up lines of investigation into the possible role of ribosomal abnormalities in another neurodegenerative disorder [20]. Taken together, the report by Pan Li. P et al., along with their earlier finding of a toxic antisense *ATXN2* transcript, presents evidence for combinatorial protein-RNA driven pathology in SCA2 (Fig.1).

Neurodegenerative disorders such as SCA2 typically exhibit age-related penetrance. The observation that ribosome biogenesis may be affected in SCA2 is intriguing and with implications for what might happen in aging brains, characterized by decreased global protein translation [21, 22]. Many age-associated neurodegenerative disorders are due to dysfunctions in core components of the translation machinery [23]. Ribosomal proteins, as well as rRNA levels, are affected during healthy aging [24] and could conceivably change ribosomal assembly as well as mRNA translation dynamics. These age-associated changes could influence ribosomal and proteasomal subunit stoichiometry, altering global protein homeostasis [25]. These age-related changes might make neurons more susceptible to the toxic effects of expanded repeat transcripts.

Another characteristic aspect of neurodegenerative disorders, such as SCA2, is degeneration of specific neuronal subtypes. Single-cell transcriptomic analyses of the aging brain are beginning to unravel tissue and cell type specificities for components of the translation machinery, such as ribosomal proteins [26, 27], suggesting distinct post-transcriptional outcomes based on neuronal cell type. This might explain the susceptibility of neuronal subtypes in specific disease contexts. In addition, high-energy neuronal subtypes, such as dopaminergic neurons, are particularly susceptible to mitochondrial dysfunction, an emerging factor in multiple neurodegenerative disorders. [28, 29]

Moreover, the most affected transcripts in the aging human cortex are involved in synaptic function [30]. Since activity-mediated dynamic regulation of mRNA translation at synapses is a key factor for neuronal function and survival [31], sequestration of crucial molecules, such as TBL3 as reported here, could be particularly impactful for synaptic

mRNA translation. Ultimately leading to neuronal death, the initial manifestations would be synaptic dysfunction and degeneration, which are widely believed to be common features of many neurodegenerative disorders.

Within the context of an aging neuronal environment, the accumulation of expanded repeat containing RNA and proteins could trigger cascades of events that affect both arms of the peptide life cycle - synthesis and degradation - precipitating neurodegeneration [32].

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Indranil Malik for assistance with the figure and Dr. Peter Todd for helpful suggestions. Dr. Geena Skariah receives support from the Claude D. Pepper Older Adults Independence Center (AG024824), Michigan Alzheimer Disease Center (AG053760) and NIH grants to Dr. Todd (R01NS086810, R01NS099280 and P50HD104463). Dr. Albin receives support from P50NS123067 and the Parkinson's Foundation.

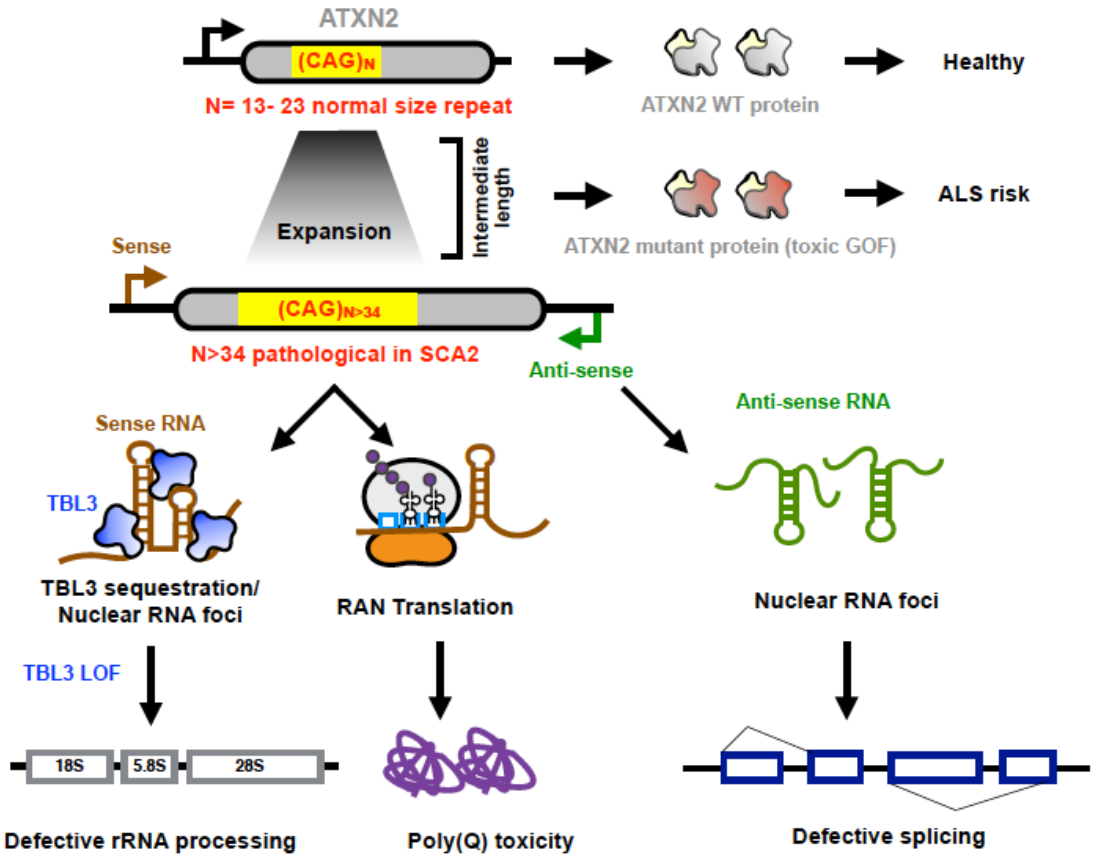

Figure 1. **Multiple modes of SCA2 pathogenesis.** Normal CAG repeat number in the *ATXN2* gene generates a WT protein that participates in a host of cellular functions. The intermediate repeat length is associated with ALS while the expanded CAG repeats in the *ATXN2* gene causes toxicity by RNA binding protein sequestration, Repeat-associated Non-AUG translation or via the *ATXN2*- Antisense (AS) transcript. Each of these aberrant processes cause dysregulation of downstream molecular pathways and could collectively contribute to neurodegeneration.

