Endocytic Vesicle Rupture in the Pathogenesis and Propagation of Neurodegenerative Proteinopathies

William P. Flavin
Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

ENDOCYTIC VESICLE RUPTURE IN THE PATHOGENESIS AND PROPAGATION OF NEURODEGENERATIVE PROTEINOPATHIES

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CELL BIOLOGY, NEUROBIOLOGY AND ANATOMY

BY
WILLIAM P. FLAVIN

CHICAGO, ILLINOIS

MAY 2017
ACKNOWLEDGEMENTS

I would first and foremost like to acknowledge my wife Ashley, for her never-ending love and support. Without her I wouldn’t be who I am today. Her constant inspiration, patience, and encouragement have helped me to overcome numerous obstacles during my academic journey, and I am forever grateful to have her as a partner who is always there for me, cheering me on through every stage of my training. I am incredibly lucky to share my life with such a caring, thoughtful, and selfless person. Our relationship is the foundation of everything we do in life, and I am very thankful to have her by my side.

I would also like to acknowledge my daughter Mae Elizabeth, who has brightened our lives with a joy that is the overflowing of love. She has made each day exciting, and gives meaning and purpose to the life we are building as a family. She is an incredible blessing, and getting to know her over the last two months has been wonderful. Spending time with Ashley and Mae is always the best part of my day, as my family is my greatest source of fulfillment in life.

I would like to acknowledge my father, Michael T. Flavin Ph.D., for the inspiration and perseverance to pursue a career in biomedical research and medicine. He was, and still is, my first science teacher, inspiring a curiosity in me that propelled me to become interested in science and technology and apply my mind toward the study of
biochemistry, cell biology, and medicine. I will never forget the passion for science he fostered in me beginning with my science fair project in 7th grade up until the present. He is always encouraging me to work hard, to broaden my perspective so as to consider diverse viewpoints, and to work for an application of research to improve human health. For his influence as an excellent role model, I am forever grateful.

I would like to acknowledge my mother Karen and siblings Marty, Pat, and Mary Clare for supporting me and motivating me to be my best at everything in life. They have been there for me throughout my development and I could not have asked for a better support system. I have my family to thank for always remembering to keep a positive outlook on life, and I am grateful for the example set by my mother who treats everyone with kindness and respect and goes out of her way to help those in need.

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I would like to acknowledge my mentor Edward M. Campbell Ph.D., for his advice, support, and teaching over the years we have worked together. He has trained me as the scientist I have become, and I am forever grateful for his example of a fun-loving attitude, willingness to collaborate widely, and perseverance to build our project
from the ground up. My experience in the lab has been amazingly educational, and I know that I will have success in the future because of the foundation I have learned under Ed’s teaching.

I would like to acknowledge David Freeman M.D., Ph.D., for his teaching and example of how to be successful in research and medical training. I am grateful for his courage to take on this project when the future was so unknown, and for the foundation he built of assays and techniques that I could learn from and build upon. I am thankful for his willingness to train me in taking on this project, and for his teaching of how to conduct experiments with both efficiency and accuracy.

I would like to acknowledge other lab members who have contributed to my project over the years, including Stratos Skarpathiotis M.D., Zachary C. Green M.S., Michael Sobieraj, Sean C. Liebscher, Oksana Zhurbich, Michael J. Chaney, Kevin Burbidge, Jonathan London M.S., and Rudy Cedillos M.S. As members of the Neuro team we have often had the added challenge of pioneering protocols in the lab, and the success of the overall project would not have occurred without the dedication of so many of these colleagues. Furthermore, I am grateful for the influence of lab members who have worked on the HIV team, including Adarsh Dharan Ph.D., Sarah Talley M.S., Sabrina Imam, Jared Weingart, Alex Simon, Zana Lukic Ph.D., Sam Choyke, Laura Johnsen M.S., Rachel Nelson M.S., and Santanu Mukherjee Ph.D. Our interdisciplinary discussions during lab meetings and in the lab have helped me to consider approaches
from beyond my own expertise, and I have grown as a scientist because of their influence.

I would like to acknowledge our collaborators, without whom our project would not have gotten off the ground and who have each elevated the quality and impact of the findings we have made. Beginning with Christopher Wiethoff Ph.D., I am grateful for his collaboration with Ed that brought the vesicle rupture assay from the world of infectious disease into the realm of prion-like neurodegenerative proteinopathies. He was also very helpful with various biochemical and protein purification approaches that benefitted our project immensely in the early years. Next, I acknowledge the help of Anurag Tandon Ph.D., whose collaboration got me started with the alpha-synuclein project after Dave completed his work. I am immensely grateful for the collaboration and expertise of Ronald Melki Ph.D. and Luc Bousset Ph.D., without whom my project would have been very limited in its scope and impact. Their friendship, teaching, and expertise in biochemical and biophysical techniques was an incredible asset to our work, and I will never forget beginning this collaboration by meeting Ronald at the Grand Challenges in Parkinson’s Disease Conference where he was so friendly and willing to work with us. Finally, I would like to acknowledge the advice, mentorship, support, and collaboration of Jeffrey H. Kordower Ph.D., who also served as a member of my dissertation committee, and Yaping Chu Ph.D., whose collaboration helped us to broaden the scope of our project by working with both induced pluripotent stem cell-derived dopaminergic neurons and with PD brain tissue. I have learned an incredible
amount about the PD field from both Jeff and Yaping, and I am grateful for their influence as experts in the field.

I would like to acknowledge the help, guidance, mentorship, and encouragement provided to me by the remaining members of my dissertation committee. Phong T. Le Ph.D. served as both the chair of my committee as well as my graduate program director in the CBNA/ICB program, and I could not have asked for a better mentor and guide through the stages of graduate education and research. Lydia L. DonCarlos Ph.D. and Michael A. Collins Ph.D. each also provided expertise and diverse perspectives from their respective neuroscience disciplines, and I am grateful for their thoughtful questions and the dedication with which they approached my mentorship.

I would like to acknowledge the guidance and encouragement provided to me by various teachers along my educational journey, without whom I would not be where I am today. Beginning at Our Lady of Peace elementary school, I am grateful to Darlene Gianotti and Patricia Krein for their science education that made the subject interesting to me. I am also grateful for the teaching of Carol Bruning, Robert Szorc, and Mary Margaret Eraci at Benet Academy, where my love for the sciences grew and I was encouraged to take an active role in exploration. Next, I began my research training under the guidance of Aye Aye Mar M.S., David Eiznhamer Ph.D., Ze-Qi Xu Ph.D., my uncle John Flavin, my aunt Nancy Tyrrell, and my grandfather Thomas Flavin at Advanced Life Sciences, Inc, where I received a foundation in research training in synthetic organic chemistry. I would not have become interested in a combined career
in research and medicine without the subsequent influence and mentorship of Mae J. Ciancio Ph.D., Mark W. Musch Ph.D., and Eugene B. Chang, M.D., who taught me about the academic approach to research in the biomedical sciences. I am grateful for the teaching of Seth N. Brown Ph.D., Olaf Wiest Ph.D., Richard Taylor Ph.D., Brian M. Baker Ph.D., Paul W. Huber Ph.D., Gregory Crawford Ph.D., Steven Wietstock Ph.D., Marvin J. Miller Ph.D., and Zachary Schafer Ph.D., who encouraged my development as a scientist at Notre Dame. Finally, I would like to acknowledge the teaching of Frederick Wezeman Ph.D., Michael Dauzvardis Ph.D., John Shea M.D., John Clancy Ph.D., John Robinson M.D., and John Lee M.D., Ph.D. at Loyola who have helped me to hone my scientific interests and deepen my knowledge of biology and medicine. I have been blessed at each step of my scientific training with incredible teachers, and I would not have made it to this point without their help.

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Medicine and Loyola University Chicago. This has been and will continue to be a wonderful experience, and I am very thankful for their help and encouragement along the way.

I would like to acknowledge the help of Ann Kennedy in the CBNA/ICB program for administrative support, as well as Linda Fox Ph.D., Karen Wielgus, Pat Simms, and Ashley Hess in Core Imaging, Animal, and Flow Cytometry facilities for their help along the way.

Lastly, I would like to acknowledge grant support from the ARCS Foundation, the Arthur J. Schmitt Foundation, and the Michael J. Fox Foundation. Thank you for believing in us and our project. We will continue to make the most of funding opportunities so that our work will benefit patients in the future.
To Ashley, for her unending love

To Lois Flavin, John Shea M.D., and all people living with Parkinson’s disease

To God, through whom all things are possible

Ad majorem dei gloriam
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<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>$\alpha$-syn</td>
<td>$\alpha$-synuclein</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>1N3R</td>
<td>tau isoform composed of 1 N-terminal insert and 3 C-terminal repeats</td>
</tr>
<tr>
<td>1N4R</td>
<td>tau isoform composed of 1 N-terminal insert and 4 C-terminal repeats</td>
</tr>
<tr>
<td>Aβ</td>
<td>$\beta$-amyloid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic-force microscopy</td>
</tr>
<tr>
<td>AGD</td>
<td>agyrophilic grain disease</td>
</tr>
<tr>
<td>ALP</td>
<td>autophagy-lysosome pathway</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>Atg</td>
<td>autophagy-related protein</td>
</tr>
<tr>
<td>Baf</td>
<td>bafilomycin A1</td>
</tr>
<tr>
<td>BiFC</td>
<td>bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSE</td>
<td>bovine spongiform encephalopathy</td>
</tr>
<tr>
<td>CatB</td>
<td>cathepsin B</td>
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<tr>
<td>CatD</td>
<td>cathepsin D</td>
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</table>
CBD
corticobasal degeneration

CDI
Cellular Dynamics International

chGal3
mCherry-galectin 3

CHMP2B
charged multivesicular body protein 2B

CJD
Creutzfeldt-Jakob disease

CK1
casein kinase 1

CK2
casein kinase 2

cm
centimeter

CMA
chaperone-mediated autophagy

CNS
central nervous system

CPP
calcium phosphate precipitates

CRD
carbohydrate recognition domain

CSF
cerebrospinal fluid

CTE
chronic traumatic encephalopathy

DA
dopamine

DABCO
1,4-Diazabicyclo[2.2.2]octane

DAMP
damage associated molecular pattern

DAPI
4′,6-diamidine-2′-phenylindole

DBS
deep brain stimulation

DLB
Dementia with Lewy Bodies

DMEM
Dulbecco’s modified Eagle’s medium

DMN
dorsal motor nucleus

DMSO
dimethyl sulfoxide

DTT
dithiothreitol

xviii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
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<tbody>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ELDR</td>
<td>endolysosomal damage response</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ESCRT</td>
<td>endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>EMCCD</td>
<td>electron multiplying charged coupled device</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>FTDP-17T</td>
<td>frontotemporal dementia and parkinsonism linked to chromosome 17</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<td>Gal3</td>
<td>galectin 3</td>
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<td>Gal8</td>
<td>galectin 8</td>
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<td>GCase</td>
<td>β-glucocerebrosidase</td>
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<td>GCI</td>
<td>glial cytoplasmic inclusion</td>
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<td>GI</td>
<td>gastrointestinal</td>
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<td>GlcCer</td>
<td>glucosylceramide</td>
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<td>globus pallidus internus</td>
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<tr>
<td>GRK2</td>
<td>G-protein-coupled receptor kinase 2</td>
</tr>
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<td>GRK5</td>
<td>G-protein-coupled receptor kinase 5</td>
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<tr>
<td>GSS</td>
<td>Gerstmann-Straussler-Scheinker</td>
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<tr>
<td>GWAS</td>
<td>genome-wide association study</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<td>H&amp;Y</td>
<td>Hoehn and Yahr</td>
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HD
Huntington’s disease

hiPSC
human induced pluripotent stem cell

hr
hour

HSC70
heat shock cognate 71 kDa protein

HSPG
heparan sulfate proteoglycan

HTTExon1-Q≥37
polyglutamine-rich huntingtin

IM
intramuscular

IP
intraperitoneal

IPTG
isopropyl-1-thio-β-D-galactopyranoside

ISF
interstitial fluid

IU
international units

IV
intravenous

K48
lysine 48

K63
lysine 63

kD
kilodalton

KO
knockout

KRS
Kufor-Rakeb syndrome

L-DOPA
levodopa (L-3,4-dihydroxyphenylalanine)

LAG3
lymphocyte-activation gene 3

LAMP1
lysosome-associated membrane protein 1

LAMP2
lysosome-associated membrane protein 2

LAMP2A
lysosome-associated membrane protein 2A

LB
Lewy body

LB
lysogeny broth
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>LC3</td>
<td>microtubule-associated protein 1A/1B-light chain 3</td>
</tr>
<tr>
<td>LLOMe</td>
<td>L-leucyl-L-leucine methyl ester</td>
</tr>
<tr>
<td>LMP</td>
<td>lysosomal membrane permeabilization</td>
</tr>
<tr>
<td>LN</td>
<td>Lewy neurite</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRRK2</td>
<td>leucine-rich repeat kinase 2</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>MAPT</td>
<td>microtubule-associated protein tau</td>
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<tr>
<td>MAOBI</td>
<td>monoamine oxidase type B inhibitors</td>
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<tr>
<td>MDS</td>
<td>Movement Disorder Society</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
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<td>milliliter</td>
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<td>millimolar</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MSA</td>
<td>multiple system atrophy</td>
</tr>
<tr>
<td>MSU</td>
<td>monosodium urate</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MVB</td>
<td>multivesicular body</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cutoff</td>
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<tr>
<td>NA</td>
<td>numerical aperture</td>
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<td>NAC</td>
<td>non-Aβ component</td>
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<td>NACP</td>
<td>non-Aβ component precursor</td>
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<tr>
<td>NCL</td>
<td>neuronal ceroid-lipofuscinosis</td>
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<tr>
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<td>normal donkey serum</td>
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<td>NHS</td>
<td>N-hydroxy succinimide</td>
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<td>NLRP3</td>
<td>nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3</td>
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<td>nm</td>
<td>nanometer</td>
</tr>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
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<td>neuropil thread</td>
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<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
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<tr>
<td>PB</td>
<td>pale body</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PDD</td>
<td>Parkinson’s disease dementia</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PFF</td>
<td>pre-formed fibril</td>
</tr>
<tr>
<td>PiD</td>
<td>Pick’s disease</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-N, N’bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PLA</td>
<td>proximity ligation assay</td>
</tr>
<tr>
<td>PLK1</td>
<td>polo-like kinase 1</td>
</tr>
<tr>
<td>PLK2</td>
<td>polo-like kinase 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PLK3</td>
<td>polo-like kinase 3</td>
</tr>
<tr>
<td>PMCA</td>
<td>protein misfolding cyclic amplification</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>polyQ</td>
<td>polyglutamine</td>
</tr>
<tr>
<td>PRNP</td>
<td>prion protein gene</td>
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<tr>
<td>PrP</td>
<td>prion protein</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;C&lt;/sup&gt;</td>
<td>cellular prion protein</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
<td>disease-associated protease-resistant prion protein</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>pS129</td>
<td>phosphorylated Serine-129</td>
</tr>
<tr>
<td>PSP</td>
<td>progressive supranuclear palsy</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>Rab7</td>
<td>Ras-related protein Rab7</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
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<tr>
<td>RBD</td>
<td>REM sleep behavioral disorder</td>
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<tr>
<td>REM</td>
<td>rapid eye movement</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SNpc</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>SOD-1</td>
<td>superoxide dismutase 1</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>SUMO</td>
<td>small ubiquitin-like modifier</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>TD</td>
<td>tangle-only dementia</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR DNA-binding protein-43</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>TFEB</td>
<td>transcription factor EB</td>
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<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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<tr>
<td>ThT</td>
<td>Thioflavin T</td>
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<tr>
<td>TLR2</td>
<td>toll-like receptor 2</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TNT</td>
<td>tunneling nanotube</td>
</tr>
<tr>
<td>TSE</td>
<td>transmissible spongiform encephalopathy</td>
</tr>
<tr>
<td>UPDRS</td>
<td>Unified Parkinson's Disease Rating Scale</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin-proteosome system</td>
</tr>
<tr>
<td>vCJD</td>
<td>variant Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td>VSV-G</td>
<td>vesicular stomatitis virus glycoprotein</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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ABSTRACT

Numerous pathological amyloid proteins spread from cell-to-cell during neurodegenerative disease, facilitating the propagation of cellular pathology and disease progression. Understanding the mechanism by which disease-associated amyloid protein assemblies enter target cells and induce cellular dysfunction is therefore key to understanding the progressive nature of such neurodegenerative proteinopathies. In this study, we utilized an imaging-based assay to monitor the ability of disease-associated amyloid assemblies to induce the rupture of intracellular vesicles following endocytosis, as well as to elucidate the cellular consequences of this damaging mechanism of invasion. We observed that the ability to induce vesicle rupture is a conserved feature of fibrillar amyloid assemblies of α-synuclein, tau, and polyglutamine-expanded huntingtin. In the case of α-synuclein amyloid assemblies, we determined that Serine 129 phosphorylation and strain conformation dictate the potency of endocytic vesicle rupture. We also demonstrated that vesicles ruptured by α-synuclein are lysosomes, and that these damaged vesicles are targeted to the autophagic degradation pathway. We observed that vesicles ruptured by α-synuclein can accumulate and fuse into large, intracellular structures resembling Lewy bodies in vitro, and showed that the same markers of vesicle rupture surround Lewy bodies in brain sections from PD patients. These data underscore the importance of this conserved
endocytic vesicle rupture event as a damaging mechanism of cellular invasion by amyloid assemblies of multiple neurodegenerative disease-associated proteins, and suggest that proteinaceous inclusions such as Lewy bodies form as a consequence of continued fusion of autophagic vesicles in cells unable to degrade ruptured vesicles and their amyloid contents.
CHAPTER ONE

REVIEW OF LITERATURE

Introduction

Neurodegenerative diseases represent one of the most formidable challenges facing biomedical science and medicine. Despite intensive study and significant advances in understanding of disease pathology and pathogenesis, patients afflicted by diseases such as Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD), amyotrophic lateral sclerosis (ALS), or Creutzfeldt-Jakob disease (CJD) are only treated symptomatically, as no disease-modifying therapy exists to target the etiology of these diseases.

Despite tremendous heterogeneity in the spectrum of clinical symptoms resulting from dysfunction and degeneration of the central nervous system (CNS) in these diseases, each similarly exhibit pathological accumulation of misfolded proteins into amyloid, a term referring to insoluble protein aggregates of a fibrillar, β-sheet-rich structure. Because each disease is characterized and defined by the aggregation of a specific disease-related protein, these disorders are collectively known as proteinopathies, and the research community has focused on understanding the mechanisms by which specific proteins become misfolded and ultimately cause neuronal dysfunction and death. Intriguingly, an explosion of insight into the
pathogenesis of neurodegenerative proteinopathies has grown from an idea that was once considered heretical.

In 1982, Stanley B. Prusiner first described the concept of a prion, a proteinaceous infectious particle [1], and determined that a protein alone was the etiological agent of transmissible spongiform encephalopathies (TSEs) such as CJD in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in cows. The idea that a misfolded protein aggregate could occupy the same infectious domain previously thought to be only that of bacteria, viruses, fungi, protozoa, and helminths was just as much controversial as it was groundbreaking [2]. In the years since this discovery, the list of proteins that behave in a similar fashion to CJD prions in converting from a normal, soluble form to a disease-associated, insoluble amyloid form and amplifying through templated recruitment of additional soluble counterparts has grown to encompass β-amyloid (Aβ) and tau in AD, α-synuclein (α-syn) in PD, polyglutamine-rich huntingtin (HTTExon1-Q≥37) in HD, and TAR DNA-binding protein-43 (TDP-43) and superoxide dismutase 1 (SOD-1) in ALS, among others [3]. Considering neurodegenerative amyloid proteins as “prion-like” in their aggregation behavior has not only yielded new knowledge of disease mechanisms, but also led to novel avenues of exploration for therapeutic development.

In addition to their misfolded amyloid structure, another key feature of all prions is their self-propagation between affected and naïve neuronal cells and brain regions, and ultimately their transmission from one infected host organism to another. While the
“prion-like” analogy for Aβ, tau, α-syn, HTTExon1-Q≥37, TDP-43, and SOD-1 does not extend so far as to say that the neurodegenerative proteinopathies caused by these misfolded amyloid proteins are transmissible between hosts, the similarity with prions spreading from cell-to-cell and between different brain regions has been demonstrated for each disease, suggesting a unifying role for prions in neurodegenerative diseases [3]. While insights into the prion-like behavior of amyloid proteins will have important therapeutic potential for preventing disease progression, much remains to be elucidated about the mechanisms by which misfolded amyloid aggregates corrupt their soluble counterparts, disrupt cellular homeostasis, and propagate their infectious pattern of protein misfolding.

In seeking to understand amyloid protein propagation more completely, and elucidate the specific steps in the life cycle of cellular infection that allow this process to occur, insights can be gained by examining other paradigms of infection in different contexts. In the situation of α-syn in PD, it is known that the process of aggregate cellular entry resembles the infectious mechanism of cellular invasion by some bacteria and viruses, which escape from the endocytic vesicle by inducing membrane rupture [4]. However, specific factors that determine the potency of this α-syn cellular invasion mechanism are unknown. Furthermore, whether amyloid assemblies of other neurodegenerative disease-associated proteins are capable of invading target cells in this manner remains to be elucidated, as does the effect of this damaging mechanism of
entry on cellular homeostatic processes, aggregate cell-to-cell propagation, and inflammatory responses.

The studies discussed in this dissertation focus on endocytic vesicle rupture as a mechanism of cellular invasion for several neurodegenerative disease-related proteins, focusing on \( \alpha \)-syn aggregates on both specific protein modifications that influence vesicle rupture potency as well as the effects of \( \alpha \)-syn-induced vesicle rupture on cellular homeostasis and aggregate propagation. A more complete appreciation of how amyloid protein aggregates invade target cells and propagate their misfolding in a prion-like manner will aid in the development of novel disease-modifying therapies to benefit millions of patients worldwide.

The Prion Principle of Infectious Proteinopathy

Protein as Infectious Agent.

At the center of Stanley Prusiner’s definition of a prion is the fact that a protein alone is the sole infectious agent causing TSEs [1]. There are several unique features of prions that confer this infectious potential. When the scrapie-associated prion protein \( \text{PrP}^\text{Sc} \) was isolated from infected animals [5], it became known that this protein is highly resilient against proteolytic digestion [6], indicating that it is composed of stable protein aggregates with few enzymatic digestion sites accessible in its assembled form. Furthermore, this aggregated, protease-resistant characteristic of \( \text{PrP}^\text{Sc} \) is necessary to maintain its infectious capability, as prolonged proteolytic digestion or disaggregation procedures sterilize prions and abolish infectivity [6, 7]. As researchers sought to learn
more about the nature and source of prions, a startling discovery was that prion protein (PrP) is encoded by a normal cellular gene (PRNP) in the host, and because this gene and protein product are not uniquely associated with infection, the prion infectious agent was no longer considered to be a foreign entity [8-10]. Hence two different forms of PrP exist, a normal “cellular” form (PrP<sup>C</sup>) that is completely degraded upon protease digestion and a disease (“scrapie”) -associated aggregated form (PrP<sup>Sc</sup>) that is resistant to protease digestion. Because the primary structure of PrP encoded by PRNP is the same in healthy and infected animals, the differences between PrP<sup>C</sup> and PrP<sup>Sc</sup> are due to post-translational events [10]. PrP<sup>C</sup> is composed mainly of α-helix (42%) and has little β-sheet secondary structure (3%), whereas PrP<sup>Sc</sup> has a much higher β-sheet content (43%) and a lower α-helical structure (30%) [11]. Limited digestion of PrP<sup>Sc</sup> by proteinase K produces an N-terminally truncated form known as PrP<sup>27-30</sup>, named for the molecular weight of this protease-resistant core present in infectious preparations, which also consists mainly of β-sheet secondary structure (54%) with low α-helical content (21%) and is known to polymerize into rod-shaped amyloid fibrils as seen in PrP amyloid plaques [11, 12]. These structural studies brought about the idea that conversion of the precursor PrP<sup>C</sup> to PrP<sup>Sc</sup> involves posttranslational modification during which the protein becomes both enriched in beta-sheet structure and resistant to limited proteolysis, and is then capable of producing infection because of these characteristics.

Further confirmation of the protein-only hypothesis is demonstrated by examining different forms of disease. Although transmissible forms of prion diseases
may be most well-known, sporadic CJD with no known cause other than the hypothesized spontaneous misfolding of PrP\textsuperscript{C} into PrP\textsuperscript{Sc} represents 85% of cases, and over 40 different mutations of \textit{PRNP} are known to cause genetic prion disease [2]. All families affected by heritable forms of prion diseases exhibit an earlier age of onset and longer duration of illness than sporadic cases [13]. One such form of heritable prion disease is known as Gerstmann-Straussler-Scheinker (GSS) syndrome, wherein affected family members inherit a leucine to proline substitution at PrP codon 102 in an autosomal-dominant fashion, leading to the development of PrP\textsuperscript{27-30} accumulation and PrP-immunoreactive amyloid plaques in the brain [14]. Transgenic animal models have also illuminated the proteinaceous source of prion diseases. One study utilized mice expressing two point mutations in mouse PrP affecting the structure of its globular domain to demonstrate that these genetic alterations in PrP result in a fully-penetrant lethal spongiform encephalopathy with cerebral PrP plaques [15]. These findings indicate that infectious prion diseases can also be genetic in nature, and suggest that mutations in the PRNP gene may cause prion disease by facilitating conversion of the protein into the prion state.

**Template for Misfolding.**

Another unique feature of prions that defines their infectious nature is the ability of misfolded, aggregated PrP\textsuperscript{Sc} to serve as a template and trigger for the further misfolding of PrP\textsuperscript{C}, facilitating amplification of the prion state and propagation of its misfolded structure through naïve counterparts. This templated conversion of PrP\textsuperscript{C} by
PrP<sup>Sc</sup> is illustrated profoundly in transgenic mice expressing both hamster and mouse prion protein, where inoculation with hamster prions greatly expands the number of hamster prions but not mouse prions, and vice versa with inoculation of mouse prions [16]. This observation reveals a species specificity of prion infectivity dictated by the PrP sequence, and indicates that interactions between PrP<sup>Sc</sup> and homologous PrP<sup>C</sup> initiate prion synthesis. Direct evidence that this interaction promotes PrP<sup>Sc</sup> production is seen when radiolabeled PrP<sup>C</sup> is incubated with PrP<sup>Sc</sup>, resulting in the formation of protease-resistant radiolabeled PrP, indicating that the protease-resistant properties of PrP<sup>Sc</sup> were imparted to incorporating PrP<sup>C</sup> counterparts [17]. Dramatically, this PrP<sup>Sc</sup>-templated conversion of PrP<sup>C</sup> into additional PrP<sup>Sc</sup> is necessary for the development of clinical disease and neuronal death, as PrP<sup>C</sup>-deficient mice are resistant to prion diseases [18-20] and only grafted neurons overexpressing PrP<sup>C</sup> incur cellular damage when implanted into the brains of infected PrP<sup>C</sup>-deficient mice [21]. This latter finding demonstrates that PrP<sup>Sc</sup> alone is not neurotoxic, but must interact with expressed PrP<sup>C</sup> and template its conversion into PrP<sup>Sc</sup> for neuronal death to occur. In addition to protease resistance, subtle conformational details of PrP<sup>Sc</sup> seeds are templated onto the incorporating naïve PrP<sup>C</sup> proteins, as evidenced by the recapitulation of the same fully penetrant lethal spongiform changes induced by mutant PrP when subsequently inoculated into mice overexpressing WT PrP, and then from these mice into normal WT mice [15]. Structural studies of infectivity and PrP<sup>C</sup> to PrP<sup>Sc</sup> conversion suggest that the PrP aggregates that most potently perform these activities are 17-27 nm in size (300-
600 kilodalton (kD), 14-28 PrP molecules), not larger fibrils or smaller oligomers (less than or equal to 5 PrP molecules)[22].