REFERENCES:

1. Pulst S-M, Nechiporuk A, Nechiporuk T, et al. Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. Nature Genetics 1996;14:269-276.
2. Imbert G, Saudou F, Yvert G, et al. Cloning of the gene for spinocerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats. Nature Genetics 1996;14:285-291.
3. Filla A, De Michele G, Santoro L, et al. Spinocerebellar ataxia type 2 in southern Italy: a clinical and molecular study of 30 families. J Neurol 1999;246:467-471.
4. Velázquez-Pérez L, Rodríguez-Labrada R, García-Rodríguez JC, Almaguer-Mederos LE, Cruz-Mariño T, Laffita-Mesa JM. A comprehensive review of spinocerebellar ataxia type 2 in Cuba. Cerebellum 2011;10:184-198.
5. Estrada R, Galarraga J, Orozco G, Nodarse A, Auburger G. Spinocerebellar ataxia 2 (SCA2): morphometric analyses in 11 autopsies. Acta Neuropathologica 1999;97:306-310.
6. Schöls L, Reimold M, Seidel K, et al. No parkinsonism in SCA2 and SCA3 despite severe neurodegeneration of the dopaminergic substantia nigra. Brain 2015;138:3316-3326.
7. Lieberman AP, Shakkottai VG, Albin RL. Polyglutamine Repeats in Neurodegenerative Diseases. Annu Rev Pathol 2019;14:1-27.
8. van Eyk CL, Richards RI. Dynamic mutations: where are they now? Adv Exp Med Biol 2012;769:55-77.
9. Li PP, Moulick R, Feng H, et al. RNA Toxicity and Perturbation of rRNA Processing in Spinocerebellar Ataxia Type 2. Mov Disord 2021.
10. Antenora A, Rinaldi C, Roca A, et al. The Multiple Faces of Spinocerebellar Ataxia type 2. Ann Clin Transl Neurol 2017;4:687-695.

11. Elden AC, Kim H-J, Hart MP, et al. Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS. Nature 2010;466:1069-1075.
12. Magaña JJ, Velázquez-Pérez L, Cisneros B. Spinocerebellar ataxia type 2: clinical presentation, molecular mechanisms, and therapeutic perspectives. Mol Neurobiol 2013;47:90-104.
13. Malik I, Kelley CP, Wang ET, Todd PK. Molecular mechanisms underlying nucleotide repeat expansion disorders. Nat Rev Mol Cell Biol 2021.
14. Jafar-Nejad P, Ward CS, Richman R, Orr HT, Zoghbi HY. Regional rescue of spinocerebellar ataxia type 1 phenotypes by 14-3-3epsilon haploinsufficiency in mice underscores complex pathogenicity in neurodegeneration. Proc Natl Acad Sci U S A 2011;108:2142-2147.
15. Scoles DR, Ho MH, Dansithong W, et al. Repeat Associated Non-AUG Translation (RAN Translation) Dependent on Sequence Downstream of the ATXN2 CAG Repeat. PLoS One 2015;10:e0128769.
16. Miller JW, Urbinati CR, Teng-Umnuay P, et al. Recruitment of human muscleblind proteins to (CUG)(n) expansions associated with myotonic dystrophy. Embo j 2000;19:4439-4448.
17. Dragon F, Gallagher JE, Compagnone-Post PA, et al. A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. Nature 2002;417:967-970.
18. Tsoi H, Chan HY. Roles of the nucleolus in the CAG RNA-mediated toxicity. Biochim Biophys Acta 2014;1842:779-784.
19. Lee J, Hwang YJ, Ryu H, Kowall NW, Ryu H. Nucleolar dysfunction in Huntington's disease. Biochim Biophys Acta 2014;1842:785-790.

20. Hetman M, Slomnicki LP. Ribosomal biogenesis as an emerging target of neurodevelopmental pathologies. J Neurochem 2019;148:325-347.
21. Tavernarakis N. Ageing and the regulation of protein synthesis: a balancing act? Trends Cell Biol 2008;18:228-235.
22. Rattan SI. Synthesis, modifications, and turnover of proteins during aging. Exp Gerontol 1996;31:33-47.
23. Kapur M, Ackerman SL. mRNA Translation Gone Awry: Translation Fidelity and Neurological Disease. Trends Genet 2018;34:218-231.
24. Curran SP, Ruvkun G. Lifespan regulation by evolutionarily conserved genes essential for viability. PLoS Genet 2007;3:e56.
25. Kelmer Sacramento E, Kirkpatrick JM, Mazzetto M, et al. Reduced proteasome activity in the aging brain results in ribosome stoichiometry loss and aggregation. Mol Syst Biol 2020;16:e9596.
26. Davie K, Janssens J, Koldere D, et al. A Single-Cell Transcriptome Atlas of the Aging Drosophila Brain. Cell 2018;174:982-998.e920.
27. Ximerakis M, Lipnick SL, Innes BT, et al. Single-cell transcriptomic profiling of the aging mouse brain. Nat Neurosci 2019;22:1696-1708.
28. Müller-Nedebock AC, van der Westhuizen FH, Kõks S, Bardien S. Nuclear Genes Associated with Mitochondrial DNA Processes as Contributors to Parkinson's Disease Risk. Mov Disord 2021;36:815-831.
29. Norat P, Soldozy S, Sokolowski JD, et al. Mitochondrial dysfunction in neurological disorders: Exploring mitochondrial transplantation. npj Regenerative Medicine 2020;5:22.
30. Dillman AA, Majounie E, Ding J, et al. Transcriptomic profiling of the human brain reveals that altered synaptic gene expression is associated with chronological aging. Sci Rep 2017;7:16890.

31. Kapur M, Monaghan CE, Ackerman SL. Regulation of mRNA Translation in Neurons-A Matter of Life and Death. Neuron 2017;96:616-637.
32. Skariah G, Todd PK. Translational control in aging and neurodegeneration. Wiley Interdiscip Rev RNA 2021;12:e1628.

ATXN2

$(CAG)_N$

N= 13- 23 normal size repeat

ATXN2 WT protein

Healthy

Expansion

Intermediate length

ATXN2 mutant protein (toxic GOF)

ALS risk

Sense

$(CAG)_{N>34}$

N>34 pathological in SCA2

Anti-sense

Sense RNA

TBL3

TBL3 sequestration/
Nuclear RNA foci

RAN Translation

Anti-sense RNA

Nuclear RNA foci

TBL3 LOF

18S 5.8S 28S

Defective rRNA processing

Poly(Q) toxicity

Defective splicing

COPYRIGHT TRANSFER AGREEMENT

Date: 8/25/2021

Contributor name: Roger L Albin

Contributor address: ralbin@med.umich.edu

Manuscript number: MDS-21-1047

Re: Manuscript entitled: Repear RNA toxicity drives ribosomal RNA processing defects in SCA2 (the "Contribution")

for publication in: Movement Disorders (the "Journal")

Published by Wiley on behalf of The International Parkinson and Movement Disorder Society (the "Owner")

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the editing and publishing process and enable the Owner to disseminate your Contribution to the fullest extent, we need to have this Copyright Transfer Agreement executed. If the Contribution is not accepted for publication, or if the Contribution is subsequently rejected, this Agreement shall be null and void. **Publication cannot proceed without a signed copy of this Agreement.**

A. COPYRIGHT

The Contributor assigns to the Owner, during the full term of copyright and any extensions or renewals, all copyright in and to the Contribution, and all rights therein, including but not limited to the right to reproduce, publish, republish, transmit, sell, transfer, distribute, and otherwise use the Contribution in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so.

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor's employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution. This reservation of rights does not affect or limit the rights assigned to Owner in Section A.

C. PERMITTED USES BY CONTRIBUTOR

1. License. The Owner grants to Contributor a non-exclusive, non-transferable and limited license to reproduce and distribute copies of the print or electronic "preprints" of the unpublished Contribution, in the original form submitted to the Journal prior to the peer review process, solely to colleagues within the Contributor's nonprofit organization or educational institution. The Contributor shall make no more than 100 printed copies of the preprints in any calendar year. Such preprints may be posted as electronic files on the Contributor's own personal website, on the Contributor's internal intranet at Contributor's nonprofit organization or educational institution, or on a secure external website at the Contributor's nonprofit organization or educational institution, provided that access is limited to employees and/or students at Contributor's non-profit organization or educational institution. Contributor shall not charge a fee for any

preprints, and Contributor's use under this Section C shall not be for any commercial purpose, or for any systematic external distribution (e.g., posting on a listserve, public website, database connected to a public access server, or automated delivery system). The license grant in this Section does not apply to for-profit corporations, and any proposed use outside of the scope of this Section C must be pre-approved in writing by the Owner. The rights granted to Contributor under this Section C do not include reproduction, distribution or any other use of rating scales, videos or other audiovisual materials associated with the Contribution.

2. Required Citation. Prior to publication, the Contributor must provide full credit and acknowledgement of the Journal in all preprints in the following format: This is a preprint of an article accepted for publication in [Journal Title], Copyright © [year] The International Parkinson and Movement Disorder Society. After publication, the Contributor must provide a citation to the Journal in all preprints in the following format: This is a preprint of an article that was published in [Journal title]: (Title of Article, Contributor, Journal Title and Volume/ Issue, Copyright © [year] The International Parkinson and Movement Disorder Society). An electronic link must be provided to the Journal's website, located at http://www.interscience.Wiley.com. The Contributor agrees not to update the preprint or replace it with the published version of the Contribution.

3. Accepted Version. Re-use of the accepted and peer-reviewed (but not the final typeset published) version of the Contribution (the "Accepted Version") is not permitted under this Agreement. There are separate arrangements with certain funding agencies governing reuse of the Accepted Version. Additional terms apply if the Contributor receives or received funding from these agencies. The details of those relationships, and other offerings allowing open web use, are set forth at the following website: http://www.wiley.com/go/funderstatement.

4. Additional Terms for Certain Funders. Certain funders, including the NIH, members of the Research Councils UK (RCUK) and Wellcome Trust require deposit of the Accepted Version in a public repository after an embargo period. Details of funding arrangements are set out at the following website: http://www.wiley.com/go/funderstatement. Additional terms may be applicable. Please contact the production editor for the journal at MDSprod@wiley.com if you have additional funding requirements.

If any Contributor receiving funds from applicable sources does not choose the Owner's OnlineOpen option, the Contributor will be allowed to self-archive by depositing the Accepted Version in a public repository after the following applicable embargo period has expired, subject to further conditions imposed by the RCUK:

a. 12 months from first publication online of the final published version of the Contribution for research funded by members of the Research Councils UK (RCUK) other than The Economic and Social Research Council (ESRC) and the Arts and Humanities Research Council (AHRC); or
b. 24 months from first publication online of the final published version of the Contribution for research funded by ESRC or AHRC.

5. Additional Terms for Certain Institutions. Wiley has arrangements with certain educational institutions to permit the deposit of the Accepted Version in the institutional repository after an embargo period. Details of such arrangements are set out at the following website: http://olabout.wiley.com/WileyCDA/Section/id-406074.html . Additional terms may be applicable.

If any Contributor affiliated with these applicable educational institutions does not choose the Owner's OnlineOpen option, the Contributor will be allowed to self-archive by depositing the Accepted Version in the educational institution's repository after the following applicable embargo period has expired. See the following website for details: http://olabout.wiley.com/WileyCDA/Section/id-817011.html.

D. CONTRIBUTIONS OWNED BY EMPLOYER

If the Contribution was written by the Contributor in the course of the Contributor's employment (as a "work-made-for-hire" in the course of employment), the Contribution is owned by the company/institution which must execute this Agreement (in addition to the Contributor's signature). In such case, the company/institution hereby assigns to the Owner, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in Section A above.

E. GOVERNMENT CONTRACTS

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government, U.K. Government, and other government employees: see notes at end.)

F. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that the Contribution is the Contributor's original work, all individuals identified as Contributors actually contributed to the Contribution, and all individuals who contributed are included. The Contribution is submitted only to this Journal and has not been published before. (If excerpts from copyrighted works owned by third parties are included, the Contributor will obtain written permission from the copyright owners for all uses as set forth in the Journal's Instructions for Contributors, and show credit to the sources in the Contribution.) The Contributor also warrants that the Contribution contains no libelous or unlawful statements, does not infringe upon the rights (including without limitation the copyright, patent or trademark rights) or the privacy of others, or contain material or instructions that might cause harm or injury. Upon request, Contributor will provide the data or will cooperating fully in obtaining and providing the data on which the Contribution is based for examination by the editors or their assignees.

G. FINANCIAL DISCLOSURES

The Contributor certifies that his/her financial and material support for this research and work, regardless of date, is clearly identified on Exhibit A to this Agreement. The Contributor has also identified on Exhibit A, all other support unrelated to this research, covering the past year from the date of submission (e.g., grants, advisory boards, employment, consultancies, contracts, honoraria, royalties, expert testimony, partnerships, or stock ownership in medically-related fields).

H. VIDEO AND PHOTOGRAPHY CONSENT

In the event that the Contribution includes, discloses or incorporates any content (including, without limitation, any video clip or photograph) which identifies any individual patient(s) ("patient identifiable content"), the Contributor obtained from such patient(s) written consent to such inclusion, disclosure or incorporation and that this consent fully complies with all legal requirements, including without limitation, all of the requirements of the laws of the jurisdiction(s) to which the patient(s) and the patient(s)' physician are subject, including the United States Health Insurance Portability and Accountability Act of 1996 ("HIPAA") if applicable. The Contributor hereby certifies that, if the patient consent form is in a language other than English, such consent form meets all of the requirements set forth in the Instructions to Authors. In addition, the Contributor hereby confirms that he/she obtained from patient(s) written consent to use the patient identifiable content in both print and online (i.e., internet/web-based) publication formats. The Contributor further certifies that the person executing any such patient consent form, to the best of his/her knowledge, had legal capacity under applicable law to execute the form on behalf of the patient.