Perhaps the strongest support of the protein-only hypothesis comes from the induction of prion disease \textit{in vivo} from PrP\textsuperscript{Sc} generated \textit{in vitro}. Soto and colleagues developed a system whereby PrP\textsuperscript{Sc} originally isolated from an infected hamster is amplified \textit{in vitro} through successive cycles of sonication and PrP\textsuperscript{C} to PrP\textsuperscript{Sc} conversion steps [23, 24]. This process, termed protein misfolding cyclic amplification (PMCA), exploits the templated misfolding and seeding property of prion proteins by fragmenting PrP\textsuperscript{Sc} aggregates into smaller seeds that can continue to amplify individually upon co-incubation with PrP\textsuperscript{C}, resulting in a PCR-like enrichment of PrP\textsuperscript{Sc} \textit{in vitro} that can be used as an inoculum \textit{in vivo}. The \textit{in vitro} generated PrP\textsuperscript{Sc} has been shown to be similar biochemically and structurally to the PrP\textsuperscript{Sc} isolated from infected hamsters, and upon inoculation of wild-type (WT) hamsters with \textit{in vitro} PrP\textsuperscript{Sc}, prion disease is induced identical to the disease induced by PrP\textsuperscript{Sc} isolated from infected brain [24]. This process of PrP\textsuperscript{Sc} fragmentation into multiple smaller seeds that independently amplify may also be important for authentic prion disease, and may represent a mechanism whereby even slowly growing PrP\textsuperscript{Sc} aggregates can have a widespread physiological impact [25].

\textbf{Propagation.}

While the propagation of protein misfolding occurs due to templated conversion of PrP\textsuperscript{C} into PrP\textsuperscript{Sc}, prions are also capable of physically propagating between cells and even between host organisms to spread infection. While spreading between cells has
been demonstrated for misfolded aggregates of amyloid proteins in many neurodegenerative diseases, bringing about the “prion-like” analogy, only prions have been demonstrated to be transmissible between hosts. One form of infectious human prion disease is known as kuru, and was transmitted by ritualistic cannibalism in New Guinea where people ate the brains of their dead relatives in an attempt to immortalize them [26]. Iatrogenic transmission of CJD has been documented through the use of contaminated neurosurgical instruments [27, 28] or human growth hormone derived from cadaveric pituitary extracts [29], or as a result of corneal transplantation [30] or dura mater grafting [31]. Finally, another form of CJD known as variant CJD (vCJD) arose through dietary exposure to prion-contaminated beef products from cattle infected with BSE, commonly known as “mad cow” disease [32-35]. Variant CJD has even been transmitted through a blood transfusion from a donor who subsequently developed vCJD [36]. These examples of disease transmission due to propagation of prions illustrate the paradigm-shifting nature of Stanley Prusiner’s definition, as they represent the only proteinaceous particles capable of transmitting neurodegenerative disease between hosts.

The spreading of prions between cell populations, organ systems, and brain regions has been intensely investigated. Neuronal prion spreading between interconnected brain regions was shown following retinal inoculation, with subsequent progressive colonization of the entire visual system from superior colliculus to lateral geniculate and terminating in the optical cortex [37]. When prions are taken up
peripherally, as in the case of non-iatrogenic infectious transmission, prions have been shown to expand and neuroinvade by way of the lymphohematopoietic system, particularly by interacting with differentiated B cells [38]. The spleen seems to represent an extraneuronal reservoir for prions, whose arrival there depends on components of the lymphohematopoietic system such as B cells; PrP transit into the spleen and further neuroinvasion requires host expression of PrP presumably because of its role as a substrate for templating of further prions [39]. Once the splenic compartment has been colonized, prions exploit an interaction between follicular dendritic cells and splenic sympathetic nerves to invade these peripheral nerve cells and eventually gain access to the CNS [40, 41]. These steps along the pathway of prion spreading in the periphery or in the nervous system represent attractive therapeutic targets for preventing the spread of infection and the onset of neurologic symptoms.

While some knowledge has been gained regarding the specific cellular mechanisms of prion cell-to-cell propagation, much remains to be elucidated about the complete life cycle of cellular infection [42]. While endogenous PrP$^C$ is known to localize to the plasma membrane, its localization there is not required for exogenous PrP$^Sc$ binding or internalization [43-45]. Instead, incoming PrP$^Sc$ interacts with glycosaminoglycans (GAGs) at the cell surface that facilitate its binding, and is internalized via nonspecific endocytic routes such as pinocytosis or transcytosis into vesicles positive for markers of late endosomes or lysosomes, but not synaptic, early endocytic, or raft-derived vesicles [43, 44]. This internalized PrP$^Sc$ is then transported
within the infected cell along neurites to positions of intercellular contact, perhaps facilitating cell-to-cell movement. Cellular uptake can be a rapid process, within 2 hours of exposure, but may not occur for all cells equivalently, as uptake depends on both the membrane microenvironment and host cell-specific factors encountered upon binding, as well as the size of the aggregates themselves, with smaller aggregates more efficiently taken up than larger ones [45]. When membrane-associated PrP<sup>C</sup> encounters exogenous PrP<sup>Sc</sup>, pathological conversion of membrane-associated PrP<sup>C</sup> by the infecting PrP<sup>Sc</sup> occurs within minutes at the cell surface in lipid rafts, followed by endocytosis of the newly formed PrP<sup>Sc</sup> into early endosomes and either recycling to the plasma membrane or retrograde transport to the Golgi and eventual lysosomal degradation, the main degradative process at this early stage of infection [46].

Once successful infection of a target cell has been established, and replicating prions evade degradative processes as they multiply, their trafficking to additional cells has been shown to occur through multiple mechanisms. Cell-to-cell spreading is facilitated by cell-to-cell contact, and tunneling nanotubes (TNTs) allow transfer of both exogenous and endogenous PrP<sup>Sc</sup> between infected and naïve neuronal cells or from infected dendritic cells to neurons as relevant for neuroinvasion from peripheral lymphoid organs [47, 48]. Significantly, because of their nature as a transient direct conduit between the cytoplasm of interconnected cells, TNTs may allow more efficient transmission of endogenous PrP<sup>Sc</sup>, which may have fewer options for cellular release and intercellular transfer compared to internalized exogenous PrP<sup>Sc</sup> [48]. In addition to
transferring through direct cell-to-cell contacts, both PrP\textsuperscript{C} and PrP\textsuperscript{Sc} are known to be released into the extracellular environment from naïve and infected neuronal cells, respectively, in association with exosomes, which can then efficiently initiate infection in uninfected cells [49, 50]. Learning more about the mechanisms of prion transmission between hosts will alleviate public health concerns, and elucidating specific steps in the pathway of intracellular and intercellular infection may allow therapeutic development for prions and other neurodegenerative disease-related amyloid proteins that share the prion-like ability to propagate between cells and brain regions.

**Strains.**

Upon pathological examination of infected brain tissue, all forms of human prion disease exhibit PrP deposition, astrocytic gliosis, and prominent vacuolation of neural tissue, the characteristic appearance of which led to the spongiform descriptor of this group of encephalopathies [2]. Despite sharing a similar pathological appearance and the same misfolded protein as the etiological agent, TSEs differ in phenotypic characteristics such as incubation time, clinical signs of disease, brain regions and cell types affected, and vacuolation and protein deposition patterns [51, 52]. When different phenotypes of disease arise from inoculation with different prion preparations, despite precisely controlling for parameters such as prion titer, tissue, infection route, PrP genotype, age, gender, and immune system status, these different disease phenotypes define unique prion strains [51]. This strain phenomenon was first observed when animals infected with the same inoculum developed prion disease with differing clinical
features [53, 54], and subsequently these observed differences in the localization and appearance of CNS spongiform degeneration are the most well-accepted criteria to distinguish strains [55, 56]. True to the templating nature of prion propagation, these distinguishing phenotypic characteristics of different prion strains are faithfully propagated to other infected animals upon serial passage [57]. Indeed, the recapitulation of strain-specific pathology allowed the identification of vCJD as the human counterpart of BSE, as characterization of the new vCJD pathology demonstrated features distinct from sporadic CJD but similar to BSE [32-35]. Strain-specific accumulation in distinct brain regions has been demonstrated using histoblot quantification of prion deposition [58, 59], and immunohistochemistry studies have revealed strain-specific cell type tropism [60]. While identifying induction of spongiform degeneration in specific brain regions has been a reliable indicator of prion strain differences, this approach is limited because prions can also accumulate in brain regions where overt degeneration is not observed, and specific cellular or structural parameters that further distinguish prion strains and that may dictate cell type and brain region tropism cannot be elucidated in this fashion.

At the structural level, prion strains have been distinguished from one another through biochemical properties such as epitope accessibility for antibody binding, differential sensitivity to proteinase K digestion, and variable sedimentation and gel electrophoretic properties [61, 62]. These results, in addition to the observation that strain-specific differences in gel migration properties were preserved through animal
transmission [63] have led to the view that strain diversity is encoded by differences in PrPSc conformation that serve as unique templates for the corruption of expressed PrPC. This structural heterogeneity of PrPSc has been seen in electron microscopic (EM) studies where fibrillar protofilaments of different strains exhibited different widths and periodicities [64], or when differential immunoreactivity is observed between strains upon incubation under denaturing conditions [65]. Another distinguishing factor between various prion strains is their conformational stability, their relative propensity for spontaneous disassociation or fragmentation, although there is some debate about whether decreasing conformational stability results in a shortening or lengthening of disease incubation time [25, 66, 67]. Similar distinguishing features have been observed between prion strains produced entirely in vitro from bacterially-derived recombinant PrP, further highlighting the idea that strain phenotypic diversity results from differences in protein aggregate conformation [66, 68-70]. Elucidating the structural differences that distinguish different prion strains and dictate their toxicity, seeding, and propagation properties may elucidate the underpinnings of differing disease phenotypes and highlight opportunities for inhibiting or preventing disease onset and progression.

**Alpha-Synucleinopathies**

**Clinical Features.**

PD, Dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) are all neurodegenerative proteinopathies that exhibit aggregation of α-syn, and hence are named α-synucleinopathies. PD is the most common and well-known α-
synucleinopathy, first described by James Parkinson in 1817 in his *Essay on the Shaking Palsy* [71]. Parkinson described the essential motor symptoms of the “paralysis agitans” disease that would later bear his name, noting an “involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported”. He also described “a propensity to bend the trunk forward, and to pass from a walking to a running pace: the sense and intellects being uninjured.” The essential characteristics of the disease were later refined by Jean-Martin Charcot, the father of modern neurology, who named the disorder Parkinson’s disease and clearly described its characteristic bradykinesia, or slowness of movement, as distinct from muscle weakness, noting “a slowness in execution of movement rather than real weakness” [72]. Charcot used the name Parkinson’s disease rather than paralysis agitans or shaking palsy because he recognized that PD patients are not very weak and do not necessarily have a tremor [73]. The Parkinson’s Disease Foundation estimates that as many as 1 million Americans and more than 10 million people worldwide are currently living with PD, although prevalence rates are projected to greatly increase as the population continues to age [74]. PD prevalence in industrialized countries is estimated at 0.3% of the entire population and about 1% of people over 60 years of age [75]. Age is known to be the greatest risk factor for the development of PD, as onset is rarely before age 50 and prevalence and incidence increase nearly exponentially with age, peaking after age 80 [75-78]. The incidence of PD is 8-18 per 100,000 person-years [75]. Gender and ethnicity also affect rates of PD, with a 3:2 higher prevalence of men affected than women and
incidence highest among Hispanics, then non-Hispanic Whites, Asians, and Blacks [77, 79]. As the second-most common neurodegenerative disease and the most common movement disorder, PD represents a significant burden of morbidity and mortality for an aging population.

Because there is no definitive test for the diagnosis of PD, the disease must be diagnosed based on clinical criteria which differ between PD, DLB, and MSA. The International Parkinson and Movement Disorder Society (MDS) Clinical Diagnostic Criteria for Parkinson’s disease (MDS-PD Criteria) place the motor syndrome at the center of disease diagnosis, characterized by bradykinesia and either rigidity, resting tremor, or both [80]. These characteristic motor features result from the death of dopaminergic neurons in the substantia nigra pars compacta (SNpc), a hallmark feature of PD observable at autopsy even at the gross anatomical level due to the loss of the dark neuromelanin “black substance” pigment that gives the substantia nigra its name (Figure 1). Without the input of dopamine (DA) from nigral projections to the striatum, the ability of the basal ganglia circuitry to control normal movement is compromised, resulting in the aforementioned motor manifestations of PD [81]. By the time these parkinsonian motor symptoms manifest, there has already been at least a 70% decrease in striatal DA, an amount that will continue to decrease over time as the disease progresses [82]. In addition to these cardinal signs of PD, loss of postural reflexes, or the inability to maintain balance, is also considered a hallmark of PD [83]. Although substantial heterogeneity can exist in the clinical phenotype of PD, other secondary
Figure 1. Degeneration of the SNpc in PD. Gross unstained cross section of human midbrain in normal case (left) and in PDD (right). Normal case (left) from 74-year old female with cause of death as multiple myeloma. PDD case (right) from 68-year old male with pathologically confirmed LBs in substantia nigra and in the cingulate cortex, in the absence of diffuse LB disease. Neuromelanized dopaminergic neurons in SNpc visible dorsal to cerebral peduncles, seen best in normal case. Neuromelanin, a byproduct of DA synthesis, is responsible for the black pigment which gives the substantia nigra its name, which in Latin means “black substance”. In the case of PDD, loss of neuromelanin results in nigral pallor (arrows), indicating dopaminergic degeneration. (Image courtesy of John Lee, MD, PhD, used with permission).

Motor features may include hypomimia (masked facies), dysarthria (slow or slurred speech), dysphagia (difficulty swallowing), micrographia (small handwriting), shuffling gait, festination (quickening and shortening of strides), freezing, or dystonia (involuntary muscle contractions causing repetitive or twisting movements) [83].

In addition to these symptoms affecting movement, it is now widely accepted that PD patients exhibit a variety of non-motor symptoms, often preceding the onset of the motor syndrome by months to decades and persisting for the duration of the disease [84-87]. Dysfunction of the autonomic nervous system is commonly experienced
as evidenced by symptoms such as constipation, postural hypotension, excessive sweating or salivation, bladder irritability, or urinary urgency [88]. Anosmia (loss of the sense of smell) is so often associated with PD that its symptomatic presence constitutes a supportive criterion in the MDS-PD Criteria alongside resting tremor and responsiveness to dopaminergic medication [80]. PD can also cause rapid eye movement (REM) sleep behavioral disorder (RBD) where patients act out their dreams [85, 88]. PD patients also experience neuropsychiatric symptoms, with anxiety present to varying degrees in 45% of PD patients and depression occurring in around 35% of patients [89-91]. Finally, cognitive decline leading to dementia commonly occurs in PD, seen in 46% of PD patients 10 years after diagnosis and 83% of patients 20 years after diagnosis [92, 93]. Although in the past the presence of dementia or the temporal relationship of its onset to the onset of parkinsonian symptoms was used to exclude a diagnosis of PD and suggest separate disorders of PD dementia (PDD) or DLB [94, 95], these conditions are considered by many to be part of the same pathological spectrum, and the MDS-PD Criteria considers DLB to be a subtype of PD [80].

Although treatment of the underlying neurodegenerative process does not exist, patient quality of life can be significantly improved for many years with symptomatic therapies. The most effective medication available for treating the motor symptoms of PD is levodopa (L-DOPA), a DA precursor, intended to more easily facilitate DA synthesis in degenerating dopaminergic neurons [96]. Additionally, DA agonists can be used to stimulate DA receptors, and monoamine oxidase type B inhibitors (MAOBI) can be used
to maintain as high a level of endogenous DA as possible by preventing its catabolism [96]. Eventually, however, these treatments begin to fail when patients report that the effects are “wearing off”, not lasting until the next dose, and eventually patients experience marked “on-off” motor fluctuations with some doses being completely ineffective [88]. In addition, when patients have been taking L-DOPA for many years, they can begin to experience involuntary movements called L-DOPA-induced dyskinesias that can be debilitating. As dopaminergic degeneration proceeds inexorably, patients then face the challenging decision to either remain on L-DOPA to experience its limited benefits despite the debilitating dyskinesias or reduce their dose of L-DOPA resulting in a concomitant reduction of dyskinesia severity but an increase in time spent in an “off” state, often one of the most frightening PD symptoms [88]. This may be the point when patients opt for symptomatic surgical intervention through deep brain stimulation (DBS), where adjustable implanted electrodes utilize high frequency electrical stimulation of brain regions such as the subthalamic nucleus (STN) and globus pallidus internus (GPI) to improve motor features and allow for a reduction in L-DOPA dosage [97]. Although almost a quarter of patients have a good outcome at 10 years after diagnosis, living free of dementia or postural instability [92], these symptoms become more prevalent and severe in the end stages of the disease, and postural instability and hypotension can cause especially severe complications for patients, with 81% experiencing falls and 23% sustaining fractures by 15 years after diagnosis [98]. Despite
these end-stage challenges, PD is a substantial contributor to death in only 20% of patients, with the most common cause instead being pneumonia [92].

Multiple system atrophy is known as a distinct α-synucleinopathy from PD because of the differences exhibited in the spectrum of patient symptoms. The average age of onset is younger for MSA compared to PD, 54 compared to 68, and is generally considered to evolve much more quickly than PD [88, 92, 99]. Autonomic dysfunction features prominently in MSA along with parkinsonism, but significant cerebellar dysfunction may also be present as distinct from PD [100], and MSA patients may not respond as well as PD patients to L-DOPA. MSA is also less common than PD, with a prevalence of 4.4 per 100,000 person-years [101]. While both PD and MSA exhibit dopaminergic degeneration of the SNpc, histopathologic examination on autopsy is the most reliable way to distinguish these disorders, as the characteristic signature of α-syn aggregates differs between the two disorders.

**Lewy Bodies.**

The histopathologic hallmark of α-synucleinopathies was first described in 1912 by Fritz Heinrich Lewy when he observed neuronal inclusions in the dorsal motor nucleus of the vagus nerve, the basal nucleus of Meynert, the globus pallidus, and the thalamus of a PD patient [102, 103]. These neuronal inclusions were later given the name “Lewy bodies” (LBs) by Konstantin Tretiakoff who reported in his doctoral dissertation the presence of “corps de Lewy” in the substantia nigra, noting a degeneration of the substantia nigra in PD and postulating a connection between this
nigral cell loss and the characteristic symptoms of rigidity and tremor seen in PD [104, 105]. LBs have subsequently been identified as widely distributed throughout multiple tissues [106]. In the central nervous system (CNS), in addition to the areas discovered by Lewy and Tretiakoff mentioned above, LBs have been found in the olfactory bulb, hypothalamus, posterior pituitary, locus ceruleus, dorsal raphe nucleus, cerebellum, amygdala, and the cerebral cortex [106]. Beyond the CNS, LBS have been identified in multiple areas of the peripheral nervous system (PNS) such as sympathetic ganglia and enteric neurons, and in tissues as diverse as heart, pelvic organs, adrenal medulla, salivary gland, and skin [106]. This widespread LB distribution underscores the multisystem nature of PD and α-synucleinopathies in general, which is also evident by the diversity of symptoms experienced by patients.

Classical, brainstem-type LBs are seen by light microscopic examination of hematoxylin and eosin (H&E)-stained sections as intracytoplasmic, single or multiple, spherical or elongated, eosinophilic masses possessing a dense core and a lighter peripheral halo [106] (Figure 2A). Antibodies for ubiquitin were initially used as the most useful marker of LBs since the fibrillar components comprising their ultrastructure were known to be heavily ubiquitinated [107, 108], but the discovery of insoluble α-syn fibrils as the main proteinaceous component of LBs led to staining for α-syn replacing staining for ubiquitin as the most sensitive and preferred method of detection for LBs [109-111]. Additionally, insoluble α-syn fibrillar aggregates were also identified as the main component of glial cytoplasmic inclusions (GCIs), or Papp-Lantos bodies, present
Figure 2. Demonstration of Lewy bodies in SNc dopaminergic neurons in sporadic PD. Conventional haematoxylin (blue) and eosin (pink) histological staining (A) reveals a spherical Lewy body (arrow) in SNc dopamine neurons with a distinct central core and a peripheral halo. Electron micrograph of a Lewy body (B) reveals that the core (c) contains granular material and the outer halo (h) is composed of radiating filaments. A standard immunohistochemical protocol shows two Lewy bodies (arrow) with ubiquitin concentrated in the core (C) and two Lewy bodies (arrow) with α-synuclein concentrated in the halo (D). Reprinted from The Lancet Neurology, Volume 3, Issue 8, C Warren Olanow, Daniel P Perl, George N DeMartino, Kevin St P McNaught, Lewy-body formation is an aggresome-related process: a hypothesis, Page 496-503 (Reference 118), Copyright 2004, with permission from Elsevier (License Number 4057260798096). http://www.sciencedirect.com/science/journal/14744422.

exclusively in oligodendrocytes that specifically characterize MSA pathology [112-114].

This difference in localization of neuronal LBs in PD and oligodendroglial LBs in MSA allows these two disorders to be definitively distinguished by pathological examination,
in combination with the differences in clinical phenotype. Not only was the α-syn protein identified in all LBs from the diverse tissues they inhabit and diseases they characterize, α-syn was not identified as a component of other neuronal or glial inclusions characteristic of other diseases such as AD, motor neuron disease, or triplet-repeat diseases [115]. It was subsequently found that the insoluble α-syn fibrils comprising the LB were the substrate for ubiquitination [116], although not all LBs exhibit the same α-syn/ubiquitin labeling pattern; some LBs show α-syn and ubiquitin evenly distributed and overlapping homogenously across the inclusion body, while others appear in a concentric pattern where α-syn is concentrated peripherally and ubiquitin inhabits the central core region [117] (Figure 2C-D). This appearance, where insoluble α-syn fibrils segregate to the periphery of LBs, agrees with electron microscopic examination of LBs demonstrating an outward-radiating filamentous halo surrounding a dense granular core [118] (Figure 2B), and is consistent with the hypothesis that insoluble α-syn is continually deposited into LB inclusions [117].

An important advance came when phosphorylation was identified as a key post-translational modification (PTM) of α-syn fibrils enriched in LB inclusions. Almost 90% of α-syn present in LBs was shown to be extensively and selectively phosphorylated at Serine 129 (pS129), whereas only 5% of α-syn in unaffected brain tissue is similarly modified [119-122]. Many other LB components have been identified including PD-linked gene products in addition to α-syn such as DJ-1, leucine-rich repeat kinase 2 (LRRK2), parkin, and phosphatase and tensin homolog (PTEN)-induced putative kinase 1
(PINK1), as well as lipids, kinases, mitochondria-related proteins, and molecules involved in protein folding, membrane trafficking, oxidative stress, the ubiquitin-proteasome system, autophagy, and aggresome formation [106, 117, 123]. This list of components can give clues to the pathological mechanisms leading to LB formation.

It was originally believed that LBs, as the histopathologic hallmark of PD and other α-synucleinopathies, are directly toxic to the cells that contain them, and their formation led to neurodegeneration. More recently, however, LB formation has been considered a protective mechanism which may occur when the cell is overwhelmed by accumulating protein aggregates that are unable to be effectively refolded by chaperones or cleared by proteasomal or autophagic mechanisms [118, 124]. As part of the cellular response to protein misfolding and to better facilitate degradation and cellular protection from aggregate toxicity, aggregates can be specifically delivered to proteinaceous perinuclear inclusion bodies called aggresomes formed at the centrosome by dynein-dependent retrograde transport on microtubules [118, 124]. The prevailing hypothesis by Kopito, Olanow and others regarding LB formation as an aggresome-related process proposes that trafficking to the perinuclear aggresome occurs when cellular quality control mechanisms such as chaperones or proteasomes are overwhelmed and become unable to cope with the increasing burden of misfolded protein “waste” [118, 124]. Hence the aggregate accumulation that is represented by inclusion formation serves to concentrate these misfolded proteins and increase the efficiency of their capture by autophagic degradative mechanisms which properly
“recycle” components of this waste for other cellular purposes [118, 124]. It is only when their recycling capacity also becomes overwhelmed and fails that aggresomes become cellular “junkyards” where continual deposition of misfolded protein waste serves to protect the cell from further aggregate toxicity but consequentially promotes abnormal growth and persistence of proteinaceous inclusions [118, 124].

This aggresome hypothesis is supported by studies of the stages of LB development. α-syn in LB-related pathology first becomes evident as diffuse or granular neuronal cytoplasmic staining in otherwise morphologically normal-looking cells, but can then begin to form thread-like or dot-like structures that first appear in neurites (Lewy neurites, LNs) and eventually inhabit the perikarya [106, 125, 126]. This pattern gives way via coalescence and incorporation of p62, ubiquitin, and other components first to irregularly shaped, uneven staining in neurons that are often poorly pigmented, indicating early cellular dysfunction, and then to discrete staining corresponding to well-defined pale bodies (PBs) which are less eosinophilic than classical LBs and do not exhibit a halo [106, 126, 127]. PBs are known to be composed of sparse granular and vesicular structures among α-syn filaments, corresponding to observations that early stages of inclusion formation involve clustering of α-syn-associated vesicles [128] as well as enrichment and aggregation of lysosomes and mitochondria in the juxtanuclear inclusion body that suggest an ongoing cellular degradation effort [129]. PBs occasionally display condensations or one or more small LBs at their periphery, eventually developing into ring-like classic LBs through a compaction mechanism [106,
Despite expressing the autophagic marker microtubule-associated protein 1A/1B-light chain 3 (LC3) [130-133], exhibiting lysosome-associated membrane protein 2 (LAMP2) expression at the periphery [133], and containing lysosomal enzymes such as cathepsins [131, 134] and glucocerebrosidase [135], LB inclusions are associated with the accumulation of abnormal autophagosomes and lysosomes consistent with aggresomes failing to fulfill their degradative intention [131, 134]. Much debate exists regarding the role of ubiquitination and S129 phosphorylation in LB inclusion formation, as studies have demonstrated conflicting reports on whether these PTMs occur at early stages of protein aggregation to promote inclusion formation, or happen after inclusion formation has already occurred and thus represent a cellular attempt to mark these inclusions for degradation [119, 136-138]. Evidence that LB inclusion formation may represent a protective cellular response was also seen when a potential therapeutic compound that promotes inclusion formation in cellular models of PD and HD prevented dysfunction and toxicity associated with misfolded protein aggregates, supporting the idea that promoting inclusion formation to potentially boost cellular mechanisms of degradation may represent a viable treatment approach for patients [139].