I. ACKNOWLEDGEMENTS

The Contributor should obtain written permission from all individuals named in the acknowledgement since readers may infer their endorsement of data and conclusions. The Contributor certifies that all individuals named in the acknowledgement section have provided written permission to be named.

J. MISCELLANEOUS

This Agreement may be amended or modified only in a writing executed by both parties. The waiver or failure of any party to exercise any rights under this Agreement shall not be deemed a waiver or other limitation of any other right or any future right. This Agreement shall inure to the benefit of, and shall be binding upon, the parties, their respective successors and permitted assigns. This Agreement may be executed in two (2) or more counterparts, each of which shall be an original and all of which taken together shall constitute one and the same agreement. Executed copies of this Agreement may be delivered by facsimile transmission, pdf/email or other comparable electronic means. If for any reason any provision of this Agreement shall be deemed by a court of competent jurisdiction to be legally invalid or unenforceable, the validity, legality and enforceability of the remainder of this Agreement shall not be affected and such provision shall be deemed modified to the minimum extent necessary to make such provision consistent with applicable law and, in its modified form, such provision shall then be enforceable and enforced. The parties agree to do such further acts and to execute and deliver such additional agreements and instruments from time to time as either may at any time reasonably request in order to assure and confirm unto such requesting party the rights, powers and remedies conferred in the Agreement. This Agreement, including any exhibits attached hereto, contains the entire agreement and understanding of the parties with respect to the subject matter hereof, and supersedes all prior agreements, negotiations, representations and proposals, written and oral, relating thereto.

All Contributors must sign below. Contributors must check one box except that NIH grantees should check both Contributor-owned work and the NIH grantee box. If your Contribution was written during the course of employment, your employer must also sign where indicated.

Please send your original completed and signed forms by fax or email a scanned copy to the Journal production editor. For production editor contact details please visit the Journal's online author guidelines. **Do not send in hard copies of these forms.**

[✓] Contributor-owned work

Roger L. Albin, MD
Contributor's signature

8/25/21
Date

Roger L. Albin, Professor
Type or print name and title

Geena S
Co-Contributor's signature

8/25/21
Date

Geena Skariah, Research Fellow
Type or print name and title

[_____] Company/Institution-owned Work (made-for-hire in the Course of employment)

_____ Company or Institution (Employer-for-Hire) _____ Date

_____ Authorized signature of Employer _____ Date

_____ Contributor's signature _____ Date

_____ Type or print name and title

ATTACH ADDITIONAL SIGNATURE PAGES AS NECESSARY

[_____] U.S. Government work

Note to U.S. Government Employees

A contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. Government publication, is called a "U.S. Government work", and is in the public domain in the United States. In such case, Paragraph A.1 will not apply but the Contributor must type his/her name (in the Contributor's signature line) above. Contributor acknowledges that the Contribution will be published in the United States and other countries. If the Contribution was not prepared as part of the employee's duties or is not an official U.S. Government publication, it is not a U.S. Government work.

[_____] **U.K. Government work (Crown Copyright)**

Note to U.K. Government Employees

The rights in a contribution prepared by an employee of a UK government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown. Contributors must ensure they comply with departmental regulations and submit the appropriate authorisation to publish. If your status as a government employee legally prevents you from signing this Agreement, please contact the Journal production editor.

[_____] **Other**

Including Other Government work or Non-Governmental Organisation work

Note to Non-U.S., Non-U.K. Government Employees or Non-Governmental Organisation Employees

If your status as a government or non-governmental organisation employee legally prevents you from signing this Agreement, please contact the Journal production editor.

Exhibit A

Financial Disclosure

The Contributor has received financial and material support for this research and work regardless of date from the following sources:

Name: P50NS123067; Parkinson's Foundation

Address: Washington, DC; New York, NY

Type of support: grants

This material will be printed with the published article.

In the past year from the date of submission, the Contributor has also received the following support unrelated to this research (e.g., grants, advisory boards, employment, consultancies, contracts, honoraria, royalties, expert testimony, partnerships, or stock ownership in medically-related fields):

Name: DSMB Service - Signal-AD; M-Star; TANGO trials

Address: Vaccinex; Biohaven; Biogen

Type of support: consulting fees

This material will be posted on the journal website and may be printed at the Editors' discretion.

ATTACH ADDITIONAL INFORMATION AS NECESSARY

TITLE: **Repeat RNA toxicity drives ribosomal RNA processing defects in SCA2.**

Authors – Geena Skariah[1] and Roger L. Albin[1,2]
Affiliations – [1]Department of Neurology, University of Michigan, Ann Arbor, Michigan, USA; [2]Neurology Service and GRECC, VAAAHS, Ann Arbor, Michigan, USA.

Address correspondence to: geskaria@med.umich.edu

Spinocerebellar Ataxia type 2 (SCA2) is an autosomal dominant ataxia with no effective treatments. It is the second most prevalent form of dominant ataxia, accounting for approximately 15% of cases worldwide [1, 2]. Clinical features of SCA2 include gait ataxia, tremor, abnormal eye movements, peripheral neuropathy, and other multisystem features. SCA2 neuropathology is characterized by olivopontocerebellar atrophy with marked loss of Purkinje cells, and inferior olivary, pontocerebellar, and substantia nigra neuron loss [3-6].

SCA2 belongs to a growing class of nucleotide repeat expansion disorders arising from unstable repeats in the genome [7, 8]. In this issue of Movement Disorders, Pan P. Li et al. add to an expanding body of literature indicating that repeat expansion disorders are often characterized by plural molecular pathogenic mechanisms [9].

SCA2 is caused by expansion of CAG trinucleotide repeats in the first exon of the *ATXN2* gene, encoding a polyglutamine (polyQ) repeat containing protein. In individuals with SCA2, repeat numbers are in the range of 37-39 repeats, as opposed to a normal range of 13-23 repeats [10]. Intermediate repeat lengths of 27-33 CAG repeats are associated with increased risk for Amyotrophic Lateral Sclerosis (ALS) [11]. The exact function of ATXN2 protein is not yet known, but it is implicated in RNA processing and translation, stress granule assembly, endoplasmic reticular (ER) calcium response, and cytoskeletal reorganization [12]. The expanded CAG repeat in ATXN2 encodes an abnormal stretch of polyglutamine (polyQ) in the N-terminal region. In multiple polyQ disorders, these canonically translated repeat harboring proteins show gain of function toxicities separate from their normal cellular functions [13]. These pathogenic protein species may exert their effects via multiple proximate mechanisms [14]. In the case of the polyQ disorder Huntington disease (HD), haploinsufficiency may also contribute to neurodegeneration [7].

A second general molecular mechanism in nucleotide expansion disorders is the phenomenon of repeat-associated Non-AUG (RAN) translation, which gives rise to toxic repeat peptides due to aberrant translation from expanded repeat regions. RAN translation, however, from the expanded SCA2 transcript appears to be minimal and may not be a significant pathogenic process in SCA2 [15].

Pan P. Li et al. evaluated a third potential pathogenic molecular mechanism involving repeat containing RNA transcripts. In some nucleotide expansion disorders, such as Type 1 Myotonic Dystrophy, expanded repeats exert neurotoxicity at the RNA level by disrupting mRNA processing. One of the mechanisms of RNA mediated toxicity is sequestration of key RNA binding proteins (RBPs), making them unavailable for their normal cellular functions [16]. RNA toxicity is often accompanied by formation of RNA foci. These authors find that expanded sense *ATXN2* RNA transcripts cause neurotoxicity and disrupt ribosomal RNA processing. They present evidence that overexpression of an expanded *ATXN2* transcript, not undergoing RAN translation, shows increased Caspase 3/7 activity in human neuroblastoma cells, and shows increased toxicity in nuclear condensation assays performed with primary mouse cortical neurons. The authors go on to show that the mutant transcripts bearing either 58 or 104 CAG triplets form more RNA foci when transfected into cells. RNA foci were also detected in cerebellar Purkinje cells of SCA2 transgenic mice and in one out of five postmortem human patient brains.

To examine the possibility of RBP sequestration, the authors performed *in vitro* pull down of the mutant *ATXN2* transcript followed by Mass Spectrometry. They identified a number of RBPs with a predominant nuclear and nucleolar localization pattern, suggesting the nucleus as the possible site for aberrant interactions. Several of the sequestered RBPs are critical for maturation of the small rRNA subunit. The authors selected TBL3 (transducing β-like protein 3) for further analysis due to its known interaction with the expanded Huntingtin transcript, implicating a possible common disease mechanism in HD and SCA2. With a series of elegant biochemical studies, the authors demonstrate that TBL3 binds to the aberrant hairpin structure formed by continuous CAG repeats in the expanded repeat *ATXN2* transcript.

The yeast homolog of TBL3 plays a role in 35S rRNA processing and 18S rRNA biogenesis [17]. Based on these findings, Pan P. Li et al tested the levels of 45S pre-rRNA (human equivalent of yeast 35S pre-rRNA) and the ratios of mature 18S and 28S rRNA after TBL3 knockdown in HEK293T cells. They observed an increase in 45S pre-rRNA levels and a decrease in the ratio of mature 18S and 28S rRNA, suggesting defective rRNA processing and maturation, respectively, in the absence of TBL3. Similar trends, albeit without statistical significance, were observed in postmortem HD and SCA2 brains. Defects in ribosome biogenesis are reported in HD, accompanied by nucleolar aggregates [18, 19]. This study opens up lines of investigation into the possible role of ribosomal abnormalities in another neurodegenerative disorder [20]. Taken together, the report by Pan Li. P et al., along with their earlier finding of a toxic antisense *ATXN2* transcript, presents evidence for combinatorial protein-RNA driven pathology in SCA2 (Fig.1).

Neurodegenerative disorders such as SCA2 typically exhibit age-related penetrance. The observation that ribosome biogenesis may be affected in SCA2 is intriguing and with implications for what might happen in aging brains, characterized by decreased global protein translation [21, 22]. Many age-associated neurodegenerative disorders are due to dysfunctions in core components of the translation machinery [23]. Ribosomal proteins, as well as rRNA levels, are affected during healthy aging [24] and could conceivably change ribosomal assembly as well as mRNA translation dynamics. These age-associated changes could influence ribosomal and proteasomal subunit stoichiometry, altering global protein homeostasis [25]. These age-related changes might make neurons more susceptible to the toxic effects of expanded repeat transcripts.

Another characteristic aspect of neurodegenerative disorders, such as SCA2, is degeneration of specific neuronal subtypes. Single-cell transcriptomic analyses of the aging brain are beginning to unravel tissue and cell type specificities for components of the translation machinery, such as ribosomal proteins [26, 27], suggesting distinct post-transcriptional outcomes based on neuronal cell type. This might explain the susceptibility of neuronal subtypes in specific disease contexts. In addition, high-energy neuronal subtypes, such as dopaminergic neurons, are particularly susceptible to mitochondrial dysfunction, an emerging factor in multiple neurodegenerative disorders. [28, 29]

Moreover, the most affected transcripts in the aging human cortex are involved in synaptic function [30]. Since activity-mediated dynamic regulation of mRNA translation at synapses is a key factor for neuronal function and survival [31], sequestration of crucial molecules, such as TBL3 as reported here, could be particularly impactful for synaptic

mRNA translation. Ultimately leading to neuronal death, the initial manifestations would be synaptic dysfunction and degeneration, which are widely believed to be common features of many neurodegenerative disorders.

Within the context of an aging neuronal environment, the accumulation of expanded repeat containing RNA and proteins could trigger cascades of events that affect both arms of the peptide life cycle - synthesis and degradation - precipitating neurodegeneration [32].

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Indranil Malik for assistance with the figure and Dr. Peter Todd for helpful suggestions. Dr. Geena Skariah receives support from the Claude D. Pepper Older Adults Independence Center (AG024824), Michigan Alzheimer Disease Center (AG053760) and NIH grants to Dr. Todd (R01NS086810, R01NS099280 and P50HD104463). Dr. Albin receives support from P50NS123067 and the Parkinson's Foundation.