**α-Synuclein Physiological Function.**

α-syn is a 140 amino-acid protein that was first identified with an antibody against synaptic vesicles from the electromotor nucleus of *Torpedo californica*; its localization to the synapse and the nuclear envelope result in the name
**Figure 3. Human ASYN.** Scheme representing the structure of human ASYN with the three distinct domains (N-terminal, NAC and C-terminal). Amino acid residues are indicated in the bottom. Brown bars inside protein domains represent the imperfect hexameric KTKEGV repeats. Arrows indicate the sites of phosphorylation and the broken lines show the mutated sites. Adapted and reprinted from PLoS Genetics, Volume 10, Issue 11, Diana F. Lazaro, Eva F. Rodrigues, Ramona Langohr, Hedieh Shahpasandzadeh, Thales Ribeiro, Patricia Guerreiro, Ellen Gerhardt, Katharina Krohnert, Jochen Klucken, Marcos D. Pereira, Blagovesta Popova, Niels Kruse, Brit Mollenhauer, Silvio O. Rizzoli, Gerhard H. Braus, Karin M. Danzer, Tiago F. Outeiro, Systematic Comparison of the Effects of Alpha-synuclein Mutations on Its Oligomerization and Aggregation, e1004741, doi:10.1371/journal.pgen.1004741 (Reference 256), Copyright 2014, reproduced under the terms of the Creative Commons Attribution License.

“synuclein” [140]. Homologs to the *Torpedo* synuclein protein were then identified in rat and human brain, as were two other members of a synuclein family of proteins, thus giving rise to the names α-, β-, and γ-synuclein for these individual isoforms [140-142].

At the time α-syn was identified in human brain, its full-length sequence was determined to be identical to the previously-identified precursor of the non-Aβ component (NACP) [142], the name of which is derived from its cleaved fragment, later determined to correspond with amino acid residues 61-95 of α-syn (Figure 3), the non-Aβ component (NAC) of AD amyloid plaques [143]. Studies of NACP, equivalent to full-length α-syn, confirmed its association with synaptic vesicles at the presynaptic terminal [142, 144]. The human α-syn gene, SNCA, maps to chromosome 4q21.3-q22 [145, 146], and while α-syn protein expression has been detected in non-neural tissues such as red
blood cells (RBCs) [147], it is abundantly expressed mainly in the nervous system where it comprises an estimated one percent of total protein in soluble cytosolic brain fractions [144]. While the endogenous state of \( \alpha \)-syn has been the subject of much debate, as some have suggested a native helically-folded tetramer as the normal form of \( \alpha \)-syn [148], most findings agree that the native form of \( \alpha \)-syn is an unfolded, unstructured monomer [149, 150].

Despite this debate about its native state, numerous findings clearly reveal that the first \(~ 100\) amino acids in the N-terminus of \( \alpha \)-syn, which contain seven imperfect 11-residue repeats with a highly conserved hexameric KTEGV motif (Figure 3), mediate a structural transition from natively unfolded to an amphipathic \( \alpha \)-helix upon association with lipid membranes [140, 143, 151-153]. \( \alpha \)-syn can adopt two distinct \( \alpha \)-helical conformations in association with lipids depending on the lipid composition or charge or on the diameter of the vesicle. In one conformation, two curved \( \alpha \)-helical regions, from Valine 3-Valine 37 and from Lysine 45-Threonine 92, are separated by a short linker region and have an antiparallel arrangement resembling a broken or bent \( \alpha \)-helix or an \( \alpha \)-helix horseshoe [154-157]. This conformation has been proposed for interactions between \( \alpha \)-syn and smaller-diameter, highly-curved vesicles for which \( \alpha \)-syn has a preferential affinity [151, 155, 156, 158]. In another conformation, an extended, uninterrupted curved \( \alpha \)-helix spans a similar stretch of N-terminal amino acids as for the two \( \alpha \)-helices in the previous conformation [156, 157]. This conformation is preferable for interaction between \( \alpha \)-syn and larger-diameter vesicles, although the two \( \alpha \)-syn
conformations may also co-exist within the same vesicle [155-157]. In both conformations, the C-terminal part of α-syn, from Aspartic acid 98 to Alanine 140 (Figure 3), is highly charged and mobile and does not associate with lipids, remaining free and unfolded [152, 155]. The N-terminal α-helical region of α-syn is key for anchoring the protein to lipid bilayers [159], possibly through interactions with lipid rafts or ganglioside GM1 [160, 161]. The various conformational transitions between natively unfolded and multiple α-helical structures are encoded within the α-syn amino acid sequence and can be triggered and tuned by lipid composition and charge as well as membrane curvature [158, 162].

The membrane binding capacity of the α-syn protein is crucial for its cellular function. α-syn knockout (KO) mice are viable and are largely normal in their brain architecture, cell bodies, fibers, and synapses, but exhibit increased DA release with some stimuli and a deficiency of undocked presynaptic vesicles in the reserve pool, pointing to a role for α-syn as a pre-synaptic regulator of vesicle mobilization and DA neurotransmission [163-165]. Through its binding affinity for membranes of high curvature, α-syn is targeted to negatively-charged endocytic or post-Golgi vesicles [166], possibly through interacting with membrane-associated GTP-bound Rab3a rather than cytosolic GDP-Rab3a [167]. In line with a role in vesicle trafficking, the synuclein family is required for the fast kinetics of synaptic vesicle endocytosis [168], and has been shown to induce the clustering of synaptic vesicles [169]. Furthermore, α-syn has been shown to act as a chaperone for the folding and refolding of soluble N-ethylmaleimide–
sensitive factor attachment protein receptor (SNARE) proteins, binding to synaptobrevin-2/vesicle-associated membrane protein 2 (VAMP2) and promoting assembly of the SNARE complex [170, 171]. This function is clearly important for proper neuronal homeostasis, as KO mice lacking the synuclein family develop neurological impairments with age and died prematurely [171]. Recently, additional functions have been proposed for α-syn that do not necessarily involve its lipid-binding functionality. α-syn can bind to tubulin to facilitate microtubule nucleation and increase growth rate and catastrophe [172], and α-syn can also interact with ATP synthase in mitochondria to increase efficiency of ATP synthesis [173]. While the function of α-syn is still incompletely understood, and its role is continuing to be elucidated [174], it clearly influences very important cellular processes, leading some to hypothesize that α-syn-induced pathology may be a toxic combination of the gain-of-function induced by α-syn aggregation and the loss-of-function caused by displacement from its native role following sequestration into insoluble protein aggregates [175, 176].

**α-syn Pathological Aggregation.**

**Characterization and process.** To learn more about how the α-syn protein aberrantly abandons its native conformation and role to become an aggregated, insoluble fibrillar component of LB inclusions, researchers have focused on characterizing α-syn aggregates from human brain and *in vitro* systems and on modeling the aggregation process. Characterizing α-syn aggregates found in patients has yielded many clues as to the mechanisms of fibril and inclusion formation. In addition to the
fibrillar composition and detergent insolubility identified from studies of LB inclusions in situ, α-syn fibrils isolated from brains of patients are partially resistant to proteinase K digestion [177] and exhibit a consistent orientation of monomeric constituents as evidenced by antibodies against the C-terminal region labeling filaments along their entire length whereas antibodies for the N-terminal region label only one filament end [178]. In addition to the filamentous α-syn pathology observed in LBs, smaller pre-fibrillar α-syn oligomers have been identified in brains of transgenic mice expressing human α-syn prior to neurological symptom onset; as opposed to α-syn fibrils, these oligomers are detergent-soluble, proteinase K-sensitive, and are found both in regions containing LB inclusions as well as histologically unaffected regions [179]. However, oligomers from inclusion-bearing regions accelerate in vitro α-syn aggregation and cause neurodegeneration, while oligomers from unaffected regions are not toxic and delay in vitro α-syn aggregation [179]. These various properties unique to α-syn oligomers and distinct from native and fibrillar α-syn suggest that oligomers represent a separate α-syn species that may evolve from monomer and develop into more mature fibrils and inclusions, and further imply that different functional conformations of oligomers may exist, each with their own specific cellular consequences. Employing the proximity ligation assay (PLA) for the specific detection of oligomeric forms of α-syn in human brain revealed previously unrecognized extensive diffuse deposition of α-syn oligomers, often in brain areas mildly affected by PD, and preferentially labeling early-stage, diffuse or loosely compacted lesions such as PBs rather than more heavily
compacted mature LBs [180]. These findings indicate that oligomeric forms of α-syn may precede the development of classical PD pathology, and in demonstrating that oligomeric α-syn exhibits an intermediate proteinase K resistance different from the proteinase K sensitivity of native α-syn and resistance of α-syn in LBs, this work supports the view that oligomers represent an α-syn species distinct from native and fibrillar forms [180]. These studies of brain-derived α-syn aggregates illuminate potential mechanisms of α-syn aggregation in authentic disease processes, and are essential to establish the physiological relevance of in vitro characterization methods.

Studies of α-syn aggregation from in vitro experiments or molecular simulations have highlighted the essential role of the central hydrophobic domain of α-syn, known as NAC for its accumulation as the non-Aβ component of amyloid plaques in AD, in promoting the aggregation process. Although native, monomeric α-syn is essentially disordered, studies of its conformation under physiological cell conditions have demonstrated that the central hydrophobic NAC domain is shielded from exposure to the cytoplasm, perhaps counteracting spontaneous aggregation [181]. However, molecular simulations have revealed that α-syn can sample conformations that place the NAC domain in a solvent exposed and extended conformation that can form cross-β structure, and that frequent long-range contacts formed between the α-syn N-and C-termini promote this solvent-exposed orientation of the NAC domain [182] (Figure 4). In addition to spontaneous conformational changes into aggregation-prone states, decreasing pH or increasing temperature have been shown to transform α-syn into a
Figure 4. Protein misfolding and aggregation into infectious fibrillar assemblies.
Natively unfolded polypeptide chain (sphere) undergoes conformational changes that lead to distinct abnormal (cube or cylinder) forms. The rates depend on the propensity of the polypeptide to populate a given conformation. According to the “template assistance” model, the abnormal folding intermediates interact with the native form of the infectious protein and convert it into abnormal forms. The distinct abnormal forms of the polypeptide interact transiently with like conformers and establish longitudinal or lateral interactions following the “seeded polymerization” model. The oligomeric species are unstable and dissociate because the inter-molecular interactions do not outweigh the entropic cost of binding. Once longitudinal and lateral interactions have been established between abnormal forms of the polypeptide, distinct stable seeds are formed (in brackets). These seeds grow indefinitely by incorporation of like molecules, yielding fibrils of distinct physical properties. The different fibrils can break into smaller fragments because, amongst other things, of Brownian movement. The rate of breakage depends on the number of bonds established between the molecules. Each resulting fragment acts as seed. Reprinted from Journal of Parkinson’s Disease, Volume 5, Ronald Melki, Role of Different Alpha-Synuclein Strains in Synucleinopathies, Similarities with other Neurodegenerative Diseases, Pages 217-227, doi: 10.3233/JPD-150543 (Reference 304), Copyright 2015, reproduced under the terms of the Creative Commons Attribution Non-Commercial License.
partially folded conformation [183], and native interactions between \(\alpha\)-syn and lipids at the plasma membrane or on/in vesicles or exosomes may also promote its initial misfolding and aggregation [184-187]. Finally, abnormal cytosolic interactions between \(\alpha\)-syn and histones released from nuclei due to disruption of nuclear membrane integrity during apoptosis may also contribute to de novo aggregation of \(\alpha\)-syn [188, 189].

While the specific events leading to initial \(\alpha\)-syn misfolding remain to be elucidated, the NAC domain clearly plays an integral role in aggregation, as a 12-amino acid stretch (Valine 71-Valine 82) in the middle of this hydrophobic region is necessary and sufficient for fibrillization [190]. Introduction of a single charged amino acid into this 12-amino acid stretch significantly slows aggregation, deletion of this stretch completely abolishes aggregation, and synthetic peptides of these 12 amino acids are sufficient to form filaments in vitro which promote the aggregation of full-length \(\alpha\)-syn [190]. Even a deletion of two amino acids, Alanine 76 and Valine 77, dramatically impairs fibril polymerization due to the combination of the location and hydrophobicity of these residues within the \(\alpha\)-syn protein sequence as key factors contributing to its aggregation propensity [191]. Crystals formed of amino acid residues Glycine 68 to Alanine 78 in the NAC region were used for structure determination by micro-electron diffraction, revealing protofibrils built of pairs of face-to-face \(\beta\)-sheets, a pattern very similar to fibrils of full-length \(\alpha\)-syn [192], known to contain parallel-in-register \(\beta\)-sheets and hydrophobic core residues [193]. The hydrophobic NAC region comprises the highly-
ordered core of α-syn fibrils, and while the N and C termini are unfolded and proteinase K sensitive in the fibrillar state, the NAC domain imparts its proteinase K resistance to the larger fibrils [177, 194]. In vitro studies of α-syn aggregation are made possible through the propensity of the α-syn protein to form filaments resembling those isolated from diseased brain [195], as well as by amyloidogenic dyes such as Thioflavin T (ThT) or K114 that allow quantitative monitoring of α-syn fibril formation through their amyloid-sensing fluorescence [196]. These studies have resulted in molecular insights that have informed research efforts to better model the stages of the aggregation process from monomer to LB inclusion.

Following the first step in the aggregation process, the conversion of the native unfolded α-syn monomer to an aggregation-competent form [183], the next step involves the misfolded monomer serving as a nucleus and template for the further misfolding of additional soluble α-syn counterparts that become recruited to the seed nucleus [197, 198] (Figure 4). This nucleation-dependent growth is known to follow first-order kinetics with respect to α-syn concentration and to occur via a stop-and-go mechanism, either extending at a homogenous rate or stopping for variable intervals [197, 199]. When pre-formed seeds of fibrillar α-syn are exogenously introduced into cultured neuronal cells, these seeds are taken up into target cells by adsorptive-mediated endocytosis and trigger intracellular α-syn aggregation by recruiting expressed α-syn into insoluble, hyperphosphorylated, and ubiquitinated inclusions that resemble LBs and LNs in PD [200-204]. These findings demonstrate that once the
internalized misfolded α-syn pre-formed fibril encounters the cytosolic expressed α-syn, it serves as a template for the conversion of native α-syn into its same misfolded conformation, an event which triggers fibril growth and eventual inclusion formation. Furthermore, this seeding process eliminates the lag phase of aggregate growth dependent on the slow, energetically-unfavorable conversion of native to aggregation-competent α-syn monomer, and dramatically accelerates fibril formation [197, 205]. Amyloid self-replication through this seeding process has been shown to be tightly dependent on the molecular sequence compatibility between the fibrillar seed and the native protein, such that cross-seeded aggregation between human and mouse α-syn is bi-directionally restricted [206]. In a direct analogy to prion infectivity, seeding of the misfolded α-syn conformation by exogenous α-syn aggregates is the basis for amplification and transmission of α-syn misfolding.

As the initial misfolded α-syn monomeric seed nucleus grows by recruiting additional soluble α-syn monomers, the growing aggregate is now considered an oligomer, a term encompassing a wide variety of α-syn aggregates but defined simply by a composition of multiple α-syn monomers, as few as two or as many as a hundred or more [205, 207]. Oligomers exhibit an intermediate β-sheet content between that of monomers and fibrils, and may initially be somewhat disordered structures that undergo slow conversion to more compact proteinase-K resistant oligomers [207-209]. Oligomers can then undergo another conversion to more mature fibrils that can elongate by further monomeric addition, indicating that oligomers represent a
continuum of species ranging from unstable low molecular weight particles to stable elongated oligomers to mature fibrils [209, 210] (Figure 4). The prion-like capacity of α-syn aggregates to seed further α-syn misfolding can occur at any stage along this continuum, with aggregation-competent monomers, various forms of oligomers, and fibrils all demonstrating seeding activity, albeit to varying degrees. The α-syn fibrillar conformation is regarded as most effective for seeding, and if larger amyloid-like fibrils are fragmented into smaller components of 50 nm or less that maintain the β-sheet-rich composition characteristic of fibrillar architecture, these short, fragmented fibrils most efficiently promote cellular accumulation of pS129 α-syn and represent the key pathogenic seeds that trigger α-syn misfolding [209-211]. Certain forms of larger stable α-syn oligomers also possess seeding capacity, although to a lesser degree than α-syn fibrils, whereas smaller more disordered unstable oligomers are unable to induce seeding and may instead inhibit fibril formation because their “off-pathway” conformation does not lead to fibril formation [208, 210]. In some circumstances, larger α-syn fibrils may fragment into multiple smaller fibrillar or stable-oligomeric components that can each serve as independent seeds, representing a mechanism for the amplification of misfolding [207] (Figure 4). While it seems feasible that even a single misfolded α-syn seed is capable of nucleation-dependent growth and amplification through fragmentation and propagation resulting in an overall acceleration of aggregation and genesis of α-syn-induced neurodegenerative pathology, other unknown factors may influence this process such that a sufficiently high amount
of α-syn misfolding must occur for pathology to ensue. Indeed, one study found that a very high number of oligomeric or fibrillar seeds, on the order of $10^4$, was required for efficient seeding that bypasses the slow rate of aggregation through spontaneous primary nucleation [209]. Because much lower numbers of α-syn seeds also generate cellular dyshomeostasis by increasing reactive oxygen species (ROS), other factors such as cellular stress may be required to synergize with seed concentration to achieve sufficiently potent α-syn seeding [209]. The templated seeding ability of misfolded α-syn aggregates plays an essential role in disease pathology, as numerous animal models in mice, rats, and monkeys have demonstrated that the introduction of exogenous aggregated α-syn triggers the conversion and misfolding of endogenous α-syn to an aggregated form identical to the exogenous seed, propagating and accelerating α-syn protein misfolding pathology [212-219].

**Regulating factors. Genetic variants.** A major breakthrough in the understanding of PD pathogenesis came with the identification of missense mutations in the α-syn protein that cause autosomal-dominant, early-onset familial PD. These mutations include A53T [220], A30P [221], E46K [222], and the more recently identified G51D [223], H50Q [224], A18T [225], A29S [225], and A53E [226]. These mutations clearly demonstrate, through their recapitulation of the α-syn LB pathology and PD motor syndrome characteristic of idiopathic PD, that α-syn misfolding induced by missense mutations cause PD. Furthermore, when A53T was identified as the first of what would eventually become the aforementioned several known α-syn missense mutations, α-syn
was soon after identified as the main component of LBs, firmly establishing the α-syn protein as central to PD pathogenesis. Additionally, increasing the baseline level of α-syn expression through SNCA gene locus triplication or duplication also causes PD, exhibiting a clear α-syn dose-dependence for age of onset and disease progression, with triplication patients developing a rapidly-progressive PD much earlier than the slowly-progressing, late onset of PD in duplication patients [227, 228]. Importantly, the presence of α-syn-rich LB inclusions in these patients indicates that increasing the expression level of α-syn promotes its pathological aggregation. Despite the causal relationship between α-syn mutation or multiplication and inherited PD, only 10-15% of PD cases have a familial cause, whereas 85-90% of cases are idiopathic, exhibiting inclusions of WT α-syn expressed at a normal level [229, 230]. The link between α-syn and idiopathic PD came with genome-wide association studies (GWAS) that recognized single nucleotide polymorphisms (SNPs) in the SNCA gene or its promoter or enhancer elements that increase risk for PD [231-234], firmly establishing the role of α-syn pathology in all cases of PD, not just rare familial forms.

Because all familial missense mutations in the α-syn protein occur within the N-terminal amphipathic α-helical domain responsible for lipid binding (Figure 3), much effort has been made to understand the effects of these mutations on the membrane binding of monomeric and fibrillar α-syn as well as to establish a link between these missense substitution mutations and alterations in native protein function and/or pathological aggregation. Despite early reports that the A30P and A53T point mutations
had little or no effect on lipid binding or \( \alpha \)-helicity [235, 236], later findings agree that the A30P mutant exhibits defective binding to phospholipid membranes perhaps because of a perturbation in the helical structural around the site of the mutation [237-240]. A30P was shown to interrupt a helix turn, destabilizing the preceding one as well as shifting the helix register after the mutation disturbing the proper amphipathic sequence of polar and hydrophobic residues for at least two turns [239]. The A53T and E46K mutations, by contrast, exhibit stronger interactions with lipids because of stabilizing hydrogen bonds that form between these protein variants and the lipid bilayer [238, 240, 241]. Finally, the recently described G51D and A53E mutations have been shown to exhibit decreased lipid binding affinity compared to WT, behaving similarly to the A30P mutation [242, 243]. Interestingly, the weakly-membrane binding A30P and G51D mutants were shown to populate an exposed membrane-bound form where the NAC domain is dissociated from the bilayer more than in the WT form, resulting in a greater membrane-induced aggregation propensity [244]. \( \alpha \)-syn missense mutations clearly alter membrane affinity, and may be relevant for promoting pathological instead of native conformations and increasing susceptibility to aggregation.

Studies of the effects of \( \alpha \)-syn mutations on aggregation behavior paralleled investigation of WT \( \alpha \)-syn aggregation, as the discovery of the first known mutation, A53T, was the first to implicate \( \alpha \)-syn as the aggregation-prone pathogenic protein in PD. Early studies of the \textit{in vitro} aggregation behavior of WT and mutant \( \alpha \)-syn
demonstrated their shared ability to abandon a natively disordered state to self-aggregate and assemble into insoluble filaments that resembled those in LB inclusions, and suggested that both A53T and A30P mutations accelerated this aggregation process [245-247]. Later findings then clarified the nuanced influence of these mutations on the various stages of the aggregation process; both A30P and A53T share the ability to accelerate oligomerization by consuming their component monomers faster than WT monomers, but this translates into an acceleration of fibrillization only for A53T, whereas A30P was shown to ultimately fibrillize more slowly than WT α-syn [248]. The observation that A53T α-syn accelerates oligomerization and fibrillization correlates with the increased severity of disease experienced by transgenic mice expressing A53T compared to WT α-syn, where A53T mice developed age-dependent filamentous intracytoplasmic neuronal α-syn inclusions, motor impairment, paralysis, and death [249]. The E46K mutation increases the rate of α-syn assembly to a similar extent as the A53T mutation [241], and because the artificial E46A, E83K, and E83A mutations exhibit a similar acceleration, glutamic acid residues residing within N-terminal KTKEGV repeats critical for amphipathic α-helical structure significantly modulate α-syn fibrillar assembly [250]. Additionally, E46K fibrils seeded aggregation more efficiently than WT fibrils, whereas A30P fibrils were less efficient seeds for aggregation [251]. The G51D and A53E mutations are known to attenuate α-syn aggregation relative to WT [223, 242, 243, 252, 253], whereas the H50Q mutation accelerates aggregation [252, 254, 255]. While these studies provide key insights into the pathogenic effects of these PD-causing
mutations, they are also limited in their comparative value because of variability in approach. Several recent studies have sought to systematically compare the effects of each disease-causing mutation within the same experimental system, either in vitro, with cell-free protein expression, or within living cells. These studies have concluded that α-syn missense mutation does indeed significantly impact on seeding capacity, oligomerization, fibrillization, and inclusion formation, and they reveal molecular insights into the initiation and spreading of α-syn aggregation [256-258].

**Serine 129 phosphorylation.** α-syn undergoes multiple PTMs such as phosphorylation, ubiquitination, sumoylation, acetylation, nitration, and truncation, but S129 phosphorylation represents the modification most preferentially and consistently up-regulated in PD and other α-synucleinopathies [259, 260] (Figure 3). α-syn S129 phosphorylation was first identified as a constitutive modification in immortalized cell lines [261], but the discovery that S129 phosphorylation affected 90% of insoluble α-syn in LBs compared to only 5% of normal soluble α-syn raised the possibility that this modification influences α-syn aggregation or toxicity [119-122]. Phosphorylation of α-syn S129 not only occurs in brains of PD and MSA patients [119-122, 125, 262, 263], but also in α-syn overexpressing mouse models of PD and MSA [262, 264] and in dopaminergic neurons of the monkey SNpc with normal aging [265]. While insoluble pS129 α-syn increases, the initially elevated levels of soluble α-syn decrease over the course of PD [266], suggesting that α-syn phosphorylation level depends directly on the amount of substrate available, where high initial levels of soluble α-syn enhance S129
phosphorylation and consume soluble α-syn into insoluble inclusions [265, 267]. It
remains unclear, however, if S129 phosphorylation is a cause or a consequence of this
transition from soluble to insoluble α-syn. The existence of a low level of normal soluble
α-syn phosphorylated at S129 suggests that this LB-associated form is produced during
normal metabolism of α-syn and may promote aggregation and inclusion formation
[121, 122]. However, α-syn could also be phosphorylated after fibrillization or inclusion
formation [122, 268-270] as a mechanism to identify and promote the clearance of
misfolded proteins [271], a possibility supported by the buildup of pS129 α-syn
following proteasome inhibition and elevated autophagic or proteasomal degradation of
α-syn induced by overexpressing specific kinases such as polo-like kinase 2 (PLK2) or G-
protein-coupled receptor kinase 5 (GRK5) [272-274]. Investigation into the identities of
the responsible kinases as well as studies of the influence of S129 phosphorylation on α-
syn membrane binding, aggregation, and toxicity have worked toward a higher level of
clarity regarding these two opposing possibilities.

When pS129 was first identified as an α-syn PTM, it was noted that this site is
located within a consensus recognition sequence of casein kinase 1 (CK1) [261], and it
was later found that casein kinase 2 (CK2) could also be responsible for S129
phosphorylation that increased under oxidative or proteasomal stress [275].
Additionally, G-protein-coupled receptor kinase 2 and 5 (GRK2, GRK5) preferentially
phosphorylate α-syn at S129 [137, 276, 277]. Finally, members of the polo-like kinase
(PLK) family such as PLK1, PLK2, and PLK3 are also capable of α-syn S129
phosphorylation, and unlike other kinases that only partially phosphorylate α-syn at S129, these PLK family members induce complete phosphorylation [268, 278]. Studies of PLK2 have found an increased expression within neurons of older monkeys and in α-synucleinopathy patient brains, perhaps contributing to the increase in pS129 α-syn [265, 273]. While several responsible kinases have been identified, the overall effects of their action remain to be determined.