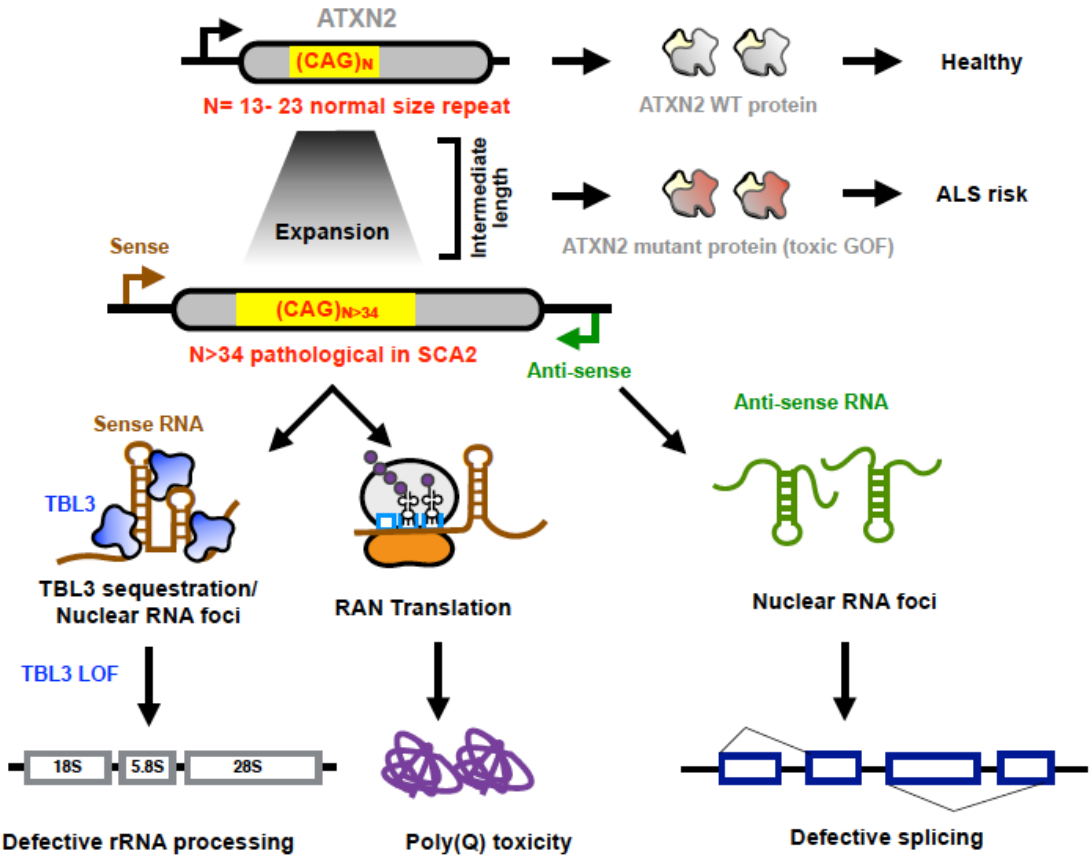

Figure 1. **Multiple modes of SCA2 pathogenesis.** Normal CAG repeat number in the *ATXN2* gene generates a WT protein that participates in a host of cellular functions. The intermediate repeat length is associated with ALS while the expanded CAG repeats in the *ATXN2* gene causes toxicity by RNA binding protein sequestration, Repeat-associated Non-AUG translation or via the *ATXN2*- Antisense (AS) transcript. Each of these aberrant processes cause dysregulation of downstream molecular pathways and could collectively contribute to neurodegeneration.

REFERENCES:

1. Pulst S-M, Nechiporuk A, Nechiporuk T, et al. Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. Nature Genetics 1996;14:269-276.
2. Imbert G, Saudou F, Yvert G, et al. Cloning of the gene for spinocerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats. Nature Genetics 1996;14:285-291.
3. Filla A, De Michele G, Santoro L, et al. Spinocerebellar ataxia type 2 in southern Italy: a clinical and molecular study of 30 families. J Neurol 1999;246:467-471.
4. Velázquez-Pérez L, Rodríguez-Labrada R, García-Rodríguez JC, Almaguer-Mederos LE, Cruz-Mariño T, Laffita-Mesa JM. A comprehensive review of spinocerebellar ataxia type 2 in Cuba. Cerebellum 2011;10:184-198.
5. Estrada R, Galarraga J, Orozco G, Nodarse A, Auburger G. Spinocerebellar ataxia 2 (SCA2): morphometric analyses in 11 autopsies. Acta Neuropathologica 1999;97:306-310.
6. Schöls L, Reimold M, Seidel K, et al. No parkinsonism in SCA2 and SCA3 despite severe neurodegeneration of the dopaminergic substantia nigra. Brain 2015;138:3316-3326.
7. Lieberman AP, Shakkottai VG, Albin RL. Polyglutamine Repeats in Neurodegenerative Diseases. Annu Rev Pathol 2019;14:1-27.
8. van Eyk CL, Richards RI. Dynamic mutations: where are they now? Adv Exp Med Biol 2012;769:55-77.
9. Li PP, Moulick R, Feng H, et al. RNA Toxicity and Perturbation of rRNA Processing in Spinocerebellar Ataxia Type 2. Mov Disord 2021.
10. Antenora A, Rinaldi C, Roca A, et al. The Multiple Faces of Spinocerebellar Ataxia type 2. Ann Clin Transl Neurol 2017;4:687-695.

11. Elden AC, Kim H-J, Hart MP, et al. Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS. Nature 2010;466:1069-1075.
12. Magaña JJ, Velázquez-Pérez L, Cisneros B. Spinocerebellar ataxia type 2: clinical presentation, molecular mechanisms, and therapeutic perspectives. Mol Neurobiol 2013;47:90-104.
13. Malik I, Kelley CP, Wang ET, Todd PK. Molecular mechanisms underlying nucleotide repeat expansion disorders. Nat Rev Mol Cell Biol 2021.
14. Jafar-Nejad P, Ward CS, Richman R, Orr HT, Zoghbi HY. Regional rescue of spinocerebellar ataxia type 1 phenotypes by 14-3-3epsilon haploinsufficiency in mice underscores complex pathogenicity in neurodegeneration. Proc Natl Acad Sci U S A 2011;108:2142-2147.
15. Scoles DR, Ho MH, Dansithong W, et al. Repeat Associated Non-AUG Translation (RAN Translation) Dependent on Sequence Downstream of the ATXN2 CAG Repeat. PLoS One 2015;10:e0128769.
16. Miller JW, Urbinati CR, Teng-Umnuay P, et al. Recruitment of human muscleblind proteins to (CUG)(n) expansions associated with myotonic dystrophy. Embo j 2000;19:4439-4448.
17. Dragon F, Gallagher JE, Compagnone-Post PA, et al. A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. Nature 2002;417:967-970.
18. Tsoi H, Chan HY. Roles of the nucleolus in the CAG RNA-mediated toxicity. Biochim Biophys Acta 2014;1842:779-784.
19. Lee J, Hwang YJ, Ryu H, Kowall NW, Ryu H. Nucleolar dysfunction in Huntington's disease. Biochim Biophys Acta 2014;1842:785-790.

20. Hetman M, Slomnicki LP. Ribosomal biogenesis as an emerging target of neurodevelopmental pathologies. J Neurochem 2019;148:325-347.
21. Tavernarakis N. Ageing and the regulation of protein synthesis: a balancing act? Trends Cell Biol 2008;18:228-235.
22. Rattan SI. Synthesis, modifications, and turnover of proteins during aging. Exp Gerontol 1996;31:33-47.
23. Kapur M, Ackerman SL. mRNA Translation Gone Awry: Translation Fidelity and Neurological Disease. Trends Genet 2018;34:218-231.
24. Curran SP, Ruvkun G. Lifespan regulation by evolutionarily conserved genes essential for viability. PLoS Genet 2007;3:e56.
25. Kelmer Sacramento E, Kirkpatrick JM, Mazzetto M, et al. Reduced proteasome activity in the aging brain results in ribosome stoichiometry loss and aggregation. Mol Syst Biol 2020;16:e9596.
26. Davie K, Janssens J, Koldere D, et al. A Single-Cell Transcriptome Atlas of the Aging Drosophila Brain. Cell 2018;174:982-998.e920.
27. Ximerakis M, Lipnick SL, Innes BT, et al. Single-cell transcriptomic profiling of the aging mouse brain. Nat Neurosci 2019;22:1696-1708.
28. Müller-Nedebock AC, van der Westhuizen FH, Kõks S, Bardien S. Nuclear Genes Associated with Mitochondrial DNA Processes as Contributors to Parkinson's Disease Risk. Mov Disord 2021;36:815-831.
29. Norat P, Soldozy S, Sokolowski JD, et al. Mitochondrial dysfunction in neurological disorders: Exploring mitochondrial transplantation. npj Regenerative Medicine 2020;5:22.
30. Dillman AA, Majounie E, Ding J, et al. Transcriptomic profiling of the human brain reveals that altered synaptic gene expression is associated with chronological aging. Sci Rep 2017;7:16890.

31. Kapur M, Monaghan CE, Ackerman SL. Regulation of mRNA Translation in Neurons-A Matter of Life and Death. Neuron 2017;96:616-637.
32. Skariah G, Todd PK. Translational control in aging and neurodegeneration. Wiley Interdiscip Rev RNA 2021;12:e1628.

COPYRIGHT TRANSFER AGREEMENT

Date: 8/27/2021

Contributor name: Geena Skariah

Contributor address: geskaria@med.umich.edu

Manuscript number: MDS-21-1047

Re: Manuscript entitled: Repeat RNA toxicity drives ribosomal RNA processing defects in SCA2 (the "Contribution")

for publication in: Movement Disorders (the "Journal")

Published by Wiley on behalf of The International Parkinson and Movement Disorder Society (the "Owner")

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the editing and publishing process and enable the Owner to disseminate your Contribution to the fullest extent, we need to have this Copyright Transfer Agreement executed. If the Contribution is not accepted for publication, or if the Contribution is subsequently rejected, this Agreement shall be null and void. **Publication cannot proceed without a signed copy of this Agreement.**

A. COPYRIGHT

The Contributor assigns to the Owner, during the full term of copyright and any extensions or renewals, all copyright in and to the Contribution, and all rights therein, including but not limited to the right to reproduce, publish, republish, transmit, sell, transfer, distribute, and otherwise use the Contribution in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so.

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor's employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution. This reservation of rights does not affect or limit the rights assigned to Owner in Section A.

C. PERMITTED USES BY CONTRIBUTOR

1. License. The Owner grants to Contributor a non-exclusive, non-transferable and limited license to reproduce and distribute copies of the print or electronic "preprints" of the unpublished Contribution, in the original form submitted to the Journal prior to the peer review process, solely to colleagues within the Contributor's nonprofit organization or educational institution. The Contributor shall make no more than 100 printed copies of the preprints in any calendar year. Such preprints may be posted as electronic files on the Contributor's own personal website, on the Contributor's internal intranet at Contributor's nonprofit organization or educational institution, or on a secure external website at the Contributor's nonprofit organization or educational institution, provided that access is limited to employees and/or students at Contributor's non-profit organization or educational institution. Contributor shall not charge a fee for any

preprints, and Contributor's use under this Section C shall not be for any commercial purpose, or for any systematic external distribution (e.g., posting on a listserve, public website, database connected to a public access server, or automated delivery system). The license grant in this Section does not apply to for-profit corporations, and any proposed use outside of the scope of this Section C must be pre-approved in writing by the Owner. The rights granted to Contributor under this Section C do not include reproduction, distribution or any other use of rating scales, videos or other audiovisual materials associated with the Contribution.

2. Required Citation. Prior to publication, the Contributor must provide full credit and acknowledgement of the Journal in all preprints in the following format: This is a preprint of an article accepted for publication in [Journal Title], Copyright © [year] The International Parkinson and Movement Disorder Society. After publication, the Contributor must provide a citation to the Journal in all preprints in the following format: This is a preprint of an article that was published in [Journal title]: (Title of Article, Contributor, Journal Title and Volume/ Issue, Copyright © [year] The International Parkinson and Movement Disorder Society). An electronic link must be provided to the Journal's website, located at http://www.interscience.Wiley.com. The Contributor agrees not to update the preprint or replace it with the published version of the Contribution.

3. Accepted Version. Re-use of the accepted and peer-reviewed (but not the final typeset published) version of the Contribution (the "Accepted Version") is not permitted under this Agreement. There are separate arrangements with certain funding agencies governing reuse of the Accepted Version. Additional terms apply if the Contributor receives or received funding from these agencies. The details of those relationships, and other offerings allowing open web use, are set forth at the following website: http://www.wiley.com/go/funderstatement.

4. Additional Terms for Certain Funders. Certain funders, including the NIH, members of the Research Councils UK (RCUK) and Wellcome Trust require deposit of the Accepted Version in a public repository after an embargo period. Details of funding arrangements are set out at the following website: http://www.wiley.com/go/funderstatement. Additional terms may be applicable. Please contact the production editor for the journal at MDSprod@wiley.com if you have additional funding requirements.

If any Contributor receiving funds from applicable sources does not choose the Owner's OnlineOpen option, the Contributor will be allowed to self-archive by depositing the Accepted Version in a public repository after the following applicable embargo period has expired, subject to further conditions imposed by the RCUK:

a. 12 months from first publication online of the final published version of the Contribution for research funded by members of the Research Councils UK (RCUK) other than The Economic and Social Research Council (ESRC) and the Arts and Humanities Research Council (AHRC); or
b. 24 months from first publication online of the final published version of the Contribution for research funded by ESRC or AHRC.

5. Additional Terms for Certain Institutions. Wiley has arrangements with certain educational institutions to permit the deposit of the Accepted Version in the institutional repository after an embargo period. Details of such arrangements are set out at the following website: http://olabout.wiley.com/WileyCDA/Section/id-406074.html . Additional terms may be applicable.

If any Contributor affiliated with these applicable educational institutions does not choose the Owner's OnlineOpen option, the Contributor will be allowed to self-archive by depositing the Accepted Version in the educational institution's repository after the following applicable embargo period has expired. See the following website for details: http://olabout.wiley.com/WileyCDA/Section/id-817011.html.