One tool to examine the specific consequences of S129 phosphorylation on α-syn behavior has been in vitro biochemical assays. In vitro studies of membrane binding suggest that the pS129 modification of WT α-syn does not perturb α-syn membrane affinity [270, 272, 279, 280], but S129 phosphorylation can increase membrane affinity of the normally weakly-binding A30P α-syn and decrease the normal binding affinity of A53T α-syn [272, 280]. These results implicate long-range interactions between pS129 in the unstructured α-syn C-terminal tail with the membrane binding ability of the N-terminal amphipathic α-helix and the familial mutations that are be present there in affected patients [236, 280]. In vitro study of the influence of pS129 on α-syn aggregation has yielded inconsistent results, with some studies demonstrating increased aggregation [119, 280], others showing decreased aggregation [279], and still others showing no effect with S129 phosphorylation [281]. These inconclusive results are mirrored by similarly varied findings when pS129 levels are modulated in cells or animals by expression of kinases capable of performing S129 phosphorylation. Some findings using this strategy to upregulate pS129 α-syn demonstrate an increase in
aggregation and inclusion formation [277, 282-284] while others demonstrate a
decrease or no effect [136, 137, 279, 285]. Finally, utilization of artificial mutants to
mimic (S129D, S129E) or prevent (S129A) phosphorylation at the S129 position generally
revealed that mimicking pS129 decreases membrane affinity while preventing S129
phosphorylation has the opposite effect, indicating that pS129 may decrease membrane
binding [286-288]. These findings are inconsistent with those obtained by in vitro
methods perhaps because of the limited ability of amino acid substitutions to truly
mimic the effects of phosphorylation or its inhibition on α-syn behavior [279].

Furthermore, phosphorylation mimic or null mutants had a differential effect on α-syn
aggregation propensity. Some studies demonstrated that preventing S129
phosphorylation with S129A expression rescues neuronal loss caused by WT α-syn that
is capable of being phosphorylated, whereas S129D α-syn increases pathology [137,
282]. Other studies had the opposite finding, where S129A α-syn caused severe
pathology that was absent or prevented with S129D α-syn expression, and still other
studies found no difference in pathology between S129A or S129D α-syn expressing
systems [281, 289]. These various approaches to study the consequences of S129
phosphorylation are each limited in their own way; biochemical approaches omit
important cellular factors that impinge on phosphorylation and aggregation processes,
and model systems that rely on kinase or α-syn overexpression may be confounded by
potential off-target effects. Nevertheless, dissecting the influence of this highly
prevalent S129 phosphorylation PTM remains a high priority for its potential therapeutic
implications to intervene on a process that directly modulates α-syn aggregation and toxicity.

*Solution conditions.* Several factors in the cytosolic milieu of dopaminergic neurons in the SNpc also influence the tendency of α-syn to form insoluble fibrils and inclusions. One factor present in cells of this region is DA itself, the synthesis of which is required for nigrostriatal signal transduction. DA synthesis is known to be highly oxidative in nature, and upon its oxidative ligation to α-syn, DA inhibits conversion of α-syn protofibrils to fibrils resulting in accumulation of protofibrillar species [290]. DA, L-DOPA, other catecholamines, and their respective oxidative breakdown products have been shown to inhibit *in vitro* α-syn fibrillization or dissolve α-syn fibrils already formed by creating an environment where formation of non-amyloidogenic protofibrils or oligomers is favored over fibril formation [291-294]. In cellular models of α-syn aggregation, increasing DA levels has a similar effect as observed *in vitro*, inhibiting aggregate formation and instead promoting formation of non-toxic oligomeric intermediates [294, 295]. These results implicate decreased DA levels in SNpc neurons as a factor promoting α-syn aggregation in PD, which could then result in a positive feedback loop of α-syn-induced dopaminergic degeneration and additional α-syn aggregation [292]. Moreover, the lysosomal protease cathepsin B (CatB) triggers intracellular α-syn aggregate formation [296], as do cellular stressors such as oxidation or cytochrome-c released from mitochondria [297, 298]; these factors would all be
increased with $\alpha$-syn-mediated cellular toxicity, and would feed into the same positive feedback loop promoting $\alpha$-syn aggregation.

Finally, the morphology of $\alpha$-syn and the aggregates formed are highly sensitive to solution conditions such as ionic strength and pH [299, 300]. At mildly acidic pH such as would be encountered in the environment of endosomes or lysosomes, the rate of secondary nucleation processes such as fragmentation and surface-assisted nucleation is dramatically faster than at physiological pH, leading to an increase in both aggregate size as well as total number [301]. $\alpha$-syn remains unfolded at neutral pH, but acidic pH is associated with rigidification and compaction of the highly hydrophobic and negatively-charged C-terminal region leading to its increased interaction with the NAC domain rather than long-range contacts with the N-terminus [302]. These conformational changes may underlie an increased propensity to become misfolded or a stabilization of $\alpha$-syn oligomeric intermediates and a concomitant decreased energetic barrier to fibrillization [303]. While the process of $\alpha$-syn aggregation is a multifactorial equation, factors such as genetic variants in protein sequence or expression, PTMs such as S129 phosphorylation, and solutions conditions represent some of the most important influences.

**Strains.** Just as prions exhibit the structural heterogeneity to fold into multiple self-replicating strains that produce distinct disease phenotypes [52], $\alpha$-syn has also been shown to adopt distinct strain conformations, perhaps underlying the phenotypic diversity of $\alpha$-synucleinopathies caused by aggregates of a single protein [304]. The
strain phenomenon for $\alpha$-syn was first elucidated by Ronald Melki and colleagues, who demonstrated that two distinct conformations of $\alpha$-syn fulfilled the molecular criteria to be designated as different strains by exhibiting differences in structure, level of toxicity, and in vitro and in vivo seeding and propagation properties [305] (Figure 4). Subsequently, these two strains, named $\alpha$-syn fibrils and ribbons for their different conformational architectures, were shown to induce distinct histopathological and behavioral phenotypes when injected into rat brain [218]. Both $\alpha$-syn strains faithfully seeded aggregation and templated their unique conformations onto expressed $\alpha$-syn to amplify in vivo, but $\alpha$-syn fibrils exhibited a higher level of toxicity with accompanying progressive motor impairment and cell death compared to $\alpha$-syn ribbons, which caused a different phenotype with characteristics resembling both PD and MSA [218]. Multiple different $\alpha$-syn strains, or polymorphs, have been produced in vitro by varying solution conditions during aggregation, and they have been rigorously characterized by solid-state nuclear magnetic resonance (NMR) spectroscopy and atomic-force microscopy (AFM) to differ by morphology and physical properties [306-310]. The existence of different $\alpha$-syn strains was confirmed in other studies by demonstrating differences in cross-seeding efficiency between different $\alpha$-syn strains and tau protein [311], by detecting differences in biochemical properties between in vitro-prepared $\alpha$-syn strains and $\alpha$-syn extracted from PD brain [311], and by transmitting a distinct $\alpha$-syn pathology from MSA brain samples to $\alpha$-syn overexpressing mice different from that induced by injection of PD brain samples or brain homogenate from diseased mice [312-314]. The
conditions under which α-syn aggregates are formed can therefore significantly impact the conformation of the resulting fibrils and the pathological phenotype they induce. Additionally, the presence of the bacterial endotoxin lipopolysaccharide (LPS) during the α-syn aggregation process produces a structurally distinct fibril strain that causes a unique pattern of α-synucleinopathy, raising the possibility that exposure to exogenous pathogens may also influence the aggregation process and contribute to the diversity of disease phenotype [315]. Finally, α-syn S129 phosphorylation also induces formation of a distinct α-syn strain with a different structure, propagation pattern, and higher cytotoxicity compared to WT α-syn, indicating that this highly prevalent PTM enriched in LB inclusions strongly influences strain conformation and increases aggregate toxicity [316]. Overall, the concept that α-syn aggregates can adopt unique strain conformations has only recently become apparent, but it has an incredible potential to explain the wide variety of disease phenotypes experienced with α-synucleinopathies and may underlie unique aspects of α-syn pathological behavior.

**Degradation and Disease-Induced Dysfunction.**

Maintaining homeostasis in cells burdened by misfolded protein aggregates is the role of degradative processes tasked with both the clearance of aberrant proteins and the recycling of their individual amino acid constituents. Both the ubiquitin-proteosome system (UPS) and the autophagy-lysosome pathway (ALP) are involved in the degradation of misfolded α-syn, although the work of degrading higher-molecular weight aggregates of α-syn is mainly performed by the ALP, the only known mechanism
in eukaryotic cells to degrade protein aggregates or damaged organelles that cannot be processed by the proteasome [317]. Autophagy, which means “self-eating” in Greek, was originally viewed as only active in response to nutrient deprivation, but more recently has been appreciated as playing a key constitutive role in promoting neuronal survival through its basal level of activity [318]. Consequently, dysfunction in this process can also contribute to neurodegeneration [319, 320]. Since the discovery that α-syn is contained within autophagosomes [321], the double-membrane structures that engulf cytoplasmic contents and eventually fuse with lysosomes to degrade the cargo, investigation of this process has elucidated the circular mechanisms of autophagy-mediated α-syn degradation and α-syn-induced autophagic dysfunction, and focused on promoting degradation through the ALP as a possible therapeutic approach for PD.

Within the ALP, there are three autophagic pathways through which substrates destined for degradation can be processed. The first type of autophagy is known as microautophagy, where substrates are directly taken up into lysosomes via invagination of the lysosomal membrane and promptly degraded by proteolytic enzymes, although this form of autophagic degradation has not been demonstrated for α-syn [317, 318]. The second form of autophagic degradation, chaperone-mediated autophagy (CMA), is known to be important for α-syn degradation [322, 323]. CMA substrates such as α-syn are first bound at the consensus KFERQ motif by the chaperone heat shock cognate 71 kD protein (HSC70) which induces the unfolding of the target [317]. Substrates then bind the CMA receptor lysosomal-associated membrane protein type 2A (LAMP2A) at
the lysosomal membrane which induces membrane translocation and subsequent proteolytic digestion of the target [324]. Despite the importance of CMA for degradation of misfolded α-syn monomers and dimers, it is not capable of degrading α-syn oligomers or higher molecular weight aggregates because of the requirement to translocate across the lysosomal membrane [324, 325]. Thus, it is the role of the third form of autophagic degradation, macroautophagy, to degrade larger α-syn aggregates that accumulate in diseased neuronal cells, and because this process is the most well-characterized of the three forms of autophagy and most important for bulk degradation of dysfunctional organelles or misfolded protein aggregates, the use of the general term “autophagy” most often refers to this process of macroautophagy, a convention that will also be used here in the discussion that follows.

Autophagy involves the engulfment of cytoplasmic cargo by a double-membrane phagophore, further expansion into an autophagosome, and fusion with a lysosome to become an autophagolysosome where the cargo is degraded by proteolytic enzymes active in this low pH compartment [317]. This process plays an essential role in degrading protein complexes like oligomers and aggregates that fail to pass through the proteasome barrel or translocate through the lysosomal membrane in CMA [326]. When the UPS is inhibited, low pH compartments containing cathepsin D (CatD) are increased, representing a compensatory increase in autolysosomes and lysosomes that degrade misfolded α-syn [327]. Inhibiting autophagic or lysosomal degradation results in an increase in large cytoplasmic α-syn inclusions, whereas activation of autophagy causes
dissolution of these inclusions and increases the number of smaller \( \alpha \)-syn aggregates [327]. Autophagic components are known to be significantly increased in the brains of DLB patients and transgenic mice overexpressing mutant \( \alpha \)-syn, indicating that autophagy is induced to facilitate clearance of \( \alpha \)-syn aggregates [328]. Moreover, overexpression of transcription factor EB (TFEB), a master regulator of the autophagy-lysosomal pathway, or Ras-related protein Rab7 (Rab7), a regulator of endosomal and autophagosomal trafficking, reduced accumulation of aggregated \( \alpha \)-syn by inducing its autophagic clearance, further supporting a role for this pathway in degradation of \( \alpha \)-syn [329, 330]. PLK2 and PLK3, kinases involved in phosphorylation of \( \alpha \)-syn S129, enhance \( \alpha \)-syn autophagic degradation by forming a complex with pS129 \( \alpha \)-syn and acting as a target for polyubiquitination, allowing the selective recognition of the complex by the autophagic machinery [273, 331]. Additionally, phosphorylation of S129 is implicated in targeting \( \alpha \)-syn for autophagic degradation because cellular expression of the S129A mutant failed to cause the induction of autophagy seen with expression of WT \( \alpha \)-syn [271]. Once the \( \alpha \)-syn cargo encounters the environment of the lysosome, the actions of proteolytic enzymes such as CatD have been shown to be effective in degrading \( \alpha \)-syn [332, 333], although mature fibrillar \( \alpha \)-syn inclusion bodies have been shown to be refractory to lysosomal or autophagic clearance [134, 334]. Lysosomal clearance of \( \alpha \)-syn has been demonstrated \textit{in vivo} by the detection of \( \alpha \)-syn within the lumen of lysosomes isolated from mouse midbrain, colocalization in nigral tissue of \( \alpha \)-syn with LAMP2A staining, and elevation of LAMP2A expression in \( \alpha \)-syn overexpressing mice.
These findings implicate the ALP as a key mediator of α-syn degradation and a first-line strategy for maintaining cellular homeostasis in the face of increased protein aggregation.

Given the importance of the ALP for degradation of α-syn aggregates, one of the most challenging aspects of PD pathology is the circular positive-feedback effect of α-syn aggregates inducing dysfunction in ALP degradation processes. This α-syn-induced dysfunction occurs in both CMA and macroautophagy. In the CMA pathway, α-syn familial mutants A53T and A30P as well as DA-modified α-syn are known to bind the LAMP2A receptor on lysosomal membranes with increased affinity compared to WT α-syn and act as uptake blockers, impairing their own degradation as well as that of other autophagic substrates [322, 325, 336]. CMA inhibition by α-syn is known to produce a compensatory increase in macroautophagy, but this autophagic mechanism is also dysfunctional because of α-syn aggregation. Nigral dopaminergic neurons of PD patients have been shown to undergo both apoptosis and autophagic degeneration, characterized by an increased number of autophagic vacuoles found in diseased neurons [337], and the level of LC3 is known to be elevated in insoluble fractions from brains of patients with DLB [132]. This abnormal accumulation of autophagosomes and increase in LC3 is also seen in cell culture models of PD [338, 339], indicating a combination of both upregulation due to increased burden of misfolded proteins and persistence due to dysfunctional clearance. While an increased abundance of autophagosomes may represent ongoing cellular attempts to combat accumulation of
misfolded proteins, a more detailed examination reveals that these accumulating autophagosomes are abnormal and dysfunctional [131, 339]. In cell culture models and in brains of α-syn transgenic mice and DLB patients, the accumulating autophagosomes are significantly enlarged compared to normal autophagosomes and irregular in appearance, some of which only having a single membrane rather than the characteristic double-membrane structure [131, 134, 339].

There are several mechanisms whereby accumulation of α-syn induces dysfunction in autophagy. One cause of compromised autophagy is the inhibition by α-syn of Rab1a, which then causes mislocalization of autophagy-related protein (Atg) 9, a key regulator of membrane transport to the degradation site, preventing proper autophagosome formation [340]. Another reason for the accumulation of autophagosomes unable to degrade their cargo is the permeabilization of lysosomal membranes by mitochondrial-derived ROS, induced by parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or α-syn, and the subsequent release of lysosomal proteases into the cytosol [130]. This lysosomal depletion results in defective clearance of autophagosomes since fusion with lysosomes for final degradation is impaired. Additionally, excessive α-syn in nigral dopaminergic neurons is associated with cytoplasmic retention of TFEB by sequestration into LBs, resulting in an inability of this key transcriptional regulator of the ALP to translocate into the nuclear compartment [341]. Finally, α-syn aggregates are known to activate Toll-like receptor 2 (TLR2) [342], and TLR2 activation in neurons results in inhibition of autophagy through
activation of AKT and mammalian target of rapamycin (mTOR) [343]. Thus, an additional positive-feedback loop exists whereby activation of TLR2 by α-syn aggregates impairs autophagy and results in accumulation of additional α-syn aggregates. In addition to impairing autophagy, α-syn aggregation induces dysfunction in lysosomal degradation as well. In a similar situation to that of autophagosomes, lysosomes are abundant in cells exhibiting α-syn aggregation as a compensatory effort to degrade the burden of misfolded proteins [344, 345], but these lysosomes are dysfunctional as evidenced by decreases in lysosome-associated membrane protein 1 (LAMP1) and CatD in human and rat nigral neurons containing α-syn inclusions [346]. These several mechanisms by which α-syn impairs the ALP illustrate the difficulty in maintaining proteostasis when the buildup of protein aggregates directly contributes to dysfunction in the autophagic and lysosomal degradative machinery.

While α-syn aggregation clearly induces ALP dysfunction, and one result of this dysfunction is a further accumulation of α-syn aggregates [323, 328, 334, 347-349], additional insight into functional and dysfunctional α-syn degradation can be gained by examining genetic diseases that exhibit impairments in degradative processes. Homozygous loss-of-function mutations in the GBA1 gene encoding the lysosomal hydrolase β-glucocerebrosidase (GCase) cause Gaucher’s disease, the most common lysosomal storage disorder, characterized by a toxic buildup of the GCase substrate glucosylceramide (GlcCer) [350, 351]. Interestingly, mutations in GBA1 are also considered the most common genetic risk factor for PD, such that PD patients are more
than 5 times more likely to carry a GBA1 mutation than normal healthy controls [350, 352]. The association between α-synucleinopathy and GBA1 mutation status is even more pronounced for DLB, where DLB patients are more than 8 times more likely than controls to carry a GBA1 mutation [353]. GBA mutation-associated PD and DLB patients have an earlier age of disease onset than non-carriers, are more likely to have affected relatives, and exhibit a more rapid progression of motor impairment and cognitive decline [352-354]. GCase enzymatic activity was lower in Gaucher’s patients with a homozygous GBA1 mutation than in carriers with only a heterozygous mutation, although the heterozygous mutant GBA1 patients exhibited a lower GCase activity than non-carriers [355]. When all PD patients were compared with controls, GCase activity was diminished in diseased patients even in idiopathic PD, when GBA mutants were excluded, and after adjusting for age and gender, suggesting that both GBA mutation carriers and PD patients without GBA mutations exhibit loss of GCase function, and that this diminished lysosomal degradative activity may contribute to PD pathogenesis [355].

The molecular explanation for this unexpected link between PD and Gaucher’s disease was established when it was discovered that loss of GCase function results in compromised lysosomal degradation and a consequential accumulation of α-syn [356, 357]. Moreover, the GCase substrate GlcCer also accumulates, and can stabilize α-syn oligomers to promote amyloid formation [356]. Finally, the accumulating α-syn inhibits the activity of normal GCase, forming a positive feedback loop of compromised lysosomal degradation, accumulation of α-syn and GlcCer, and further degradative
impairment which leads to a progressive neurotoxicity in both GBA1 mutation carriers and idiopathic PD patients [356]. Recently, the disruptive influence of α-syn accumulation in sporadic PD was more completely understood with the observation that α-syn disrupts the proper trafficking of multiple lysosomal hydrolases by interfering with the normal association between GM130 and Rab1a and inhibiting their cis-Golgi tethering activity, critically impairing Golgi structure and vesicular trafficking [358]. Consistent with the view that α-syn aggregation impairs GCase and overall lysosomal degradative capacity, reductions in GCase expression and activity are seen in brain tissue of idiopathic PD patients just as in that of GBA1-mutation carrying PD patients [359-361], and GCase depletion often occurs in brain regions burdened by increased α-syn levels [360]. Other regulatory factors may certainly impinge on the bidirectional interplay between α-syn and GCase, as decreases in GCase mRNA have been documented in SN of PD and DLB patients [361] and GCase activity gradually diminishes with age in the SN and putamen of healthy controls to a level comparable with that of GBA1-mutation carrier PD patients [362]. Impaired GCase results in increased GlcCer and αsyn, perturbations in autophagic degradation, and dysfunctional and enlarged lysosomes in affected neurons [360, 363-366]. While additional factors, perhaps dependent on age, may influence GCase activity and alter the threshold for disease initiation, α-syn aggregation has been firmly established as a primary cause of decreased GCase activity, initiating a positive-feedback cycle of degeneration central to PD pathogenesis.
Other genetic diseases that exhibit a relationship with α-syn through impairment of degradative processes include Kufor-Rakeb syndrome (KRS) and neuronal ceroid-lipofuscinosis (NCL). KRS is an autosomal recessive form of early-onset familial parkinsonism with pyramidal degeneration and dementia caused by homozygous loss-of-function mutations in a late-endosomal and lysosomal transmembrane P-type ATPase known as ATP13A2 [367-369]. Even single heterozygous mutations in ATP13A2 have etiological relevance for young onset PD [368]. Decreased ATP13A2 function results in impaired lysosomal acidification, reduced proteolytic processing of lysosomal enzymes and degradation of substrates, diminished clearance of autophagosomes, and accumulation of abnormal lysosomes [369, 370]. ATP13A2 has also been shown to be decreased in dopaminergic nigral neurons from patients with PD, where it instead accumulates within LBs, implicating ATP13A2-mediated lysosomal dysfunction in settings other than just its genetic mutation [370]. In vitro studies have demonstrated that loss of ATP13A2 function leads to accumulation of α-syn and α-syn-mediated neurotoxicity through impairment of its degradation [371], but animal models with deficient ATP13A2 observed defects in protein trafficking, accumulation of ubiquitinated protein aggregates, and endolysosomal abnormalities occurring in the absence of α-syn pathology, suggesting an additional contribution by α-syn-independent mechanisms to endolysosomal dysfunction and neurotoxicity [372]. NCL is a lysosomal storage disorder characterized by early onset, progressive neurodegeneration with gliosis and autofluorescent deposits of lipofuscin known to be caused by homozygous deficiency of
CTSD, the gene encoding the lysosomal protease CatD [372, 373]. CatD is instrumental for the degradation of α-syn in the lysosome, and thus expectedly its pharmacological inhibition, introduction of a catalytically inactive mutant form, or loss-of-function genetic mutation result in α-syn misprocessing and accumulation [333, 373, 374]. Although complete loss of CatD elicits the most profound inhibition of lysosomal function, heterozygous mutations that result in a partial loss of CatD still reduce lysosomal activity and cause accumulation of intracellular α-syn aggregates [373]. In addition to highlighting the key role of the ALP in degradation of α-syn, these studies demonstrate that slight perturbations in lysosomal acidification or a partial loss of CatD activity, occurring even in the absence of ATP13A2 or CTSD mutations, may be sufficient to cause a reduction in lysosomal function and result in α-syn aggregation in PD. This, coupled with the dysfunction in degradative processes induced by α-syn aggregates, results in a vicious cycle of protein aggregate accumulation and degradative dysfunction.

Given the dysfunction induced by α-syn in degradative processes, and the critical role of these processes in clearance of α-syn aggregates, some therapeutic approaches have sought to augment autophagic or lysosomal degradation as a strategy to ameliorate α-syn induced dysfunction, reduce the burden of misfolded α-syn, and prevent neurotoxicity. Expression of the autophagy related protein beclin 1 was shown to activate autophagy and lysosomes, reduce accumulation of α-syn, and ameliorate neuritic pathology caused by α-syn overexpression [339]. Likewise, overexpression of the transcription factor TFEB, known to be aberrantly retained in the cytosol of PD
neurons, reverses the PD-linked decline in lysosome function and facilitates neuroprotection via clearance of α-syn aggregates [341]. Overexpression of LAMP2A attenuates α-syn-induced dysfunction in CMA by upregulating this degradative process, reducing both total and misfolded α-syn levels, and preventing α-syn-mediated neurotoxicity [375]. Finally, boosting expression or activity of GCase in the lysosomal compartment has been shown to ameliorate histopathological abnormalities such as accumulation of GlcCer, ubiquitin, tau, and α-syn, and to reverse perturbations in hydrolase trafficking and lysosomal dysfunction caused by the previously accumulating α-syn [357, 376, 377]. Correcting the defects in α-syn degradation have the therapeutic potential to break the cycle of protein aggregation and impaired degradation, and allow both reversal and prevention of dysfunctional α-syn proteostasis.

**Cytotoxicity.**

Given all the pathologic events associated with α-syn aggregation, there are many possibilities for how protein aggregation contributes to neuronal cell death in PD. Aside from the accumulation of misfolded α-syn into LB inclusions, the histologic hallmark of PD is dopaminergic neurodegeneration in the SNpc. Aggregates of α-syn, or even just the NAC domain, induce apoptotic cell death in cultured neuronal cells [378], and gene transfer of human α-syn to rat SN results in 50% loss of dopaminergic neurons 13 weeks after infection with the gene-carrying viral vector [379], although several mechanisms have been proposed to explain this neuronal loss. One mechanism for induction of cytotoxicity may relate to the physiologic role of α-syn in associating with
pre-synaptic membranes, such that α-syn aggregation both sequesters functional forms of α-syn into insoluble aggregates and further impairs the actions of other synaptic proteins involved in membrane fusion events at synaptic terminals [176, 380]. While the knockdown of α-syn and the resulting loss of its physiologic function has been shown to be toxic for nigrostriatal dopaminergic neurons [175], α-syn overexpression and aggregation results in decreased synaptic proteins involved in exocytosis and endocytosis, enlarged synaptic vesicles, deficits in neurotransmitter release, impairments in excitability and connectivity, and eventual neurotoxicity [202, 381]. One reason for α-syn aggregates causing dysfunctional excitability and neurotransmitter release may be due to sequestration of a subunit of the Na⁺/K⁺-ATPase within membrane bound α-syn clusters, reducing local densities of this subunit and decreasing efficiency of Na⁺ extrusion following stimulus [382]. Another mechanism whereby α-syn aggregation results in neurotoxicity is its impairment of its own degradation. Not only has α-syn been causally linked to impairments in the ALP which result in toxicity [336, 356, 383], Golgi fragmentation and subsequent toxic impairments in trafficking are known to be a specific consequence of α-syn aggregation as well [129, 358]. Finally, α-syn aggregation may cause neuronal dysfunction and toxicity by disrupting normal axonal transport [384, 385], a key process for movement of signaling components and degradative vesicles throughout the long, elaborate axonal network of dopaminergic neurons. Axonal transport motor proteins such as kinesin and dynein are decreased in brains of sporadic PD patients prior to the onset of dopaminergic neurodegeneration,
with significantly greater reductions observed in nigral neurons containing \( \alpha \)-syn inclusions [386]. Kinesin motor proteins facilitate anterograde transport from the soma to the presynaptic terminal while dynein motor proteins facilitate retrograde transport in the opposite direction, from the terminals back to the soma. Animal models of PD that overexpress A53T or A30P \( \alpha \)-syn observe decreased kinesin levels and increased dynein levels in the striatum, the site where nigrostriatal dopaminergic neurons synapse, and increased kinesin levels in the SNpc where the cell bodies of nigrostriatal neurons reside [386, 387]. These findings suggest that \( \alpha \)-syn causes motor proteins to be trapped at their respective origins, unable to bind to microtubule tracks to facilitate axonal transport [384]. These effects may be due to direct actions of \( \alpha \)-syn on microtubules themselves, as \( \alpha \)-syn pathology is known to begin in axons as LNs before progressing to more mature LB pathology in the soma. \( \alpha \)-syn is known to act in its monomeric state as a microtubule-dynamase, binding tubulin and promoting microtubule nucleation and enhancing growth rate and catastrophe frequency [172], although this role may be perturbed when \( \alpha \)-syn becomes misfolded into aggregates. Indeed, tubulin levels are decreased in nigral neurons overexpressing \( \alpha \)-syn [387], and both \( \alpha \)-syn fibrils and protofibrils inhibit microtubule assembly by binding to tau and promoting its aggregation, depleting the tau available for microtubule polymerization and inhibiting its microtubule stabilizing function [388]. The great variety of neurotoxic insults brought on by \( \alpha \)-syn aggregation underscores the importance of understanding this complex process more fully.
One of the most highly debated questions regarding α-syn-induced cytotoxicity is whether smaller forms of α-syn aggregates represent the more toxic α-syn species, or if instead the larger histopathologic hallmark α-syn inclusions induce the most cellular toxicity. Although structured intermediates in the fibrillization spectrum from single monomer to elongated fibril may be known by various names such as protofibrils or oligomers, there are two key differences between the multitude of oligomeric forms and larger α-syn inclusions; the first difference seems to be the overall size of the assembly, with inclusions being many times larger than individual α-syn oligomers, and the second difference is the likelihood of α-syn self-association or clustering, which is necessary to generate a compact LB inclusion from multiple elongated α-syn fibrils. While the subject of the most toxic α-syn species is still actively contested, smaller α-syn oligomeric forms have emerged as likely candidates for pathological induction of cell death [129, 389, 390]. Smaller α-syn oligomeric or fibrillar species have been shown to directly induce cytotoxicity in a multitude of experimental settings [391-393]. In contrast, α-syn forms that fibrillize to larger sizes more quickly are less toxic [393, 394], consumption of excess soluble α-syn into fibrillar forms is neuroprotective [204], and promoting elongation and clustering of fibrils may reduce fragmentation, seeding, and the resulting toxicity [395]. These findings agree with the protective role of larger LB inclusions containing fibrillar α-syn and suggest that smaller oligomeric or fibrillar α-syn forms represent the most toxic aggregate species. While there are many possibilities for mechanisms of neurotoxicity induced by the various forms of α-syn aggregates, and
multiple mechanisms may independently or cooperatively contribute to PD, elucidating new therapeutic targets within these impaired pathways will result in disease-modifying treatments.

Cell-to-Cell Propagation

α-Synuclein.

Braak hypothesis. The foundation for investigating the prion-like cell-to-cell transmission of α-syn was formed with the pioneering work of Heiko Braak, who elucidated a neuroanatomical staging system of α-syn pathology in PD demonstrating the topographical progression of pathologic α-syn from early to advanced cases of idiopathic PD [396]. Braak noted specific disease induction sites, such as the dorsal motor nucleus (DMN) of the glossopharyngeal and vagal nerves and the olfactory bulb (OB), where LBs and LNs containing pathologic α-syn initially occur [397]. From lower brainstem or olfactory regions initially affected, α-syn lesions then progress in a topographically-predictable ascending fashion through six stages, progressively involving olfactory, autonomic, limbic, and somatosensory brain regions with little variation between affected individuals [397-399]. The early presence of α-syn inclusions in the submucosal Meissner plexus of the gastrointestinal (GI) tract, whose axons reach within the intestinal mucosa near mucosal glands, or in olfactory neurons, whose olfactory receptors reside in the nasal epithelium, highlights the exposure of both neuronal cell types to potentially hostile environmental factors, and formed the basis for what is now known as the “Braak hypothesis”, that α-syn pathology in PD originates outside the CNS.
prior to its stereotypic CNS expansion pattern [400-402]. While Braak’s original theory postulated the existence of a yet unidentified pathogen capable of crossing the GI mucosal barrier, invading post- and pre-ganglionic enteric neurons, and eventually entering the CNS by way of transneuronal and retrograde axonal transport through visceromotor projections of the vagus nerve, such a pathogen has not been shown to exist [400]. The Braak hypothesis has thus expanded to include the idea that exposure to environmental factors may induce initial α-syn misfolding in the peripheral enteric or olfactory neurons, and then set into motion the prion-like cell-to-cell propagation of α-syn aggregates through seeded templating of further aggregation in the aforementioned uninterrupted series of susceptible neurons extending from the periphery to the CNS [401, 402]. While controversial, the Braak hypothesis has the potential to explain many relevant aspects of PD pathology.

In agreement with Braak’s hypothesis and pathological staging system, LBs are frequently observed in the GI tract, in paraspinal sympathetic ganglia, in the vagus nerve, and in the spinal cord of PD and DLB patients [403-405]. GI tract immunostaining for α-syn occurs prior to the onset of first motor PD symptom, and was not observed in healthy control samples or in subjects with inflammatory bowel disease, suggesting that α-syn histopathology is not a non-specific consequence of inflammation or oxidative stress [406, 407]. Constipation and anosmia are both frequent complaints of PD patients prior to the onset of classic motor symptoms [80, 85, 88], possibly reflecting an early-stage alteration in normal gut motility and olfaction induced by α-syn accumulation in
enteric and olfactory neurons. If, as the Braak hypothesis postulates, exposure of enteric neurons to environmental factors present in the GI tract can create conditions promoting the initial misfolding of \( \alpha \)-syn, the intestinal microbiome, with its diverse ecosystem of trillions of microbes, represents one of the most prominent exposures and has the potential to influence a multitude of immunologic and metabolic processes relevant for the microenvironment of neurons in the GI tract. Analysis of intestinal microbiomes by bacterial genome sequencing revealed that bacterial populations are altered in PD patients compared to controls, with a significant reduction in Prevotellaceae and an association between the abundance of Enterobacteriaceae and the severity of postural instability and gait difficulty [408]. In addition to alterations in microbiome bacterial populations, the presence of gut microbiota is required for motor deficits, microglial activation, and \( \alpha \)-syn pathology in mice overexpressing \( \alpha \)-syn [409]. In this mouse model of PD, removal of gut microbes with antibiotic treatment or germ-free conditions reduced microglial activation, \( \alpha \)-syn inclusions, and motor deficits, whereas gut re-colonization worsens neurological disease [409]. Recolonization with microbiota derived from PD patients results in exacerbated motor symptoms compared to recolonization with healthy control microbiota, indicating that microbiome dysbiosis in the PD gut influences the development of \( \alpha \)-syn pathology in the brain [409]. While neuroinflammation has been implicated as playing a key role in gut-brain signaling and the prion-like behavior of \( \alpha \)-syn [410], identifying the specific microbiome alterations that pose an increased risk for PD as well as elucidating the events, mediators, and
routes involved in signaling are tasks for future study. Despite the lack of clarity regarding the factors that initiate α-syn pathology in the gut and/or OB, the Braak hypothesis of peripheral-to-central transmission of disease is nevertheless supported by the finding that full truncal vagotomy is associated with a decreased risk for subsequent PD [411]. This study and many others highlight the need for a deeper understanding of the mechanisms underlying disease transmission.

Because of the theory that environmental insults contribute to pathological α-syn misfolding in peripheral nervous tissue and trigger the prion-like invasion of pathology into and throughout the CNS [412], various animal models have been employed to demonstrate the feasibility of α-syn peripheral-to-central transmission. Injection of α-syn-containing PD brain lysate or recombinant α-syn aggregates into the intestinal wall leads to α-syn transport via the vagal nerve to the DMN in the brainstem in a time-dependent manner [413]. Retrograde transport on axonal microtubules has been implicated in this gut to brain translocation, both through the vagal nerve when α-syn is injected into the gut and through the sciatic nerve when α-syn is injected intramuscularly (IM) into the hind limb [413, 414]. α-syn aggregates are also capable of invading the CNS via intraperitoneal (IP) injection or crossing the blood-brain barrier following intravenous (IV) injection, although IM injection has been shown to be more efficient in inducing CNS pathology compared to IP or IV injections [218, 415, 416].

Despite this experimental evidence for the feasibility of ascending disease from PNS to CNS, another proposed theory, contrary to Braak’s hypothesis, instead postulates a
lower functional threshold for emergence of non-motor symptoms prior to classical motor symptoms, but that both CNS and PNS exhibit parallel degeneration [417]. Finally, there is also evidence that α-syn pathology can propagate in the reverse direction to that proposed by Braak, that upon its midbrain overexpression pathological α-syn can reach the medulla, then the DMN of the vagus nerve, and finally utilize vagal projections as a conduit for transmission to the GI tract [418]. Despite conflicting theories regarding the origins of α-syn pathology, these studies illustrate the centrality of α-syn cell-to-cell propagation in PD progression and underscore the importance of continued work to understand this process more completely.

**Clinical evidence of propagation.** The first studies to uncover cell-to-cell propagation of α-syn in the PD brain involved pathological examination of brain tissue from PD patients who had received grafts of fetal dopaminergic neurons into the striatum many years prior to death. Although grafted dopaminergic neurons remained viable and exhibited extensive innervation of the host striatum more than a decade following transplantation, long-term clinical benefit was not observed from this therapy and grafted nigral neurons developed pathological LB inclusions containing α-syn and ubiquitin (Figure 5C-E) identical to those observed in host brain (Figure 5A-B) [419, 420]. These matching findings from both the Kordower and Brundin groups were the first to demonstrate that ongoing α-syn pathology can propagate from affected to naïve neurons in PD brain [419, 420], although another report observing long-term dopaminergic graft survival in the absence of infiltrative α-syn pathology suggests that
Figure 5. Parkinson's disease–like pathology in long-term nigral grafts. In the nongrafted host’s nigra, typical α-synuclein (a) and ubiquitin (b) neuropathology was observed (arrows). (c) Extensive α-synuclein pathology was seen in grafted neurons, including cytoplasmic and aggregated α-synuclein (arrows) as well as α-synuclein neurites. (d,e) Pathological aggregates of ubiquitin were also seen in grafted neurons (arrows) and fibers (arrowheads). Scale bar, 40 mm. Reprinted by permission from Macmillan Publishers Ltd: Nature Publishing Group, Nature Medicine, Volume 14, Issue 5, Jeffrey H Kordower, Yaping Chu, Robert A Hauser, Thomas B Freeman, C Warren Olanow, Lewy body–like pathology in long-term embryonic nigral transplants in Parkinson’s disease (Reference 419), Copyright 2008. (License Number 4057251374706). http://www.nature.com/nm/index.html.

α-syn transmission may not occur equally in all patients [421]. While most grafted dopaminergic neurons remain unaffected by α-syn pathology, various studies have reported the presence of α-syn-containing LBs in 2%-27% of grafted neurons, illustrating varying degrees of propagation from host to graft [422-426]. LBs that form within
grafted neurons are indistinguishable from LBs in host brain, as both are composed of fibrillar pS129-α-syn and ubiquitin and exhibit positive staining for thioflavin-S [422, 423]. Furthermore, the distinct stages of LB development observed in host brain are also present in grafted tissue, which contains both loose meshwork aggregates and classical compact LBs [423]. When dopaminergic neurons are implanted into the striatum in a rat model of PD, about 6% of grafted cells contain α-syn 5 weeks following α-syn overexpression in a neighboring host brain region, indicating that α-syn host-to-graft propagation is a possible mechanism to explain graft α-syn pathology and the observed results in human grafts are not due solely to non-specific responses to possible inflammation in the graft area [424]. Despite the positive observations that graft cells exhibit long-term viability, provide DA re-innervation to a widespread area of the striatum, and in some cases result in albeit temporary major motor improvement, the lack of sustained clinical improvement following transplantation could reflect progression of degenerative changes into grafted cells induced by infiltrating α-syn pathology [425, 426].

Another set of clinical observations that support α-syn cell-to-cell propagation are those that have identified α-syn within the cerebrospinal fluid (CSF) of PD patients. The presence of α-syn in the CSF reflects a process of neuronal release that would be relevant for cell-to-cell propagation if taken up by neighboring naïve cells. Following the identification of α-syn in human CSF and blood plasma [427], further investigation revealed that α-synucleinopathy patient CSF exhibited lower total α-syn but elevated
levels of oligomeric or aggregated $\alpha$-syn compared to healthy control CSF [428-432].

Even pS129 $\alpha$-syn has been identified in CSF of PD patients [432, 433], and despite high levels of $\alpha$-syn expression in blood, the principal source of $\alpha$-syn in CSF is known to be neurons of the brain and spinal cord [434]. Additionally, $\alpha$-syn was detected in the CSF of patients with traumatic brain injury and with CJD, implicating cell death processes as an additional mechanism for neuronal release of $\alpha$-syn [429, 435]. Methods such as enzyme-linked immunosorbent assay (ELISA) and mass spectrometry (MS) have been employed for detection of $\alpha$-syn in CSF samples [427-430, 433, 435], although one recent study utilized PMCA to successfully identify PD patients with a sensitivity of 88.5% and specificity of 96.9% by detecting as little as 0.1 pg/mL of $\alpha$-syn oligomers in CSF [436]. Critically, despite $\alpha$-syn detection in the extracellular environment of the CNS and in blood, a large-scale retrospective cohort study of nearly 1.5 million blood transfusion patients over more than 50 years did not observe any evidence of PD transmission, nor transmission of any neurodegenerative disease investigated, despite 2.9% of included patients receiving a transfusion from a donor diagnosed with one of the studied neurodegenerative diseases [437]. While the presence of $\alpha$-syn in biological fluids does not result in disease transmission between hosts in the manner of prion infection, and thus is the reason its propagation is referred to as “prion-like”, $\alpha$-syn aggregates released from diseased cells into CSF certainly impact neighboring susceptible cell populations. Although the presence of $\alpha$-syn in grafted dopaminergic neurons and in the extracellular environment of the CSF implies a process for its release,
intercellular trafficking, and uptake, the specific mechanisms involved in this life cycle remain unclear (Figure 6).

**Axonal transport.** Just as in Braak’s hypothesis for transfer of pathological α-syn from peripheral neurons to the CNS, axonal transport has been a central focus for the movement of α-syn within cells and between interconnected brain regions [385, 438]. The propagation of α-syn to distant brain regions through axonal connections was first demonstrated in young asymptomatic α-syn transgenic mice following intracerebral injections of brain homogenates from older α-syn transgenic mice which had developed α-syn pathology [212]. Coincident with the propagation of pathological α-syn through axonal transport to interconnected brain regions is an acceleration of LB inclusion formation, consistent with seeded templating of endogenous α-syn by exogenous aggregates, as well as a quicker onset of neurological symptoms and reduced survival in animals receiving α-syn injections [212]. Crucially, an identical phenotype of PD-like LB formation, α-syn propagation to interconnected brain regions, neuronal loss, and eventual neurological symptoms is observed following intracerebral or intrastriatal inoculation of both α-syn transgenic and WT nontransgenic mice with synthetic α-syn fibrils assembled from recombinant α-syn [212, 213]. Because synthetic α-syn fibrils are sufficient to initiate and propagate disease, these findings implicate the prion-like cell-to-cell transmission of α-syn aggregates as underlying neuronal degeneration and clinical progression of PD.
Figure 6. Potential mechanisms mediating cell-to-cell transmission of cytosolic protein aggregates. (a,b) Misfolded protein seeds (for example, oligomers and protofibrils) first form in the cytoplasm of the releasing neuron (left), where soluble native monomers are recruited into large intracellular aggregates and a positive feedback loop can be initiated by generation of more seeds through fragmentation or secondary nucleation. A small amount of protein aggregates can be released into the extracellular space in the ‘naked’ form (a) or via membrane-bound vesicles such as exosomes (b). Free-floating seeds may directly penetrate the plasma membrane of the recipient neuron (1) or enter by fluid-phase endocytosis (2) or receptor-mediated endocytosis (3), whereas exosomes containing seeds may fuse with the membrane of the recipient neuron (4). Intercellular transfer of seeds may also occur by nanotubes that directly connect the cytoplasm of two cells (5). Internalized seeds then nucleate the fibrillization of native monomers in the cytoplasm of the recipient neuron. Reprinted by permission from Macmillan Publishers Ltd: Nature Publishing Group, Nature Medicine, Volume 20, Issue 2, Jing L Guo, Virginia M Y Lee, Cell-to-cell transmission of pathogenic proteins in neurodegenerative diseases (Reference 465), Copyright 2014. (License Number 4057270285760). http://www.nature.com/nm/index.html.
These initial observations of α-syn transmission through axonal contact have been substantiated using several other model systems. When pathological α-syn contained within LB-enriched isolates from the SN of postmortem PD brain were injected into the SN or striatum of mice or monkeys, the exogenous human α-syn was quickly taken up by host neurons where seeding of expressed α-syn then occurred [216]. This was followed by both anterograde and retrograde axonal transport to interconnected brain regions and eventual nigrostriatal degeneration that required the presence of α-syn in both PD brain extracts and host cells, implying that both exogenous and expressed α-syn play a role in transmission and toxicity [216]. Although fibrillar forms of injected α-syn induce a greater accumulation of pS129 α-syn LB pathology in rat striatum, injection of non-aggregated α-syn can also produce this effect, and both injection forms produce LBs in brain regions that supply innervation to the striatum injection site [217]. Another study revealed pS129 LB inclusions are found at first-order afferent sites in the piriform and entorhinal cortices, amygdala, and hippocampus three months following infusion of α-syn fibrils into the OB of mice, whereas areas nearby but without axonal connections to the injection site lacked LB inclusions, supporting movement of α-syn through axonal routes rather than simple diffusion [439]. Finally, targeted overexpression of α-syn using unilateral intracerebral injection of an α-syn lentiviral vector caused axonal propagation of expressed α-syn to the contralateral side along with axonal degeneration and behavioral deficits in mice [440]. These findings implicate trafficking of α-syn aggregates through axonal networks as a key mediator of
pathological propagation, and indicate the importance of elucidating both the species of α-syn involved as well as mechanisms facilitating intercellular spreading.

It is of utmost importance to elucidate the molecular form of α-syn most capable of cell-to-cell transmission so that therapeutic efforts targeting these propagating forms, such as immunotherapy, will have maximum success in halting disease progression [440]. Just as smaller forms of α-syn aggregates have emerged as the molecular species most capable of seeding further aggregation and inducing the most cytotoxicity, smaller α-syn aggregates also appear to propagate from one cell to another with much greater efficiency. Most PD models utilizing exposure to exogenous α-syn aggregates generated in vitro from purified recombinant α-syn will perform sonication to fragment larger α-syn fibrils into multiple smaller components that retain their original structure although with a much-decreased population size distribution [200, 202, 203, 212, 213, 219, 439]. These smaller fragments of previously elongated α-syn fibrils are known as “pre-formed fibrils” (PFFs) and are widely utilized to examine the impact of exogenous α-syn on seeding aggregation, intracellular toxicity, and intercellular transmission precisely because their smaller size distribution allows them to be readily taken up by recipient cells [202, 203]. The increased propagation efficiency of smaller α-syn aggregates is illustrated in recent work examining transmission of α-syn from OB to other brain structures. When monomeric, oligomeric, and elongated fibrillar preparations of α-syn aggregates were injected into the mouse OB, monomeric and oligomeric α-syn, but not the larger fibrillar forms, were readily taken up by OB neurons and transferred to
axonally interconnected brain regions within minutes to hours, although these non-fibrillar preparations are cleared from the brain within a few days following injection [441]. In contrast, when elongated α-syn fibrils were fragmented to smaller sizes by vigorous sonication to create PFFs prior to injection into mouse OB, they efficiently seed the aggregation of endogenous α-syn into pS129 LB inclusions and spread over several months via axonal connections first within the olfactory network and later in distant connected brain regions [219]. Another study found that waterbath sonication of fibrils for 1 hour significantly reduced fibril size by EM compared to 1 minute probe sonication, and this results in improved α-syn cell-to-cell transmission [439]. Finally, viral vector-based overexpression of α-syn in the medulla results in monomeric, oligomeric, and fibrillar immunoreactivity in donor neurons at this site, but only the non-fibrillar forms of α-syn were similarly detected in pontine recipient neurons, indicating that larger α-syn fibrils have significant barriers to propagation because of their larger size [442]. These studies demonstrate that smaller α-syn fibrils, because of their decreased size, are readily taken up by recipient neurons, efficiently seed aggregation of expressed α-syn, and are quickly spread along axonal connections to naïve brain regions, facilitating further aggregation and disease propagation.

Investigation into the mechanisms of α-syn axonal propagation have been ongoing, but studies utilizing microfluidic devices where axons project through small channels to terminate in a microenvironment isolated from that of their respective cell bodies have brought new insights regarding the steps in the process. α-syn fibrils have
been shown to be transferred from axons to second-order neurons following axonal transport through a process that did not require direct cell-to-cell contacts, implying the existence of an extracellular phase between donor and recipient cell [443] (Figure 6). In addition, this release of α-syn following axonal transport was not due to axonal lysis, but occurred in healthy, intact neurons [444]. Contrary to these findings, however, α-syn has also been shown to transit from donor to acceptor cells through TNTs inside lysosomal vesicles, and even to increase cellular formation of TNTs, eventually resulting in seeding of further α-syn aggregation in the cytoplasm of acceptor cells [445]. Since TNTs are F-actin containing membranous bridges that connect the cytoplasm of remote cells, this direct transfer of lysosomal vesicles and their α-syn cargo would avoid an extracellular phase in the intercellular journey [445] (Figure 6). While multiple mechanisms of transmission may play a role in α-syn cell-to-cell spreading, the specific form of propagating α-syn as well as its accessibility to therapeutic targeting during this transit are crucial factors for the success of immunotherapy treatment approaches [440].

Despite the highly reproducible nature of the findings detailing α-syn cell-to-cell propagation via axonal transport by multiple independent groups, this phenomenon has not been without contradiction. One study found that injected α-syn PFFs induced widespread LB inclusion formation only in transgenic mice expressing A53T α-syn but not in non-transgenic mice or transgenic mice expressing E46K α-syn, which instead exhibited inclusion pathology restricted to the site of injection and had no evidence of
spreading [446]. This observation suggests that alternative processes may also contribute to α-syn inclusion formation and neurotoxicity in addition to or instead of α-syn prion-like spreading. Also, seeding and propagation processes may operate independently of one another, as evidenced by the observation that a lack of endogenous α-syn expression does not counteract axonal transport and intercellular transmission of exogenous αsyn, but instead results in its more pronounced intercellular movement [442]. Although the seeding process induces neurotoxicity and explains the amplifying nature of α-syn aggregation throughout the brain, this finding indicates that seeding endogenous aggregation may also consume exogenous propagating seeds into larger aggregates that propagate less efficiently. Thus, intercellular transmission of α-syn may also occur in the absence of seeding, suggesting two separate but intertwined processes. Axonal transport has been extensively implicated in the cell-to-cell propagation of pathological α-syn, and forms the rationale for further investigation into the mechanistic steps of its intercellular life cycle.

Endocytosis. The process of endocytosis, where cells sample their extracellular environment through plasma membrane invaginations and inward budding of vesicles containing extracellular components [447], has been repeatedly implicated as a central mechanism for the cellular uptake of α-syn aggregates (Figure 6). The endocytic route of uptake features prominently in the cellular life cycle of α-syn aggregate propagation as a mechanism of entry into recipient cells [448]. The first evidence for the importance of the endocytic process in the cellular uptake of α-syn aggregates was the correlation
between neuronal cell death induced by the addition of exogenous α-syn and Rab5A-specific endocytosis [449]. Expression of a GTPase-deficient Rab5A resulted in a decrease of α-syn-induced cytotoxicity due to its incomplete endocytosis [449]. Additionally, internalization of oligomeric and fibrillar forms of α-syn aggregates could be inhibited by low temperature or expression of a dominant-negative mutant of dynamin, a component of the endocytic machinery essential for scission of endocytic intermediates to form vesicles [450]. These observations implicate endocytosis as the mechanism of internalization for α-syn aggregates, although since the cellular entry of monomeric α-syn was unaffected by these manipulations, single α-syn monomers are likely capable of entering cells via direct membrane translocation [450] (Figure 6). Several other studies have used similar methodologies in various model systems to verify the involvement of endocytosis in α-syn aggregate uptake by many different recipient cell types. Neurons, astrocytes, oligodendrocytes, and microglia all internalize α-syn aggregates through endocytosis, and inhibition of endocytic uptake results in impaired intercellular α-syn propagation, underscoring the importance of this method of cellular entry for the α-syn transmission life cycle [450-457].

Once α-syn fibrillar aggregates bind to the cellular membrane, a process that occurs through lateral binding of the fibrillar aggregate [458], endocytic vesicle-mediated internalization matches the efficacy of membrane binding [280]. α-syn aggregates gain entry to recipient cells through either fluid-phase endocytosis, where membrane binding stimulates membrane ruffling and endocytic uptake [459], or
receptor-mediated endocytosis, where specific interactions between \( \alpha \)-syn aggregates and membrane components or receptors such as heparan sulfate proteoglycans (HSPGs) or lymphocyte-activation gene 3 (LAG3) initiate endocytosis [460, 461] (Figure 6). Once inside the cell, \( \alpha \)-syn aggregates are first contained within endosomes that transition into lysosomes for degradation of the \( \alpha \)-syn cargo [450, 462, 463]. However, \( \alpha \)-syn aggregates taken up by endocytosis are well known to serve as seeds for the templated misfolding of monomeric \( \alpha \)-syn expressed in the cytosol of the recipient cell, triggering the formation of LB inclusions [202, 347, 454, 464]. The mechanism by which \( \alpha \)-syn aggregates escape the endocytic vesicle compartment to access the cytosol and proceed with seeded templating of further \( \alpha \)-syn misfolding was until recently an open question [448, 465] (Figure 6), but the demonstration that \( \alpha \)-syn aggregates induce lysosomal rupture following endocytosis provided an explanation [4]. The identification of \( \alpha \)-syn-induced lysosomal rupture following endocytosis illuminates the connection between exogenous and expressed \( \alpha \)-syn necessary for seeding to occur and, moreover, implicates \( \alpha \)-syn propagation as contributing to degradative dysfunction through inducing lysosomal damage. Cellular entry of \( \alpha \)-syn aggregates through endocytosis is an essential step in the propagation pathway, and contributes to many other aspects of prion-like neurodegeneration.