D. CONTRIBUTIONS OWNED BY EMPLOYER

If the Contribution was written by the Contributor in the course of the Contributor's employment (as a "work-made-for-hire" in the course of employment), the Contribution is owned by the company/institution which must execute this Agreement (in addition to the Contributor's signature). In such case, the company/institution hereby assigns to the Owner, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in Section A above.

E. GOVERNMENT CONTRACTS

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government, U.K. Government, and other government employees: see notes at end.)

F. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that the Contribution is the Contributor's original work, all individuals identified as Contributors actually contributed to the Contribution, and all individuals who contributed are included. The Contribution is submitted only to this Journal and has not been published before. (If excerpts from copyrighted works owned by third parties are included, the Contributor will obtain written permission from the copyright owners for all uses as set forth in the Journal's Instructions for Contributors, and show credit to the sources in the Contribution.) The Contributor also warrants that the Contribution contains no libelous or unlawful statements, does not infringe upon the rights (including without limitation the copyright, patent or trademark rights) or the privacy of others, or contain material or instructions that might cause harm or injury. Upon request, Contributor will provide the data or will cooperating fully in obtaining and providing the data on which the Contribution is based for examination by the editors or their assignees.

G. FINANCIAL DISCLOSURES

The Contributor certifies that his/her financial and material support for this research and work, regardless of date, is clearly identified on Exhibit A to this Agreement. The Contributor has also identified on Exhibit A, all other support unrelated to this research, covering the past year from the date of submission (e.g., grants, advisory boards, employment, consultancies, contracts, honoraria, royalties, expert testimony, partnerships, or stock ownership in medically-related fields).

H. VIDEO AND PHOTOGRAPHY CONSENT

In the event that the Contribution includes, discloses or incorporates any content (including, without limitation, any video clip or photograph) which identifies any individual patient(s) ("patient identifiable content"), the Contributor obtained from such patient(s) written consent to such inclusion, disclosure or incorporation and that this consent fully complies with all legal requirements, including without limitation, all of the requirements of the laws of the jurisdiction(s) to which the patient(s) and the patient(s)' physician are subject, including the United States Health Insurance Portability and Accountability Act of 1996 ("HIPAA") if applicable. The Contributor hereby certifies that, if the patient consent form is in a language other than English, such consent form meets all of the requirements set forth in the Instructions to Authors. In addition, the Contributor hereby confirms that he/she obtained from patient(s) written consent to use the patient identifiable content in both print and online (i.e., internet/web-based) publication formats. The Contributor further certifies that the person executing any such patient consent form, to the best of his/her knowledge, had legal capacity under applicable law to execute the form on behalf of the patient.

I. ACKNOWLEDGEMENTS

The Contributor should obtain written permission from all individuals named in the acknowledgement since readers may infer their endorsement of data and conclusions. The Contributor certifies that all individuals named in the acknowledgement section have provided written permission to be named.

J. MISCELLANEOUS

This Agreement may be amended or modified only in a writing executed by both parties. The waiver or failure of any party to exercise any rights under this Agreement shall not be deemed a waiver or other limitation of any other right or any future right. This Agreement shall inure to the benefit of, and shall be binding upon, the parties, their respective successors and permitted assigns. This Agreement may be executed in two (2) or more counterparts, each of which shall be an original and all of which taken together shall constitute one and the same agreement. Executed copies of this Agreement may be delivered by facsimile transmission, pdf/email or other comparable electronic means. If for any reason any provision of this Agreement shall be deemed by a court of competent jurisdiction to be legally invalid or unenforceable, the validity, legality and enforceability of the remainder of this Agreement shall not be affected and such provision shall be deemed modified to the minimum extent necessary to make such provision consistent with applicable law and, in its modified form, such provision shall then be enforceable and enforced. The parties agree to do such further acts and to execute and deliver such additional agreements and instruments from time to time as either may at any time reasonably request in order to assure and confirm unto such requesting party the rights, powers and remedies conferred in the Agreement. This Agreement, including any exhibits attached hereto, contains the entire agreement and understanding of the parties with respect to the subject matter hereof, and supersedes all prior agreements, negotiations, representations and proposals, written and oral, relating thereto.

All Contributors must sign below. Contributors must check one box except that NIH grantees should check both Contributor-owned work and the NIH grantee box. If your Contribution was written during the course of employment, your employer must also sign where indicated.

Please send your original completed and signed forms by fax or email a scanned copy to the Journal production editor. For production editor contact details please visit the Journal's online author guidelines. **Do not send in hard copies of these forms.**

[✓] Contributor-owned work

Geena S

Contributor's signature

8/27/21

Date

Geena Skariah, Research fellow

Type or print name and title

Co-Contributor's signature

Date

Type or print name and title

[_____] Company/Institution-owned Work (made-for-hire in the Course of employment)

_____ Company or Institution (Employer-for-Hire) _____ Date

_____ Authorized signature of Employer _____ Date

_____ Contributor's signature _____ Date

_____ Type or print name and title

ATTACH ADDITIONAL SIGNATURE PAGES AS NECESSARY

[_____] **U.S. Government work**

Note to U.S. Government Employees

A contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. Government publication, is called a "U.S. Government work", and is in the public domain in the United States. In such case, Paragraph A.1 will not apply but the Contributor must type his/her name (in the Contributor's signature line) above. Contributor acknowledges that the Contribution will be published in the United States and other countries. If the Contribution was not prepared as part of the employee's duties or is not an official U.S. Government publication, it is not a U.S. Government work.

[_____] **U.K. Government work (Crown Copyright)**

Note to U.K. Government Employees

The rights in a contribution prepared by an employee of a UK government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown. Contributors must ensure they comply with departmental regulations and submit the appropriate authorisation to publish. If your status as a government employee legally prevents you from signing this Agreement, please contact the Journal production editor.

[_____] **Other**

Including Other Government work or Non-Governmental Organisation work

Note to Non-U.S., Non-U.K. Government Employees or Non-Governmental Organisation Employees

If your status as a government or non-governmental organisation employee legally prevents you from signing this Agreement, please contact the Journal production editor.

Exhibit A

Financial Disclosure

The Contributor has received financial and material support for this research and work regardless of date from the following sources:

Name: Claude. D. Pepper OAIC, MADC, NIH

Address: University of Michigan, Ann Arbor, MI; Bethesda, Maryland,US

Type of support: grants

This material will be printed with the published article.

In the past year from the date of submission, the Contributor has also received the following support unrelated to this research (e.g., grants, advisory boards, employment, consultancies, contracts, honoraria, royalties, expert testimony, partnerships, or stock ownership in medically-related fields):

Name: _____

Address: _____

Type of support: _____

This material will be posted on the journal website and may be printed at the Editors' discretion.

ATTACH ADDITIONAL INFORMATION AS NECESSARY