**Exocytosis.** Given the presence of \( \alpha \)-syn in the CSF and multiple synapses separating neurons along the \( \alpha \)-syn cell-to-cell propagation pathway, the cellular release of \( \alpha \)-syn from cells burdened by its misfolding and accumulation is necessary for
cell-to-cell propagation to occur [448, 466, 467] (Figure 6). Several mechanisms have been proposed to account for the jettison of α-syn from affected cells into the extracellular environment, each a variation on the theme of exocytosis. α-syn, because its lacks a secretory signal peptide sequence, has been shown to be secreted from cells via non-classical endoplasmic reticulum (ER)/Golgi-independent exocytosis [468]. Intracellular α-syn can be contained within the lumen of vesicles, often in an oxidized and aggregation-prone state, and the fusion of these vesicles with the plasma membrane results in the release of the intralumenal abnormal α-syn contents into the extracellular environment [468, 469]. Unconventional ER/Golgi-independent exocytosis of α-syn can also be caused by exophagy, the exocytosis of autophagosomes or their contents upon fusion with the plasma membrane [470, 471]. Late endosomes or recycling endosomes may also play a role in the release of α-syn cargo through exocytosis to the extracellular environment, as evidenced by inhibition of α-syn release upon disruption of Rab27a or Rab11a which are involved in endosomal regulation [470, 472, 473]. Regardless of the identity of the intracellular vesicle compartment fusing with the plasma membrane to release α-syn, these various forms of exocytosis discharge α-syn in a free state, no longer associated with lipid membranes, given that the vesicular membrane remains associated to the plasma membrane upon fusion (Figure 6). Free α-syn outside the cell would be susceptible to cleavage by matrix metalloproteinases (MMPs) operating in the extracellular environment [474], but would also be available for uptake into neighboring cells by endocytosis to further the propagation life cycle.
Additionally, α-syn has also been shown to be released from affected cells contained within exosomes, a type of extracellular vesicle originating from the plasma membrane fusion of multivesicular bodies (MVBs) which are endosomal compartments containing multiple internal vesicles generated from inward budding [475-477] (Figure 6). Exosomes have been found to contain both monomeric and oligomeric α-syn, and exosome-associated α-syn is more likely to be taken up by recipient neurons where it causes more toxicity than free α-syn released by exocytosis in a non-membrane bound form [478, 479]. α-syn within exosomes, as well as α-syn within extracellular vesicles derived from plasma membrane blebbing and shedding [473], are immune to the hostile extracellular environment because of their protected membrane-bound state. Sorting of α-syn into exosomes may be regulated by post-translational addition of small ubiquitin-like modifier (SUMO) which facilitates α-syn interaction with the endosomal sorting complex required for transport (ESCRT) machinery and eventual extracellular release [480]. Interestingly, the ESCRT pathway is also important for α-syn transport through MVBs for degradation, but α-syn is known to promote degradation of the ESCRT protein charged multivesicular body protein (CHMP2B), resulting in impaired ESCRT activity, accumulation of α-syn unable to be degraded, and increased exocytosis of α-syn [481]. Exosomes represent another mechanism for the cellular release of α-syn via exocytosis, and given their utility for intercellular delivery of biochemical information to directly modify signaling pathways or proteostasis in receiving cells [475], elucidating the underlying mechanisms prompting their release is of utmost importance.
**Stress-induced propagation.** Despite the detrimental consequences of α-syn release for the propagation of protein misfolding and its associated neurotoxicity throughout the brain, paradoxically, the release of α-syn aggregates is also a protective, last-resort response of stressed cells overwhelmed by α-syn accumulation and their ineffective or dysfunctional degradative clearance mechanisms [318, 482, 483]. When cellular degradative mechanisms are insufficient to cope with the continued accumulation of misfolded α-syn aggregates, or increasing mitochondrial stress perturbs cellular homeostasis, this prompts α-syn clearance through cellular release instead. Pharmacological inhibition of the proteasome or of mitochondrial complex I increases the release of both monomeric and aggregated α-syn, although more aggregated forms are present in the released proteins [468, 469]. Furthermore, elevated ROS occurring as a byproduct of DA synthesis results in mitochondrial dysfunction and increased secretion of α-syn oligomers, but not monomers, into the extracellular space [294].

Perhaps the most significant stressor contributing to α-syn release and propagation in PD is dysfunction in the ALP, as this pathway is the main mechanism for degradation of α-syn aggregates. Failure of autophagic processes, modeled by pharmacological or genetic inhibition, increases the exocytosis of α-syn [484], as does the lowering of autophagosomal mobility and the prevention of fusion with lysosomes [470, 471]. Lysosomal failure has even more far-reaching consequences, as lysosomal degradation is responsible for ultimate clearance of both autophagic and endocytic compartments. Dysfunctional lysosomal degradation, which has been extensively
modeled by treatment with bafilomycin A1 (Baf) to inhibit the acidifying action of the lysosomal H^+-ATPase, also results in increased α-syn release from affected cells [469-471, 473, 479, 485]. Deficiency of lysosomal enzymes CatD or GCase, occurring profoundly when mutated in genetic forms of PD but still contributing to lysosomal dysfunction even in sporadic PD, also results in increased α-syn secretion to the extracellular environment [364, 366, 373, 486]. Lysosomal degradation is also known to be impaired during normal aging, and as a result, the rate of cell-to-cell transmission of α-syn aggregates was also increased in an aging model [487]. These findings highlight the essential nature of the ALP in the degradation of α-syn aggregates, and illustrate how its dysfunction can result in the pathological release of α-syn from affected cells as a last-resort mechanism of clearance.

**Tau.**

The microtubule-associated protein tau (MAPT), pathologically aggregated in diverse tauopathies including AD, chronic traumatic encephalopathy (CTE), tangle-only dementia (TD), Pick’s disease (PiD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), agyrophilic grain disease (AGD), and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17T), exhibits many similarities to α-syn as a prion-like amyloid protein [488]. Just as for α-syn, tau aggregation from a soluble, unfolded, phosphorylated monomer to an insoluble, hyperphosphorylated neuropil thread (NT) or neurofibrillary tangle (NFT) results in unique strain conformations, arising due to seeded templating of aggregation, which impart tropism for different cell types.
and brain regions and result in distinct patterns of pathology for human tauopathies [314, 488-492]. Although alternative mRNA splicing results in tau expression as six different isoforms, composed of 0, 1, or 2 N-terminal inserts (0N, 1N, 2N) and either 3 or 4 C-terminal repeats (3R, 4R), the various compositions of tau isoforms (1N3R vs. 1N4R, for example) allow higher-order aggregates to adopt different shapes or conformations that behave as distinct strains [488].

Tau strains propagate their conformation through seeding aggregation of additional monomeric tau, and a specific strain conformation and its associated pathological phenotype of affected cell types and brain regions can be faithfully reproduced when tau strains are re-introduced into naïve cells or successively inoculated into mice [490, 492, 493]. Distinct tau strains may also exhibit varying levels of potency in their seeding of further aggregation because of their unique conformations, as strains purified from AD brain exhibited increased seeding potency compared with synthetic tau fibrils [491]. Seeding occurs between like isoforms, as aggregation of expressed 4R tau will occur following cellular uptake of 4R fibrils but not 3R fibrils, and vice versa when 3R tau is expressed in target cells [201]. This nucleation-dependent, composition-specific aggregation of tau recapitulates Prusiner’s protein-only hypothesis by the induction of NFT insoluble aggregates following cellular uptake of tau fibrils [494]. If seeding of further aggregation is inhibited by suppressing the expression of soluble tau, cellular degradative mechanisms are no longer overwhelmed by amyloid accumulation and tau aggregates can be cleared by the ALP, just as α-syn
aggregates are cleared, although both the UPS and ALP have difficulty clearing large aggregates [495]. Tau inclusions formed within affected cells are highly dynamic structures, undergoing fission and fusion events that facilitate pathological propagation by additional seeding events [495]. The observation that different tauopathies are associated with different sets of strains [490] raises the possibility, just as for \(\alpha\)-synucleinopathies or prion diseases, that conformational diversity of tau aggregation dictates the phenotype of clinical disease [492].

Another feature of tau pathology that is analogous to \(\alpha\)-syn is in the prion-like cell-to-cell propagation of tau aggregates. Heiko Braak and colleagues also described a staging system for tau pathology in AD just as for \(\alpha\)-syn in PD, detailing six distinct stages of an evolving characteristic distribution pattern for NT and NFT pathology [496]. An ordered and predictable progression of hyperphosphorylated tau pathology was also seen in CTE co-occurring with axonal disruption and death, leading to the progressive staging of pathology through 4 stages [497]. These findings suggest, through the predictable, conserved expansion pattern of tau pathology in different tauopathies, that cell-to-cell propagation of tau aggregates may also contribute to unique clinical phenotypes and may be an etiological factor for disease progression and increasing severity. Cell-to-cell spreading of aggregated tau has been observed in numerous experimental models, where expressed tau adopts characteristics of the pathological tau seeds and spreads to neighboring cultured cells or interconnected brain regions. Extracellular tau aggregates, but not monomer, become internalized into recipient cells
where they induce formation of expressed tau aggregates which then transfer between co-cultured cells [498]. When brain extracts are prepared from mice expressing human P301S mutant tau, the tau mutation that causes FTDP-17T in humans and results in widespread hyperphosphorylated tau and neurodegeneration in transgenic mice, extract injection into the brains of transgenic WT human tau-expressing mice which do not normally develop tau filaments or neurodegeneration induces aggregation of WT tau and the spreading of these aggregates to neighboring brain regions [499]. Similar results were observed when brain extracts from humans with AD, AGD, PSP, and CBD were injected into the brain of WT mice and caused the seeding and cell-to-cell propagation of endogenous tau with a phenotype that recapitulated the hallmark disease lesions of the extract source [500, 501]. Even intracerebral injection of synthetic tau PFFs into mice overexpressing P301S mutant tau induces NFT inclusions and propagation of tau pathology to connected brain regions over time [502]. In the process of spreading between interconnected brain regions [491, 502], synaptic connections appear to enhance tau propagation [503], although transmission can also occur via non-synaptic mechanisms such as along glymphatic or CSF pathways [504, 505]. The prion-like cell-to-cell propagation of tau aggregates represents a mechanism for the amplification and dissemination of tau pathology throughout the brain of affected patients, and thus understanding the mechanisms underlying cellular uptake and release of tau aggregates are of utmost importance.
Examination of potential mechanisms of tau cellular entry and egress again reveals numerous similarities with α-syn and prions. Endocytosis is again the predominant mechanism for cellular uptake of tau aggregates as for α-syn aggregates [498, 506-508], perhaps occurring after interaction at the plasma membrane between tau aggregates and HSPGs [460]. Tau uptake depends on both the conformation and the size of the aggregates, as low-molecular weight aggregates or short fibrils, but not monomers, long fibrils, or long filaments, were capable of cellular entry via endocytosis [509]. This endocytic uptake process is regulated in part by the neuronal isoform of the BIN1 protein which serves as a negative regulator of endocytic flux, preventing tau propagation through endocytosis when functional but promoting tau propagation when downregulated in AD brain [507]. The conserved role of endocytosis in the uptake of tau and α-syn aggregates underscores the importance of this pathway in the cellular life cycle of these amyloid proteins, allowing for the propagation of misfolded structure from affected to naïve cells. Endocytosis allows for the induction of expressed tau aggregation by exogenous tau seeds, as extracellular seeds come into direct contact with intracellular tau as seen by Förster resonance energy transfer (FRET) following endocytosis [498, 510]. Just as for α-syn aggregates taken up via endocytosis, the process by which endocytosed tau aggregates escape the endocytic vesicle and thus become competent to seed the aggregation of expressed tau in the cytosol remains unanswered [465].
As for the process of tau cellular release, monomeric tau is constitutively released, even from normal healthy cells, into the interstitial fluid (ISF) and CSF for unknown reasons [511-513], although this release process can be stimulated by neuronal activity and calcium-influx and blocked by inhibiting pre-synaptic vesicle release [512]. Although the CSF level of tau correlates with the burden of hyperphosphorylated tau and NFT pathology [514], levels of tau in ISF are significantly higher than in CSF, and following spontaneous or seed-induced tau aggregation, levels of monomeric ISF tau decrease significantly as monomers are consumed into insoluble aggregates [513]. This finding is additional evidence for the seeding-dependent equilibrium between monomeric and aggregated tau occurring in both the extracellular and intracellular environments. Tau secretion can occur through a non-classical pathway that is prevented by low temperature but not blocked by inhibitors of the conventional secretory pathway [511]. This form of tau secretion results in extracellular tau that is not associated with membranes, and thus illustrates the mechanism of therapeutic efficacy underlying antibody-mediated reduction in insoluble tau aggregates and inhibition of tau seeding and propagation [510, 515]. Tau can also be released from affected cells in a membrane-bound form, either in ectosomes formed from outward budding of the plasma membrane or in exosomes released upon plasma membrane fusion of MVBs containing internal vesicles [516-519]. Similar to \( \alpha \)-syn release, tau secretion is increased under conditions of cellular stress [518]. Finally, just as for \( \alpha \)-syn and prions, TNTs have been implicated in the intercellular transmission of tau aggregates, and tau,
like α-syn, promotes the formation of TNT connections that facilitate its transmission [520, 521]. There are many similarities in the aggregation and propagation processes of tau and α-syn as prion-like amyloid proteins, and there may also be similar therapeutic approaches to arrest progression of the diverse diseases caused by these different proteins with similar pathological activities.

**Huntingtin.**

HD is among a group of neurodegenerative disorders exclusively caused by dominantly-inherited genetic mutations that expand a CAG repeat sequence in the coding region of the affected gene and result in expression of proteins with expanded tracts of uninterrupted polyglutamine (polyQ) residues that are longer than in the WT protein [522]. In the case of HD, this trinucleotide repeat expansion occurs in the gene encoding the huntingtin protein, HTTExon1, and a polyQ tract greater than 37Q causes HD [523]. When the polyglutamine expansion is in the pathogenic range, for HTTExon1-Q≥37, this alteration induces the formation of insoluble, high-molecular weight aggregates that adopt a fibrillar morphology like that of prions, α-syn, and tau [524].

Since this discovery of the effect of polyQ expansion on the structure of the huntingtin protein, several commonalities have been revealed between HTTExon1 aggregates and other prion-like amyloid aggregates such as those of α-syn and tau [522].

Just as for α-syn and tau, the aggregation process of HTTExon1-Q≥37 exhibits prion-like properties. PolyQ repeat lengths of greater than 37 increase the kinetics and thermodynamic favorability of the spontaneous misfolding reaction into amyloid
aggregates, and although shorter repeats do not undergo spontaneous aggregation, they are capable of adding to an already formed aggregate and will be recruited into insoluble aggregates when exposed to extended polyQ tracts [525, 526]. Indeed, in addition to the length- and concentration-dependence of aggregation, exposure to pre-formed seeds significantly abbreviates the normal lag phase associated with spontaneous misfolding just as for other prion-like amyloid proteins [527]. This seeded recruitment and templating of further misfolding continues the prion-like aggregation theme of α-syn, tau, and others, and just as for these other amyloid proteins that adopt β-sheet ThT-positive filamentous structures, the smaller, more finely-divided aggregates of HTTExon1 are much more efficient at recruiting additional polyQ peptides than larger aggregates [525, 527].

Again like α-syn and tau, prion-like mechanisms of cell-to-cell propagation have been implicated in the progression of HD pathology [522]. In the cell-to-cell propagation pathway, the membrane association mechanisms of monomeric and assembled HTTExon1 are of significant interest. The N-terminus of monomeric HTTExon1 binds to membranes through a charge-based mechanism where it forms an amphipathic α-helix that inserts into the lipid bilayer [528]. PolyQ expansion allows for a greater depth of α-helix membrane insertion that may perturb the function of cellular membranes and alter the normal membrane-bound cellular localization of HTTExon1 [528, 529]. Once in an aggregated form, HTTExon1 aggregates will become internalized into recipient cells following binding of cell surface membrane proteins that uniquely recognize its fibrillar
amyloid structure [530]. This membrane binding occurs, as for \( \alpha \)-syn aggregates, through lateral association between the fibrillar aggregate and the cell membrane [458], although HSPGs do not seem to be involved for binding and internalization of HTTExon1 aggregates as they were for aggregates of \( \alpha \)-syn and tau [460]. Once bound to the membrane, HTTExon1 aggregates are internalized via endocytosis and directed to the lysosome [531], although the process of escaping the vesicle to gain access to the cytosol following endocytic uptake remains unclear [522, 531]. Despite the question of the specific vesicle escape mechanism remaining open just as for \( \alpha \)-syn and tau, exogenous HTTExon1 aggregates do gain entry to the cytoplasm following internalization where they become sequestered into aggresomes and recruit additional soluble homologous counterparts through seeded templating [531-533]. The technique of bimolecular fluorescence complementation (BiFC) illustrates that exogenous HTTExon1 aggregate seeds contact cytosolic HTTExon1 and induce its misfolding in the cell-to-cell transmission pathway [534]. Cell-to-cell propagation of HTTExon1 aggregates can occur through direct cell-to-cell contact, where TNT-mediated transmission is implicated and expression of mutant HTT can, like \( \alpha \)-syn and tau, increase formation of TNTs to facilitate transmission [535]. HTTExon1 aggregates can be transported within the cell in both anterograde and retrograde directions, and can also be secreted from axons of affected cells following anterograde transport to spread trans-neuronally in the absence of axonal lysis [444]. These various prion-like characteristics of HTTExon1 aggregates illustrate similarities with aggregates of \( \alpha \)-syn and tau, and highlight the
importance of more clearly understanding steps in the intercellular propagation pathway so that new effective therapeutic strategies can be developed.

**Vesicle Rupture**

**Membrane Pores.**

In considering the means through which prion-like amyloid aggregates of α-syn escape the vesicular compartment following their endocytosis to proceed with propagating protein misfolding, investigation of their effects on vesicle membranes begins with the proposed physiological role of monomeric α-syn, involving interaction through its amphipathic N-terminal domain with phospholipid membranes in synaptic vesicles [143]. α-Syn has been shown to induce membrane thinning and curvature by inserting its N-terminal amphipathic domain as a wedge into the membrane, resulting in the conversion of large low-curvature vesicles into highly curved membrane tubules and vesicles [536-540]. This curvature and membrane tubulation is caused only by monomeric α-syn [538], and can result in the disruption of membrane integrity [536, 540]. While it is tempting to speculate that these same membrane curvature mechanisms underlie cellular invasion by α-syn amyloid fibrils at the plasma or endocytic membrane, the actions of an unfolded or α-helical α-syn monomer on biological membranes are not likely to translate to higher-order β-sheet amyloid assemblies of α-syn, as assembly of α-syn monomers into an insoluble fibrillar conformation involves both multimerization and a conformational change.
Once aggregation of \( \alpha \)-syn begins, however, aggregates do not lose their affinity for membranes. Specific pre-fibrillar intermediates called protofibrils are \( \beta \)-sheet-rich annular ring-like or wreath-like assemblies resembling a pore-forming bacterial toxin, and these \( \alpha \)-syn protofibrillar assemblies have been shown to permeabilize vesicle membranes via a pore-forming mechanism that allows passage of ions or low-molecular mass structures [541-545]. The pore-like structure of \( \alpha \)-syn protofibrils containing a central channel illustrates how membrane penetration of the annular protofibrils results in perforation of the lipid bilayer [546, 547]. Protofibril membrane insertion and subsequent membrane pore formation results in an increase in the conductance of the lipid bilayer, eliciting ion-channel currents as a result of the non-selective passage of ions [548, 549]. Especially critical for the survival of neurons, a dysregulated influx of \( \text{Ca}^{2+} \) ions through \( \alpha \)-syn membrane pores results in cell death [550-552]. Alternatively, other studies have found an \( \alpha \)-syn oligomer-mediated increase in plasma membrane ion conductance in the absence of pore formation [553], possibly occurring through a thinning of the plasma membrane hydrophobic core resulting from oligomer insertion between tightly packed lipids [554]. In any case, these studies examine the actions of \( \alpha \)-syn monomers or oligomers on lipid membranes, and while related, they do not necessarily apply to the actions of \( \alpha \)-syn amyloid fibrils on biological membranes. While some characteristics of \( \alpha \)-syn monomers or oligomers may remain upon fibril formation, it is doubtful that the same membrane association properties of \( \alpha \)-syn monomers or oligomers will be maintained following fibril assembly. Thus, membrane perturbations
induced by α-syn monomers, oligomers, protofibrils, and fibrils are likely distinct processes. Whereas monomers can induce membrane curvature possibly due to their physiological role and oligomers can form membrane pores, the vesicular escape of α-syn fibrillar assemblies likely occurs via a different mechanism due to their larger size.

**Amyloid-Induced Vesicle Rupture.**

Investigation into the effects on biological membranes of amyloid proteins in the fibrillar assembly conformation is most relevant for aggregate cell-to-cell propagation and has yielded some new insight, but the understanding of how these membrane interactions relate to disease pathogenesis remains largely incomplete. To determine the specific effect of fibrillar amyloid forms on membrane structure and integrity, amyloid fibrils generated *in vitro* are often incubated with artificial liposome suspensions. Fibrillar forms of amyloid proteins, just like monomeric and oligomeric forms, are known to associate with lipid membranes, and *in vitro* association of α-syn or HTTExon1 fibrils with artificial liposomes is known to induce permeabilization or destruction of the liposomal membrane [555, 556]. Membrane disruption or permeabilization of artificial liposomes and natively derived membranes was enhanced when fragmentation of fibrils was performed, suggesting an increased toxic action on membrane integrity by smaller fibrillar amyloid forms rather than larger entangled amyloid inclusions [557]. In addition, the binding of amyloid fibrils to the plasma membrane is known to induce cellular Ca^{2+} influx through a mechanism that has yet to be defined, but may be similar to its lipid permeabilization of artificial liposomes that
allows the release of intravesicular indicator dyes [210, 556]. While these models of \( \alpha \)-syn fibril association with lipids are certainly relevant for membrane interactions that may occur within the endocytic compartment during pathological cell-to-cell propagation, the mechanisms of membrane perturbation by \( \alpha \)-syn fibrils on the external face of artificial liposomes or at the plasma membrane may not directly relate to the process of intravesicular escape and cellular invasion.

The most authentic representation of endocytic vesicle integrity relies on the identification of specific factors enriched within intact endocytic vesicles or that become enriched in the endocytic compartment upon loss of membrane integrity. Our group was the first to apply one such approach to the study of cellular invasion by \( \alpha \)-syn aggregates. By exploiting the ability of cytosolic galectin 3 (Gal3) to become enriched at the site of intracellular vesicle damage due to its binding of target \( \beta \)-galactoside residues exposed as a result of damage, we determined that aggregates of \( \alpha \)-syn are capable of inducing membrane rupture of lysosomes following their endocytic uptake [4, 280].

Utilizing both the cellular uptake of exogenous \( \alpha \)-syn aggregates prepared \textit{in vitro} from recombinant purified protein as well as the cell-to-cell transmission of \( \alpha \)-syn aggregates from over-expressing donor cells to naïve recipient cells, this study demonstrated induction of vesicle rupture through the colocalization between \( \alpha \)-syn aggregates and distinct Gal3 puncta formed at the site of vesicle rupture (Figure 7). These findings illuminated a potential mechanism for exogenous \( \alpha \)-syn fibrillar amyloid assemblies to escape the endocytic compartment to access the cytosol, and did so in a model that
Figure 7. Vesicle rupture following the cell to cell transfer of α-synuclein. N27 cells stably expressing α-synuclein and treated with MPP+ for 24 hours. N27chGal3 cells were then added to the culture and co-cultured for 48 hours. Cells were then fixed and stained for α-synuclein (green). The boxed areas in the left panel are enlarged to allow visualization of α-synuclein and chGal3 colocalization. Reprinted from PLoS One, Volume 8, Issue 4, David Freeman, Rudy Cedillos, Samantha Choyke, Zana Lukic, Kathleen McGuire, Shauna Marvin, Andrew M. Burrage, Stacey Sudholt, Ajay Rana, Christopher O’Connor, Christopher M. Wiethoff, Edward M. Campbell, Alpha-Synuclein Induces Lysosomal Rupture and Cathepsin Dependent Reactive Oxygen Species Following Endocytosis, e62143, doi:10.1371/journal.pone.0062143 (Reference 4), Copyright 2013, reproduced under the terms of the Creative Commons Attribution License.
recapitulates both the neuronal endocytic membrane components and the proper orientation of escape, from intravesicular to cytosolic, that are involved in pathologic α-syn amyloid cell-to-cell propagation.

α-Syn-induced lysosomal rupture not only allowed cellular invasion of misfolded amyloid fibrils, but also resulted in a CatB-dependent increase in ROS in target cells following proteolytic enzyme leakage from damaged lysosomes. A later study found that the induction of cytosolic α-syn misfolding by exogenous α-syn seeds was enhanced by the actions of CatB, proposed to increase the amount of smaller α-syn seeds in the lysosome through partial digestion that could then trigger intravesicular aggregation by recruiting additional membrane-bound monomers and, through further growth within the vesicular compartment, result in vesicle rupture and escape [296]. While CatB may perform this facilitative role for α-syn aggregation in the lumen of the lysosome prior to rupture, its leakage from the lysosome following rupture may also result in its action on expressed monomeric α-syn in the cytosol, perhaps accounting for its enhancement of cytosolic aggregate formation. This process of intravesicular aggregation and eventual vesicle rupture due to the large size of the resulting aggregate is one mechanism proposed for the induction of α-syn amyloid-mediated vesicle rupture. Additionally, recruitment of α-syn monomers away from the vesicular membrane and into a growing intravesicular aggregate may result in membrane destabilization through lipid extraction, another possible mechanistic explanation for α-syn fibril-mediated rupture [558]. Finally, uptake of rigid α-syn amyloid fibrils into endocytic vesicles could result in
physical-mechanical perturbations of the vesicle membrane as another mechanistic possibility, due to trafficking of intracellular vesicles through the dense cytoplasmic environment carrying their rigid fibrillar cargo [559].

Endocytic vesicle rupture has also been suggested as a mechanism of cellular invasion for fibrillar assemblies of other amyloid proteins. Tau aggregates have been proposed to rupture the endocytic membrane during cellular uptake, although the evidence supporting this conclusion is limited by a lack of specificity in defining tau aggregates that cause this damage [507, 560]. Exposure of artificial liposomes to fibrillar assemblies of Aβ has been shown to result in vesicle permeabilization [561], and endocytic uptake of degradation-resistant Aβ1-42 into target cells results in lysosomal permeabilization indicated by cytosolic release of the endocytic tracer Lucifer Yellow as well as the lysosomal enzymes CatD and β-hexosaminidase [562]. Finally, amyloid fibrils composed of β2-microglobulin, associated with dialysis-related amyloidosis, have also been shown to disrupt lipid bilayers in vitro resulting in membrane leakage, and this effect was enhanced by low pH conditions such as those in the endocytic pathway [563, 564]. While less is known about the actions of amyloid fibrils on biological membranes compared to monomers or oligomers of these proteins, insights into membrane association in vitro or upon cellular uptake have postulated a vesicle rupture mechanism for the cellular invasion of amyloid fibrils through the endocytic compartment.
Utility and Role of Galectin Proteins.

Galectin family proteins derive their name from their function as galactoside-binding lectins, and β-galactoside recognition by the galectin carbohydrate recognition domain (CRD) mediates their diverse functions in cell communication, inflammation, development, and differentiation [565-569]. One such function, critical for innate immunity, is the action of cytosolic galectins as membrane sensing pattern recognition receptors (PRRs) or danger receptors that continually ensure intracellular endosomal and lysosomal membrane integrity through their lack of binding to carbohydrate targets that are sequestered within intact intracellular vesicles and thus absent from the cytosol [570, 571]. The ability of cytosolic galectins to recognize exposed β-galactoside targets upon vesicular membrane permeabilization highlights their utility for studying the vesicle membrane rupture ability of fibrillar amyloid aggregates following endocytosis. Galectins detect vesicle rupture induced by the cellular invasion of numerous pathogens, such as Salmonella enterica serovar Typhimurium, Shigella flexneri, Listeria monocytogenes, Mycobacterium tuberculosis, Burkholderia cenocepacia, Ureaplasma parvum, and non-enveloped viruses such as adenovirus [572-575], that each damage the endocytic compartment to gain entry to the cytoplasm as part of their infectious life cycle. Following the rupture of the endocytic compartment that formed around the pathogen during host cell uptake, galectins become selectively, specifically, and instantly recruited to the site of vesicle damage, serving as a novel tool to spot vesicle
rupture and to identify the molecular and cellular mechanisms of rupture during pathogenic invasion [570, 576-580].

In addition to this utility for the study of vesicle rupture mechanisms, the recruitment of cytosolic galectins to the site of intracellular vesicle damage also plays a physiologic role in innate immune signaling and inflammation [581-586], initiated as a response to vesicle rupture by any cause; bacteria, virus, or amyloid fibril alike. Within affected cells stimulated by ischemia or α-syn aggregate exposure, a resulting increase in Gal3 expression and/or activity prompts a signaling cascade that is required for proper microglial activation and proliferation [587-589]. Additionally, Gal3 expression is markedly increased in prion-infected brain tissue after central or peripheral inoculation, where it plays a detrimental role for survival as evidenced by Gal3 KO mice exhibiting prolonged survival times [590]. Gal3 KO mice also had decreased LAMP2 protein levels, suggesting that a regulation of lysosomal function may underlie the detrimental role of Gal3 in prion infections [590]. In additions to its actions within affected cells, Gal3 can also be secreted from cells through a non-classical leaderless secretory pathway where now in the extracellular environment it acts as an endogenous danger signaling molecule producing proinflammatory responses from nearby cells [591-593]. Extracellular Gal3, released in a paracrine fashion in response to diverse stimuli such as ischemia, LPS exposure, or traumatic brain injury (TBI), has been proposed to act as an alarmin, promoting inflammation and neuronal loss through its binding to Toll-Like Receptor 4 (TLR4) [594-596]. Gal3-mediated TLR4 activation could result in excessive
and prolonged microglial activation with resulting inflammation becoming detrimental [594], a view supported by the neuroprotective phenotype in Gal3 KO or Gal3 neutralizing antibody-treated animals despite TBI insult [596]. These actions not only highlight the potential application of galectin proteins to the study of endocytic vesicle rupture by invading fibrillar amyloid protein assemblies, but also portend the consequences of such vesicle damage in the resulting intracellular and extracellular galectin-mediated inflammatory responses that could contribute as one of many causes for neurotoxicity.

**Infectious Pathogens and Sterile Damage.**

Insight into the mechanisms and consequences of amyloid protein-mediated vesicle rupture can be gained from examining other paradigms of cellular infection by pathogens that are also confronted with this vesicular escape hurdle *en route* to cellular invasion and continuation of their infectious life cycle. While physical damage to the endocytic vesicle membrane allows invading pathogens to subvert lysosomal degradation and escape into the cytosol where replication proceeds, this mechanism of cellular invasion is particularly damaging to intracellular vesicles and induces their autophagic degradation. Once a pathogen breaks the vacuolar membrane to gain access to the cytosol, a diverse set of cellular responses ensues. PRRs, with cytosolic galectins being some of many, respond to danger by identifying both pathogen-associated molecular patterns (PAMPs) associated with the invading bacterium or virus as well as damage-associated molecular patterns (DAMPs) associated with the damaged vesicles
The vesicular membrane remnants act as a signaling node for PRR activity, triggering both galectin- and polyubiquitination-mediated autophagic degradation [570, 576, 579, 597-600]. This upregulation of autophagic degradation mechanisms not only helps to remove and recycle vesicular debris, but can also serve an antibacterial function to restrict intracellular proliferation of invading pathogens through their degradation [570, 579, 601, 602]. Even in the absence of pathogen invasion, sterile vesicle membrane damage caused by transfection reagents such as calcium phosphate precipitates (CPPs) or latex beads coated with Effectene also result in ubiquitination and subsequent autophagic degradation of the damaged vesicles [597, 603]. In addition to binding exposed β-galactosides, galectins 3 and 8 are an essential component of the pathway facilitating recruitment of the autophagic machinery to the site of vesicle damage [570, 579, 602, 603]. Autophagy-mediated degradation of invading pathogens and ruptured vesicular debris is one adaptive mechanism for cells to survive these forms of stress.

One specific example of a pathogen infection that closely resembles the mechanism of α-syn cellular invasion is that of adenovirus, where both the process of vesicular escape and the consequences of lysosomal rupture are shared. As a non-enveloped virus, adenovirus enters cells via endocytosis where it must then escape from the endocytic compartment [575]. Upon exposure to the low pH of the late endosome or early lysosome, the viral capsid undergoes a conformational change that allows a membrane-perturbing protein known as protein VI to associate with the lipid membrane
and generate a positive membrane curvature, resulting in the physical rupture of the endosome [604, 605]. This vesicular membrane fragmentation ability of adenoviral protein VI closely resembles that of \( \alpha \)-syn monomers, perhaps mediated by their shared amphipathic nature. Endocytic vesicle rupture induced by adenovirus also results in Gal3 recruitment to the site of rupture, occurring for adenovirus most often in the early stages of the endosomal pathway for the evolutionary reason that adenovirus thus avoids enzymatic digestion in the lysosome [606, 607]. Adenovirus, like \( \alpha \)-syn, has also been shown to induce small plasma membrane lesions through the actions of protein VI, resulting in \( \text{Ca}^{2+} \) influx and lysosomal exocytosis to repair plasma membrane piercing [608]. Endocytic vesicle rupture by adenovirus, like \( \alpha \)-syn, results in release of activated cathepsins and a consequent upregulation in mitochondrial ROS [609]. Examining the process of cellular infection by various pathogens or sterile damage in endocytic vesicle membranes has illuminated similarities with the cellular invasion of \( \alpha \)-syn aggregates, as well as highlighted several detrimental cellular consequences resulting from endocytic vesicle rupture.

**Lysosomal Membrane Permeabilization.**

Lysosomal rupture induced by \( \alpha \)-syn aggregates after their endocytic uptake is one of many diverse causes of lysosomal membrane permeabilization (LMP), a common programmed cell death pathway induced by various cytotoxic stimuli and stressful cellular conditions [610, 611]. Just as observed in response to \( \alpha \)-syn endocytic uptake and lysosomal rupture, the LMP pathway causes the release of lysosomal cathepsins
and other hydrolases from the lumen to the cytosol, causing aberrant digestion of vital proteins, oxidative stress, and inflammatory sequelae [610]. In addition to endocytic vesicle rupture by fibrillar assemblies of amyloid proteins or invading pathogens, other stimuli such as ROS, endogenous cell death effectors like Bax, lysosomotropic compounds with detergent activity like L-leucyl-L-leucine methyl ester (LLOMe), high doses of the quinolone antibiotic ciprofloxacin, uptake of mineral crystals such as silica and monosodium urate (MSU), and even adherent noningested bacteria like Type 1-fimbriated *Escherichia coli* also induce LMP [610, 612-615]. There is additional evidence that the nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome, activated in response to many of the same stimuli as LMP, may also contribute to its induction [616].

The ectopic presence of lysosomal proteases in the cytosol resulting from LMP causes caspase activation indirectly through cathepsin-mediated cleavage and activation of Bid, inducing mitochondrial outer membrane permeabilization (MOMP), cytochrome c release and caspase activation during apoptotic cell death [610]. This induction of the mitochondrial apoptotic pathway through Bid activation synergizes with a concomitant degradation of antiapoptotic Bcl-2, Bcl-xL, or Mcl-1 by lysosomal proteases [614]. However, if LMP is massively increased, cell death can occur through necrosis in the absence of caspase activation [610, 617]. This LMP cell death pathway can be regulated under physiologic conditions or can contribute to cell death in pathological situations, including PD [611, 618]. In PD pathogenesis, lysosomal depletion is known to be caused
by ROS-mediated LMP, which results in defective clearance and subsequent accumulation of autophagosomes and dopaminergic cell death mediated by the released lysosomal proteases [130]. Additionally, dysfunctional lysosomal degradation and the resulting accumulation of autophagosomes leads to LB inclusion formation, as LBs contain markers of autophagosomes in PD brain [130]. These findings highlight the important role of LMP in neurodegenerative pathology as a consequence of amyloid-mediated lysosomal rupture, oxidative stress, or a combination of the two.

LMP has been noted, similar to pathogen-induced endocytic vesicle rupture, through a variety of techniques that rely on detecting either the release of normal lysosomal factors, such as cathepsins or fluorescent dextran, or the localized accumulation of membrane damage sensors upon permeabilization, like the enrichment of galectin puncta [617, 619-623]. These methods have been employed to determine that cells can survive limited LMP that occurs at a basal, resting state, but can also be overwhelmed by LMP and succumb to this insult following more significant incidence [620]. Following membrane permeabilization, damaged lysosomes are selectively engulfed by autophagosomes following the recruitment of autophagic machinery to the site of damage, resulting in their recovery of low pH and degradation capacity [615]. This autophagosomal clearance of damaged lysosomes begins with lysine 63 (K63)-linked ubiquitination of damaged membranes and the recruitment of p62, followed by translocation of the ubiquitin-directed AAA-ATPase p97, which cooperates with cofactors UBDX1, PLAA, and the deubiquinating enzyme YOD1 as components of the
endolysosomal damage response (ELDR) to remove lysine 48 (K48)-linked ubiquitin conjugates and promote autophagosome formation [560]. If autophagic degradation is compromised in the setting of LMP, this causes inhibition of lysosomal biogenesis since functional, degradation-competent lysosomes cannot be regenerated through autophagy [615]. Particularly relevant for the cell-to-cell propagation of amyloid fibrils following entry via endocytic vesicle rupture, elevated intracellular Ca\(^{2+}\) induced by exogenous amyloid proteins [210, 382, 550, 551, 556, 624] triggers lysosomal exocytosis to repair membrane ruptures, releasing lysosomal contents to the extracellular environment [478, 512, 625]. Autophagy, too, may be involved in the unconventional secretion of leaderless cytosolic proteins such as \(\alpha\)-syn and Gal3, as lysosomal damage leads to the recognition of unconventional secretory cargo by specialized cytosolic cargo receptors and a dedicated SNARE system to facilitate secretion [626]. Overall, LMP has important consequences for invading fibrillar amyloid assemblies that are relevant to both their impaired degradation and their pathological release from the cells into which they have invaded.

**Concluding Remarks**

The prion-like characteristics of amyloid proteins in various neurodegenerative diseases not only provide a platform for in-depth examination of disease pathogenesis, but also raise the possibility that similar therapeutic strategies may be effective to combat shared mechanisms of neurotoxicity in this group of progressive, currently intractable diseases. Misfolded aggregates of \(\alpha\)-syn in PD, tau in AD, and HTTExon1 in
HD are considered “prion-like” for their many shared characteristics with prions but also for the important lack of transmissibility between host organisms only seen with true prions. Even so, considering the pathogenesis of these diverse proteinopathies from the cell biological perspective of host-pathogen interactions yields novel insight into disease mechanisms that were previously poorly understood. Although the “pathogen” is derived from pathological aggregation of a host protein, many aspects of its cellular life cycle such as cellular invasion, amplification, and propagation are similar to an infection by a foreign agent.

Three aspects of prion-like amyloid proteins make combating cellular “infection” by these protein aggregates particularly challenging. First, the ability of misfolded amyloid proteins to serve as both seeds and templates for further misfolding of naïve counterparts represents the mechanism by which a pathologic protein aggregate can replicate and amplify its protein misfolding pathology [197, 200-202, 212, 213, 347, 490, 492, 525, 526, 533]. Second, the ability of misfolded amyloid proteins to interfere with the very mechanisms that cells employ to degrade them represents a positive feedback mechanism for degradative dysfunction and amyloid protein accumulation [130, 340, 341, 356, 358]. Finally, the ability of misfolded amyloid proteins to propagate from affected to naïve cells represents the mechanism of disease progression and dissemination [212, 213, 419, 420, 498, 502, 535].

Endocytic vesicle rupture, an event occurring during cellular invasion by fibrillar amyloid protein assemblies, exerts influence on all three challenging aspects of prion-
like pathology. Rupture of endocytic vesicles represents the mechanism of amyloid protein vesicular escape following uptake, an essential step in the cell-to-cell propagation pathway, which induces degradative dysfunction through damage to lysosomal membranes and allows seeding of further aggregation by the escaped seed nucleus. As an event that impacts these critical pathological activities of misfolded protein aggregates, a clear understanding of its mechanisms and consequences is essential for a complete understanding of disease pathogenesis.

The goal of this dissertation is to better understand endocytic vesicle rupture as a mechanism of cellular invasion for several amyloid proteins, focusing for α-syn on specific modifications that influence vesicle rupture potency as well as the effects of α-syn-mediated rupture on cellular homeostasis and aggregate propagation. Taken together, these studies demonstrate the ability of fibrillar amyloid protein assemblies to induce endocytic vesicle rupture, and provide a clear understanding of the impact of this damaging mechanism of cellular invasion on the pathogenesis and progression of proteinopathies.
CHAPTER TWO
MATERIALS AND METHODS

Cell Lines and Media

The human neuroblastoma cell line SH-SY5Y was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The rat dopaminergic neuronal cell line N27 was a kind gift from Dr. Anumantha Kanthasamy [627]. SH-SY5Y cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum (FBS) (HyClone) and 100 IU/mL penicillin, 100 µg/mL streptomycin, and 10 µg/mL ciprofloxacin. N27 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media with 10% FBS and the same additives used for SH-SY5Y cells. Human embryonic kidney 293T cells were cultured in DMEM supplemented with 10% FBS and the same additives used for SH-SY5Y cells. SH-SY5Y and N27 cells were plated on fibronectin (Sigma)-treated glass coverslips or Delta T dishes (Bioptechs) at least 12 hr prior to experiments. Human dopaminergic neurons differentiated from human induced pluripotent stem cells (hiPSC-derived dopaminergic neurons) were obtained from Cellular Dynamics International (iCell DopaNeurons, CDI, Madison, WI). hiPSC-derived dopaminergic neurons were grown on dishes pre-coated with poly-L-ornithine and laminin according to the supplier’s instructions. One half of the hiPSC-derived dopaminergic neuron complete maintenance media was changed at
24 hr post thaw and every other day thereafter until the start of experiments, between 4-14 days post thawing. Cells were maintained in a 37°C incubator with 5% CO₂.

**Generation of Stable Cell Lines**

The mCherry-galectin 3 (chGal3) lentiviral plasmid was generated by inserting the galectin 3 open reading frame (ORF) into a mCherry-pLVX backbone (Takara Bio USA, Mountain View, CA). Lentiviruses for transduction were produced by polyethylenimine (PEI) (molecular weight, 25,000; Polysciences) transfection of 293T cells seeded at 60% confluency in a 15 cm dish with 8.33 µg PLVX mCherry-Gal3, 8.33 µg vesicular stomatitis virus glycoprotein (VSV-G), and 8.33 µg PsPax2 (lentiviral packaging plasmid, AIDS Reagent Repository). Fresh media was added 24 hr after transfection and viruses were harvested 48 hr after transfection. Following filtration through a 0.45-µm filter (Millipore), viruses were used to transduce SH-SY5Y, N27, and hiPSC-derived dopaminergic neurons. Twenty-four hours after infection, vector was removed and replaced with fresh media. Forty-eight hours after transduction, SH-SY5YchGal3 or N27chGal3 cells were selected in media containing 5 µg/mL puromycin (Sigma-Aldrich).

The yellow fluorescent protein (YFP)-LC3 lentiviral plasmid was generated by inserting the YFP-LC3 open reading frame into pLVX (Takara Bio USA, Mountain View, CA). Lentiviruses for transduction were prepared in an identical fashion to above, and used to transduce SH-SYSYchGal3 cells previously transduced to express chGal3 with a retroviral vector [4]. SH-SYSYchGal3 YFP-LC3 cells were selected in media containing 400 µg/mL Geneticin (G418, Gibco) and 5 µg/mL puromycin.
Amyloid Proteins

Alpha-Synuclein.

Wild type and familial mutant alpha-synuclein assemblies. A pET-28a plasmid containing human WT α-syn cDNA was generously provided by Dr. Anurag Tandon [280]. Human α-syn cDNAs for familial mutants (A53T, A30P, E46K, and G51D) were subcloned into this pET-28a plasmid using Ncol and HindIII restriction sites. α-syn was purified as previously described [628]. Briefly, α-syn was overexpressed in Escherichia coli BL21 via an isopropyl-1-thio-β-D-galactopyranoside (IPTG)-inducible T7 promoter. The bacterial pellet was resuspended in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The bacterial pellet was then sonicated for 15 sec several times, boiled for 15 min, and centrifuged at 10,000 x g for 20 min. The supernatant was then ultracentrifuged at 150,000 x g for 30 min. The supernatant containing the heat-stable α-syn was dialyzed against 50 mM Tris, pH 8.3, loaded onto a Q-Sepharose column (GE Healthcare), and eluted with a 0-500 mM NaCl linear gradient. Ion exchange fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue (CBB) staining and western blotting for α-syn to confirm purity. Appropriate ion exchange fractions were pooled, dialyzed against 20 mM Tris, pH 7.4, sterile filtered, flash frozen in liquid nitrogen, and stored at -80 °C. Protein concentration was determined by spectrometer absorbance at 280 nm using an extinction coefficient of 5960 M⁻¹cm⁻¹.
α-syn samples were prepared for aggregation by standardizing protein and solute concentrations, as well as total sample volume, across all samples according to our previously published aggregation conditions [4, 280]. Specifically, 200 µL of purified α-syn (1 mg/mL) was prepared in a pH 7.4 buffer containing the following solute concentrations: 323.3 mM NaCl, 20 mM Tris-HCl, 9 mM Na₂HPO₄, 2.43 mM KCl, and 1.62 mM KH₂PO₄. Sample preparations were then incubated for 3 days at 37 °C under constant agitation (300 revolutions per minute (rpm), Benchmark Incu-Shaker Mini), followed by flash freezing in liquid nitrogen and storage at -80 °C. Assemblies were fluorescently labeled with DyLight488 N-hydroxysuccinimide (NHS) ester fluorophores (Thermo Scientific) according to the manufacturer’s protocol prior to use. Briefly, 100 µL of aggregated protein at 1 mg/mL was dialyzed into 0.1 M sodium phosphate buffer, pH 8.0, for 2 hr at 4°C using 10,000 molecular weight cutoff (MWCO) minidialysis units (Thermo Scientific). The protein solution was then transferred to the vial containing the dye and was labeled as per the supplier’s instructions for 30 min at room temperature (RT). After incubation, the labeling reaction was quenched with 40 mM Tris (final concentration), and extensive dialysis was performed at 4 °C using 10,000 MWCO dialysis units into a buffer containing 40 mM Tris and 150 mM NaCl for about 24 hr to remove excess unlabeled dye. Following labeling, samples were flash frozen in liquid nitrogen and stored at -80°C.

**Phosphorylated serine-129 alpha-synuclein aggregates.** Recombinant WT, A30P, and A53T α-syn were expressed in *E. coli* with or without PLK2, purified as described
previously [628], and stored in 20 mM Tris-Cl, pH 7.4 at -80 °C. Samples containing monomeric WT, WT pS129, A30P, A30P pS129, A53T, and A53T pS129 α-syn were aggregated under identical protein and solute concentrations and in the same volume as for WT and mutant α-syn assemblies above, followed by storage at 4 °C. Fluorescent labeling was also performed in a manner identical to that of WT and mutant α-syn assemblies as above, followed by storage at 4 °C.

**Alpha-synuclein polymorphic assemblies.** To generate distinct fibrillar polymorphs of α-syn, the WT protein was expressed in *Escherichia coli* strain BL21(DE3) (Stratagene, La Jolla, CA) transformed with the expression vector pET3a (NovagenTM) encoding wild-type, full-length α-syn. The expression of α-syn was induced by 0.5mM IPTG for 2 hr when the bacteria grown in lysogeny broth (LB) medium at 37 °C reached an optical density of 1.0 at 660 nm. Soluble, monomeric α-syn was purified from the bacteria lysate as previously described [629]. α-syn concentration was determined spectrophotometrically using an extinction coefficient of 5960 M⁻¹cm⁻¹ at 280 nm. Pure α-syn (0.2-0.5mM) in 50mM Tris-HCl, pH 7.5, 150mM KCl was filtered through sterile 0.22-µm filters and stored at -80 °C. To obtain on-fibrillar assembly pathway α-syn oligomers, α-syn was incubated at 800 µM in 50mM Tris-HCl, pH 7.5, 150mM KCl at 4°C, without shaking, for 7 days, followed by separation from monomeric α-syn by size exclusion chromatography using a Superose6 HR10/30 column (GE Healthcare) equilibrated in PBS buffer [210, 382, 556, 630]. To obtain the polymorph “fibrils”, α-syn (400 µM) was assembled in 50 mM Tris-HCl, pH 7.5, 150 mM KCl as described [305]; to
obtain the polymorph “ribbons”, α-syn (400 µM) was dialyzed overnight against 5 mM Tris-HCl pH 7.5 prior to assembly [305]; to obtain the polymorph “fibrils-91”, α-syn (400 µM) was dialyzed overnight against 20 mM KPO₄ pH 9.1 prior to assembly as described [309]; to obtain the polymorph “fibrils-65”, α-syn (400 µM) was dialyzed for 3 hours against 20 mM MES pH 6.5, 150 mM NaCl prior to assembly. Assembly was achieved by incubating the samples for one week at 37 °C under continuous shaking in an Eppendorf Thermomixer set at 600 rpm. Fibrillar assemblies were spun at 15,000 x g for 10 min and resuspended in PBS. Labeling of all polymorphs was achieved by addition of 2 molar equivalent of lysine reactive ATTO-488 (ATTO-TEC, GMBH) for 1 hr at RT. The unreacted fluorophore was removed from fibrillar preparations by a cycle of two centrifugations at 15,000 x g for 10 min and resuspensions of the fibrillar pellets in PBS. Unreacted fluorophore was removed from oligomeric preparations by size exclusion chromatography. Fibrillar preparations homogenous in size were obtained through sonication by fragmenting fibrillar polymorphs for 20 min at a constant temperature (20°C) in 2 mL Eppendorf tubes in a VialTweeter powered by an ultrasonic processor UIS250v (250 watts, 24 kHz, Hielscher Ultrasonic, Teltow, Germany) set at 75% amplitude, 0.5 sec pulses every 1 sec. On-fibrillar assembly pathway oligomeric α-syn was not fragmented.

**Alpha-synuclein pre-formed fibrils.** α-syn monomer to generate PFFs (Proteos, Inc, Kalamazoo, MI) was prepared according to the manufacturer’s protocol [203]. Briefly, α-syn was assembled in PBS at 5 mg/mL by incubation for 7 days at 37 °C under
constant agitation (300 rpm, Benchmark Incu-Shaker Mini), followed by flash freezing in liquid nitrogen and storage at -80°C. Samples were fluorescently labeled with Dylight488 or Dylight650 NHS ester fluorophores as above, with the exception that pre-labeling dialysis was omitted as samples could be sufficiently labeled in PBS buffer, and post-labeling dialysis was performed using PBS at RT. Following labeling, samples were flash frozen in liquid nitrogen and stored at -80°C. Immediately prior to use, PFFs were generated from aliquots of Dylight488 or Dylight 650-labeled α-syn assemblies by diluting each sample to 0.1 mg/mL in PBS and sonicating with 60 pulses at 20% power for 0.5 sec each using a Sonics Vibra Cell sonicator (VCX 130PB, Sonics and Materials Inc, Newtown, CT).

**Tau.**

Full-length human tau 1N3R and tau 1N4R cloned in pET14b vector were expressed in *E. coli* BL21 DE3 codon + cells (Stratagene). Cells were grown in LB medium to an optical density of 0.8 absorbancy units at 600nm. Tau isoform expression was induced by 0.5 mM IPTG for 3 hr. The cells were then centrifuged (4,000 x g, 10 min), resuspended in lysis buffer (20 mM MES pH 6.8, 500 mM NaCl, 1 mM EGTA, 0.2 mM MgCl₂, 5 mM dithiothreitol (DTT), 1 mM PMSF + 1 tablet of Complete (Roche)) per liter and lysed by sonication. Bacterial homogenates were clarified by centrifugation at 14,000 x g for 30 min. The supernatant was heated to 80 °C for 20 min and centrifuged at 14,000 x g for 30 min. The supernatant was dialyzed against 100 volumes of buffer A (20 mM MES pH 6.8, 50 mM NaCl, 1 mM EDTA, 1 mM MgCl₂, 2 mM DTT, 0.1 mM PMSF)
at 4 °C. The dialyzed protein mixture was loaded on SP Sepharose column (60 mL bed volume). Proteins were separated with a linear gradient of 0 to 100% buffer B (20 mM MES pH 6.8, 1 M NaCl, 1 mM EGTA, 1 mM MgCl₂, 2 mM DTT, 0.1 mM PMSF). Fractions were analyzed on SDS-PAGE stained with CBB. Fractions containing human tau isoforms were pooled and dialyzed against 100 volumes of PBS buffer containing 1 mM DTT. The protein concentration was determined spectrophotometrically using an extinction coefficient of 7450 M⁻¹cm⁻¹ at 280nm. Tau 1N3R and tau 1N4R at a concentration of 50 to 100 µM were aliquoted and stored at -80 °C. Tau 1N3R and tau 1N4R (40 µM) were assembled in the presence of 10 µM heparin at 37 °C in an Eppendorf Thermomixer set at 600 rpm for 4 days. Fibrils were spun for 20 min at 20 °C, 16,000 rpm. Tau assemblies were spun at 15,000 x g for 10 min and resuspended in PBS, followed by fluorescent labeling in an identical fashion to labeling of α-syn polymorphic assemblies. Fibrillar tau preparations homogenous in size were obtained through sonication as for α-syn polymorphic assemblies.

**Huntingtin.**

Recombinant HTTExon1 with a polyQ stretch of 45 glutamine residues (HTTExon1-Q45) was expressed and purified as described [631]. For fibril formation, HTTExon1-Q45 was incubated in assembly buffer B (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 10% glycerol) at 37 °C without agitation. HTTExon1-Q45 assemblies were spun at 15,000 x g for 10 min and resuspended in PBS, followed by fluorescent labeling in an identical fashion to labeling of α-syn polymorphic assemblies. Fibrillar HTTExon1-Q45
preparations homogenous in size were obtained through sonication as for α-syn polymorphic assemblies.

**Electron Microscopy**

WT and familial mutant α-syn assemblies were diluted to 0.1 mg/mL and added to carbon-coated, 200 mesh copper grids (Electron Microscopy Sciences) for 60 sec. Grids were stained with 2% uranyl acetate for 5 seconds. Grids were dried overnight and transmission electron microscopy (TEM) performed on a Hitachi H-600 transmission electron microscope.

The morphology of α-syn, tau and HTTExon1-Q45 polymorphs was assessed by TEM in a Jeol 1400 transmission electron microscope following adsorption onto carbon-coated 200 mesh grids and negative staining with 1% uranyl acetate. The images were recorded with a Gatan Orius CCD camera (Gatan).

**mCherry-Galectin 3 Relocalization Assay**

At least 12 hr following the plating of SY5YchGal3 or N27chGal3 cells onto fibronectin-coated glass coverslips or dishes, or at least 48 hr following chGal3 lentiviral transduction of hiPSC-derived dopaminergic neurons, amyloid assemblies were added to the cell culture media. For comparisons of the effect of α-syn missense mutation and S129 phosphorylation, WT and familial mutant α-syn assemblies and pS129 α-syn aggregates were added at a concentration of 200 nM. For comparisons of the effect of amyloid strain conformation, α-syn monomer, oligomers, fibrils, ribbons, fibrils-65, fibrils-91, and assemblies of HTTExon1-Q45, tau 1N3R and tau 1N4R were added at a
concentration of 660 nM. Following 24-48 hrs of treatment time as specified for each experiment, cells were either fixed to be mounted on coverslips or imaged directly via live cell imaging.

**Immunofluorescence Microscopy**

Cells were fixed with 3.7% formaldehyde (Polysciences) in 0.1 M piperazine-N,N’bis(2-ethanesulfonic acid) (PIPES) buffer, pH 6.8, for 5 min. When immunofluorescent staining was performed, cells were stained with rabbit anti-human LAMP1 antibodies (1:1000, ab24170, Abcam, Cambridge, MA) in PBS with 10% normal donkey serum (NDS) and 0.1% saponin (Sigma-Aldrich) for 1 hr. Primary antibodies were then secondarily labeled for 20 mins with fluorophore-conjugated donkey anti-rabbit 647 antibodies (1:400, Jackson ImmunoResearch) at the same time as 4’,6-diamidine-2’-phenylindole (DAPI) staining. When vesicle rupture was examined in the absence of immunofluorescent staining, cells were stained with DAPI alone for 20 min in PBS. Coverslips were then mounted on glass slides using Fluoro-Gel with Tris Buffer (Electron Microscopy Sciences) and allowed to dry before image acquisition. Images were collected with a DeltaVision wide-field deconvolution microscope (Applied Precision) equipped with a digital camera (CoolSNAP HQ or Cascade 2 Electron Multiplying Charge Coupled Device (EMCCD); Photometrics), using either a 1.4-numerical aperture (NA) 100X oil-immersion objective lens or a 1.42-NA 60X oil-immersion objective lens with immersion oil (N=1.514, Applied Precision) at RT. Images were then acquired and constrained iterative deconvolution performed using SoftWoRx software (Applied
Precision). Tif images and quantification data were collected from each image data file using Imaris software (Bitplane). To exclude the possibility that the image deconvolution process affects the quality of images or localization pattern of any fluorescent signal, an image from Figure 15 is displayed again in Figure 33 where a single field, in addition to enlarged inset images, is displayed as overlay or individual fluorescent channels in both an undeconvolved and deconvolved setting.

**Live Cell Imaging**

Images and videos were acquired on a DeltaVision wide-field deconvolution microscope (Applied Precision) equipped with a Weather Station™ chamber utilized to maintain cells at 37 °C in 5% CO₂. Images were acquired using either a 1.4-NA 100X oil-immersion objective lens or a 1.42-NA 60X oil-immersion objective lens and a Cascade 2 EMCCD (Photometrics) digital camera. Acquisition was performed at 37 °C using immersion oil (N=1.520, Applied Precision), and images were deconvolved as described above. In some experiments LysoTracker Deep Red (Thermo Scientific) was utilized to identify acidic organelles in live cells. 60 min prior to imaging, cells were incubated in the presence of 20 nM LysoTracker Deep Red for 30 min, washed with fresh media, and imaged 30 min later.

**Image Analysis**

Deconvolved images of experiments utilizing WT and familial mutant α-syn assemblies as well as polymorphic assemblies of α-syn, tau, and HTTExon1-Q45 were analyzed for chGal3+ puncta formation by blinded manual quantification using the
Imaris software program (Bitplane). Each experiment collected at least 20 images per treatment type. Values for number of chGal3 puncta per cell were pooled across all experiments for statistical analysis. Values for mean number of chGal3 puncta per cell induced by each treatment type from at least three independent experiments were compared with one another and to unexposed cells by use of student’s t-test or one-way analysis of variance with Tukey’s post-hoc multiple comparison test as appropriate for each data set, and expressed as fold increases relative to unexposed cells which were normalized to 1.

Deconvolved images of experiments utilizing pS129 α-syn aggregates were analyzed for puncta formation by use of the Surpass Mode of the Imaris software package (Bitplane). Specifically, a three-dimensional surface was created around chGal3 puncta by designing an algorithm for each experiment that specifically detected punctate events that increased in intensity sufficiently above background fluorescence (Figure 34). This same algorithm was uniformly applied to each image in the data set, and the number of surfaces created by the algorithm was divided by the number of cells in the field to measure puncta per cell. Each experiment collected at least 20 images per treatment type. Algorithm-detected values for number of chGal3 puncta per cell were pooled across all experiments for statistical analysis. Values for mean number of chGal3 puncta per cell induced by each α-syn treatment type from at least three independent experiments were compared with one another and to untreated cells by use of one-way analysis of variance and Tukey’s post hoc multiple comparison test. When mean number
of chGal3 puncta per cell values from each experiment are normalized for pS129 α-syn isoforms relative to their non-pS129 counterparts in the same experiment (set to 1), unpaired t tests were performed to compare each α-syn pS129 isoform to its non-pS129 counterpart.

**Parkinson’s Disease Brain Tissue Imaging**

Brains were obtained at autopsy from 5 subjects (4 male, 1 female) with a clinical and neuropathological diagnosis of PD. All patients with PD were diagnosed by neurologists in the Section of Movement Disorders in the Department of Neurological Sciences at Rush University Medical Center. Post mortem, the clinical diagnosis was confirmed by neuropathologists at Rush University Medical Center. For PD, inclusion criteria included a history compatible with idiopathic PD and at least two of the four cardinal signs (rest tremor, rigidity, akinesia/bradykinesia, and gait disturbance/postural reflex impairment). The Unified Parkinson's Disease Rating Scale (UPDRS “on”) and Hoehn and Yahr staging (H&Y “on”) were recorded, and for this study, tissue sections from patients at H&Y Stage 5 were used. The pathological diagnosis was based on finding Lewy bodies in catecholamine nuclei such as the SN. Exclusion criteria included familial PD, dementia with Lewy bodies, the Lewy body variant of AD, or the combination of PD and AD.

At autopsy, the brains were removed from the calvarium and processed as described previously [346, 632]. Briefly, each brain was cut into 1 cm coronal slabs using a plexiglass brain slice apparatus and then hemisected. The slabs were fixed in 4%
paraformaldehyde for 48 hr at 4 °C. The left side brain slabs were used for pathological diagnoses. The right side brain slabs were cryoprotected in 0.1 M PBS (pH 7.4) containing 2% dimethyl sulfoxide (DMSO), 10% glycerol for 48 hr followed by 2% DMSO, 20% glycerol in PBS for at least 2 days prior to sectioning. The fixed slabs containing the SN were cut into 18 adjacent series of 40 µm thick sections on a freezing sliding microtome for this study. All sections were collected and stored in a cryoprotectant solution prior to processing.

A double-label immunofluorescence procedure was employed to determine whether Gal3 colocalized with α-syn in LBs. The sections through the SN from each brain were blocked for 1 hr in a solution containing 5% goat serum, 2% bovine serum albumin (BSA), and 0.3% TritonX-100 in Tris buffered saline (TBS) and then incubated with α-synuclein (pS129) (EP1536Y, ab51253; 1:1000; Abcam, Cambridge, MA) rabbit monoclonal antibody overnight at RT. Following 6 washes in TBS, the sections were sequentially incubated with goat anti-rabbit antibody coupled to Cy2 (1:200; Jackson ImmunoResearch, West Grove, PA) for 1 hr. After 6 washes in TBS, the sections were blocked again for 1 hr in a solution containing 5% goat serum, 2% BSA, and 0.3% Triton X-100 in TBS. Sections were then incubated with Gal3 (1:300; 556904, BD Biosciences, San Jose, CA) mouse monoclonal antibody overnight at RT. After 6 washes, the sections were sequentially incubated in goat anti-mouse antibody coupled to Cy5 (1:200; Jackson ImmunoResearch) for 1 hr. The sections were mounted on gelatin-coated slides and allowed to air dry overnight. To block autofluorescence, the sections were rinsed in
distilled water, dehydrated in 70% alcohol for 5 min, incubated in the autofluorescent eliminator reagent (Millipore) for 5 min, and immersed in three changes of 70% alcohol. After rinsing in distilled water, the sections were coverslipped using polyvinyl alcohol with 1,4-Diazabicyclo[2.2.2]octane (DABCO) (Sigma-Aldrich).

Autofluorescence represents a potentially confounding factor using immunofluorescence techniques in this tissue. However, we employed two means to effectively minimize autofluorescence. First, Cy5 is a fluorophore in the infrared range, and the autofluorescence from lipofuscin is minimized within this range of the visual spectrum. Second, use of the autofluorescent eliminator reagent further blocked autofluorescence in the tissue [346]. Immunohistochemical control experiments included omission of the primary antibodies (which control for the specificity of the staining procedure and the secondary antibody) and inclusion of images taken in the A594 channel which is between Cy2 and Cy5 emission spectra (to control for signal bleed-through to adjacent channels). The control sections were processed in a manner identical to that described above. All control experiments resulted in the absence of specific staining.

Brain sections were imaged using a DeltaVision wide-field deconvolution microscope equipped with a digital camera (CoolSNAP HQ; Photometrics), using a 1.42-NA 60X oil-immersion objective lens and N=1.515 immersion oil (Applied Precision) at RT. Images were acquired and deconvolved and tif images generated as before. At least
25 images of LBs were collected from each PD patient, and images were analyzed using the Imaris software (Bitplane).
CHAPTER THREE

SERINE-129 PHOSPHORYLATION AND STRAIN CONFORMATION DETERMINE ENDOCYTIC VESICLE RUPTURE POTENCY OF ALPHA-SYNUCLEIN AMYLOID ASSEMBLIES

Rationale

As previously discussed, the prion-like cell-to-cell propagation of α-syn aggregates underlies the spreading of PD pathology throughout the brain and results in the inexorable progression of clinical symptoms for affected patients. Understanding the mechanism by which disease-associated α-syn aggregates enter target cells and induce cellular dysfunction is therefore key to understanding the progressive nature of PD as a neurodegenerative disease, and for developing effective disease-modifying therapeutic strategies. Although much has been learned regarding the life cycle of α-syn aggregates as they accumulate in diseased cells, traverse the extracellular environment, and come to invade recipient cells to propagate protein misfolding and neurotoxicity, much remains to be elucidated about this pathological disease transmission [380, 448, 465, 466, 633-637].

One central aspect of α-syn aggregate-induced pathology that defines the prion-like designation of α-syn aggregates is their ability to serve as seeds and templates for further α-syn misfolding and assembly, and that internalization of α-syn seeds results in the propagation and amplification of protein misfolding from diseased cells to their
normal neighbors when the seed encounters the naïve, expressed α-syn in the cytoplasmic environment [200, 202, 212, 213, 347, 454]. While endocytosis is the predominant process for cellular uptake of α-syn aggregates [280, 450, 454], the mechanisms by which misfolded extracellular α-syn aggregates then escape this endocytic compartment to access the cytosol and directly interact with normal intracellular α-syn remained poorly understood [448, 465].

By noting the striking similarity between α-syn infection and the infectious mechanism of pathogens like adenovirus, Salmonella, or Shigella, our group contributed an important mechanistic advancement in demonstrating that α-syn aggregates induce lysosomal vesicle rupture following endocytosis, triggering cathepsin-mediated oxidative stress and inflammasome activation [4]. Although this finding provided the mechanism of contact between extracellular α-syn aggregates internalized in the endocytic vesicle and soluble α-syn monomers in the cytosol, and linked α-syn spread with known lysosomal, autophagic, oxidative, and mitochondrial stress in PD [326, 346, 638, 639], numerous questions remain about this damaging mechanism of cellular invasion and its contribution to PD pathology. These previous studies did not elucidate the molecular determinants of α-syn-induced vesicle rupture potency or pinpoint the specific aggregate species of α-syn responsible for this vesicle damage, both of which are mechanistic questions essential for a more complete understanding of endocytic vesicle rupture in the cell-to-cell propagation pathway.
With the aim of determining specific modifications to α-syn assemblies that increase vesicle rupture potency, we sought to examine the impact of familial missense mutation, S129 phosphorylation, and strain conformation on α-syn-induced vesicle rupture, hypothesizing that these disease-associated modifications will increase potency of vesicle rupture induced by α-syn assemblies. To measure the influence of these modifications on the ability of α-syn to access the cytoplasm of cells following endocytosis, we employed the chGal3 relocation assay, as previously described [4, 280], to demonstrate enrichment of chGal3 at the site of intracellular vesicle rupture induced by α-syn aggregates carrying each individual modification. This assay, as described earlier, which we and others have also used to study both cellular entry by pathogenic organisms [576-578, 606] and the LMP cell death pathway [560, 615, 620], exploits the ability of chGal3 to bind target β-galactosides present exclusively on the outer leaflet of the plasma membrane or the topologically-equivalent inner leaflet of an internalized vesicle. In the context of an intact vesicular membrane, these β-galactoside targets remain unavailable for binding and the cytoplasmic localization of chGal3 remains diffuse. In a situation of vesicle rupture following endocytosis of an invading pathogen or α-syn fibril, these sugars are now exposed to the cytoplasmic chGal3 protein, resulting in the accumulation of chGal3 puncta that are visible by fluorescent microscopy as distinct from the diffuse chGal3 cytoplasmic expression (Figure 8). In the sections described below, we note vesicle rupture induced by fluorescently-labeled α-syn assemblies carrying each modification as indicated by colocalization between chGal3
Figure 8. Schematic of chGal3 relocalization assay. Galectin 3, fused to an mCherry-fluorophore, recognizes through the CRD its target β-galactoside sugars present on the extracellular leaflet of the plasma membrane or the inner leaflet of an internalized vesicle. In the situation of an intact vesicle (left), the cytoplasmic localization of chGal3 is diffuse. In the situation of a ruptured vesicle (right), luminal β-galactosides are now exposed to the cytosolic milieu, allowing chGal3 binding and the resulting localization of chGal3 is punctate, with discrete puncta localizing to the site of vesicle rupture.

puncta and α-syn fluorescence. Upon quantification of vesicle rupture induced by each α-syn assembly type, we find that S129 phosphorylation and strain conformation dictate the potency of α-syn-induced vesicle rupture. These results indicate the importance of these α-syn modifications for influencing α-syn-induced vesicle rupture damage during cell-to-cell propagation, and illuminate more details of how endocytic vesicle rupture is involved in PD pathology.
WT and Mutant Alpha-Synuclein Assemblies Exhibit Similar Potency of Intracellular Vesicle Rupture

We first sought to determine if α-syn mutations associated with familial forms of disease conferred to high-molecular weight assemblies of this protein an increased ability to induce vesicle rupture. We hypothesized that assemblies of mutant forms of α-syn associated with familial, autosomal dominant, early-onset PD would demonstrate an increased potency of endocytic vesicle rupture compared to WT assemblies, thereby accounting for their increased pathogenicity. To test this hypothesis, we utilized purified, recombinant α-syn, including WT α-syn and familial mutants A53T [220], A30P [221], E46K [222] and G51D [223]. To assess the ability of disease-associated α-syn assemblies to induce vesicle rupture, we generated α-syn aggregates by shaking recombinant α-syn for 3 days at 37 °C under identical protein and solute aggregation conditions, followed by labeling of these assemblies with amine-reactive fluorophore, a commonly utilized technique to follow α-syn microscopically [218, 454, 640]. Electron microscopic examination of the reaction products revealed that following aggregate formation and fluorophore labeling, fibrillar species were present in preparations of WT and mutant α-syn associated with familial forms of disease, and α-syn aggregate populations exhibited a similar size distribution (Figure 9).

Following the standardized in vitro aggregation and fluorophore labeling just described, we investigated the relative abilities of these α-syn assemblies to induce vesicle rupture following endocytosis. To test this hypothesis, we employed the chGal3
Figure 9. Structural characterization of WT and familial mutant α-syn assemblies. Morphologies of WT, E46K, A53T, A30P, and G51D α-syn assemblies. Representative negatively-stained TEM of α-syn assemblies before and after Dylight488 labeling. Assemblies were aggregated for 3 days at 37 °C under constant agitation followed by fluorescent labeling with DyLight488 NHS ester fluorophores as described in the Materials and Methods. Images are magnified 30,000X, scale bar = 1 µm.
relocalization assay in SH-SY5YchGal3 human neuroblastoma cells following 24 hr exposure to WT, A53T, A30P, E46K, and G51D α-syn assemblies in the culture media. While untreated SH-SY5YchGal3 cells maintained a diffuse cytosolic localization of the chGal3 marker of vesicle rupture (Figure 10), cells that were exposed to assemblies made of WT or mutant α-syn associated with familial forms of PD demonstrated pronounced relocalization of chGal3 to discrete punctate or annular structures that colocalized with α-syn fluorescence (Figure 10, arrowheads). This punctate chGal3 phenotype and colocalization with α-syn is indicative of endocytic vesicle rupture induced by α-syn assemblies. Moreover, consistent with the ability of α-syn to associate with vesicular membranes, Dylight488-labeled α-syn aggregates can frequently be seen adopting a curved or arc-shaped localization at the periphery of chGal3+ ruptured vesicles. Quantification of this vesicle rupture by α-syn assemblies revealed that WT and mutant α-syn associated with familial forms of PD induced vesicle rupture to the same extent (2.5-3 fold increase relative to unexposed cells, p<0.0001) (Figure 11). These results demonstrate that endocytic vesicle rupture induced by α-syn assemblies is not tightly dependent on mutations associated with early disease onset.

**Serine-129 Phosphorylation Increases Potency of Alpha-Synuclein-Induced Vesicle Rupture**

The experiments above suggest that individual familial disease-associated mutations do not substantially influence the ability of aggregated α-syn to induce vesicle rupture following endocytosis. However, α-syn can also be modified by multiple
SY5YchGal3 cells were subjected to 24 hr exposure in the culture media to 200nM exogenous Dylight488-labeled assemblies of WT, E46K, A53T, A30P, or G51D α-syn, followed by fixation and DAPI staining in PBS. α-Syn-induced vesicle rupture (arrowheads) identified by chGal3 puncta colocalizing with α-syn. Left panel inset box is enlarged and separated in right panels. Images representative of at least 20 images per treatment in 4 independent experiments. Scale bar=10 µm (left), 1 µm (right 3 panels).

**Figure 10. Vesicle rupture induced by WT and familial mutant α-syn assemblies.**
Figure 11. WT and familial mutant α-syn assemblies exhibit similar potency of vesicle rupture. Manual quantification of fold change in mean number of chGal3 puncta per cell +/- standard error of the mean (SEM) induced by each treatment type relative to unexposed cells, with N>190 cells per type in total from 4 independent experiments. ANOVA p <0.0001, * denotes significance (p<0.0001) compared to unexposed cells as determined by Tukey’s post-hoc multiple comparison test.

covalent PTMs and truncations that may affect both aggregation and cell-to-cell propagation by influencing α-syn conformation and modifying membrane association, complex formation, and degradation [259]. Perhaps the most ubiquitous PTM is S129 phosphorylation [260, 641], and given the fact that almost 90% of α-syn in LBs is phosphorylated at S129 while only 5% of α-syn in normal brains carries this PTM [119-122], S129 phosphorylation is tightly associated with pathological α-syn forms. We therefore utilized the chGal3 relocalization assay to investigate the ability of
endocytosed non-phosphorylated and phosphorylated α-syn to rupture vesicular membranes and to test the hypothesis that this PTM alone or in combination with disease-associated familial missense mutations can differentially affect the potency of α-syn entry via this disruptive mechanism. We again generated α-syn aggregates by shaking recombinant WT, A30P, or A53T α-syn in either their non-phosphorylated or pS129 forms for 3 days at 37 °C under identical protein and solute aggregation conditions, followed by labeling of these assemblies with amine-reactive fluorophore. SY5YchGal3 cells were then exposed in the cell culture medium for 24 hr to these labeled non-pS129 or pS129 α-syn aggregates, followed by an assessment of the formation of chGal3 puncta as a measurement of vesicle rupture induction. Again, each type of α-syn was able to induce chGal3 relocalization to intracellular punctate structures when compared with the diffuse cytoplasmic localization in unexposed cells (Figure 12), and the profound co-localization of α-syn and chGal3 puncta implicates α-syn as the causative agent of membrane permeabilization. α-Syn aggregates again adopted curved or arc-shaped appearances at the periphery of chGal3+ ruptured vesicles in many instances.

To quantify vesicle rupture in a semi-automated, unbiased fashion and to compare the relative abilities of each α-syn species to cause this membrane permeabilization, algorithm-assisted identification of chGal3 puncta was employed in unexposed and exposed cells. This algorithm reliably identified cytoplasmic chGal3 puncta (Figure 34). When the mean number of chGal3 puncta per cell values were
Figure 12. Vesicle rupture induced by WT and familial mutant α-syn assemblies with or without S129 phosphorylation. SY5YchGal3 cells were subjected to 24 hr exposure in the culture media to 200nM exogenous Dylight488-labeled aggregates of WT, A30P, and A53T α-syn with and without pS129, followed by fixation and DAPI staining in PBS. α-Syn-induced vesicle rupture (arrows) identified by chGal3 puncta colocalizing with α-syn. Left panel inset box is enlarged and separated in right panels. Images representative of at least 20 images per treatment in 3 independent experiments. Scale bar=10 µm (left), 3 µm (right 3 panels). Reproduced with permission from [280].
normalized so that pS129 α-syn isoforms were expressed as a fold change relative to their non-pS129 counterparts within the same experiments, all pS129 α-syn types show an approximately 50% increase in the mean number of chGal3 puncta per cell relative to their non-pS129 counterparts (Figure 13). Although pS129 A30P αsyn was the only pS129 α-syn aggregate to induce a statistically significant increase in chGal3 puncta per cell relative to its non-pS129 counterpart, both pS129 WT α-syn and pS129 A53T α-syn showed trends above their non-pS129 counterparts, although not reaching statistical significance. This finding suggests that S129 phosphorylation impacts the potency of vesicle rupture following endocytosis regardless of the influence of familial missense mutation, and thus significantly contributes to neurotoxicity in the α-syn cell-to-cell propagation pathway.

**Alpha-Synuclein Strain Conformation Dictates Vesicle Rupture Potency**

While previous results suggest that S129 phosphorylation significantly increases the potency of α-syn-induced vesicle rupture, and that α-syn familial mutation has little effect on membrane damage following endocytosis even when combined with S129 phosphorylation, the size and structural heterogeneity of the aggregates generated in the manner described above prevents the identification of specific high-molecular weight oligomeric or fibrillar species capable of inducing vesicle rupture. To more precisely define α-syn high-molecular weight species responsible for endocytic vesicle rupture, we utilized structurally well-defined WT α-syn assemblies and the chGal3 relocalization assay in SY5YchGal3 cells following 24 hr exposure to α-syn assemblies in
Figure 13. S129 Phosphorylation increases potency of α-syn-induced vesicle rupture. Algorithm-based quantification of fold change in mean number of chGal3 puncta per cell +/- SEM induced by each treatment type relative to non-pS129 counterpart, with N> 200 cells per type in total from 3 independent experiments. * denotes significance (p<0.05) compared to non-pS129 counterpart as determined by unpaired t test. Reproduced with permission from [280].

the culture media. Generated in vitro by varying the aggregation conditions for recombinant monomeric α-syn, the 7-day aggregation protocol results in fully elongated α-syn assemblies of varying strain conformations which are then fragmented by sonication, resulting in structurally well-characterized, homogenous assembly populations of a smaller size distribution capable of cellular uptake by endocytosis (Figure 14). This preparation protocol resembles that of α-syn PFFs, whose fibrillar conformation and smaller size distribution make it the α-syn species most efficient for
**Figure 14. Structural characterization of WT α-syn polymorphs.** Morphologies of α-syn oligomers and fibrillar assemblies/strains. Representative negatively stained TEM of α-syn on-fibrillar assembly pathway oligomers, fibrils, ribbons, fibrils-65, and fibrils-91, shown before and after fragmentation for all except oligomers which were not fragmented. Fibrillar assemblies were fragmented for 20 min at a constant temperature (20 °C) in 2 mL Eppendorf tubes in a VialTweeter powered by an ultrasonic processor UIS250v (250 watts, 24 kHz, Hielscher Ultrasonic, Teltow, Germany) set at 75% amplitude, 0.5 s pulses every 1 s. Scale bar, 200 nm.
inducing seeding, propagating from cell-to-cell, and causing neurotoxicity [129, 200, 202-204, 209-213, 219, 389-395, 439, 441, 442]. When α-syn monomers and on-fibrillar assembly pathway oligomers were assessed in the chGal3 relocalization assay, these α-syn forms were unable to induce significant vesicle rupture. Cells exposed to these forms of α-syn exhibited a predominantly diffuse phenotype of chGal3 fluorescence resembling that of untreated cells (Figure 15).

α-syn has been previously shown to assemble into fibrils that exhibit different intrinsic structures, surfaces, seeding and persistence propensities [305]. These distinct fibrillar polymorphs yield different synucleinopathies when injected into recipient animals [218]. We hypothesized that differences in the surface and physical properties of the distinct fibrils will influence their vesicle rupture potency. We therefore examined the relative ability of four different α-syn fibrillar polymorphs, fluorescently labeled and fragmented to produce fibrillar assemblies with uniform size distribution (Figure 14), to induce vesicle rupture following endocytosis. We observed that α-syn fibrils, ribbons, fibrils-65, and fibrils-91 potently induced endocytic vesicle rupture in target SY5YchGal3 cells as demonstrated by chGal3 relocalization to punctate or annular structures that colocalize with α-syn fluorescence (Figure 15, arrowheads). Interestingly, quantification of chGal3+ ruptured vesicles per cell revealed that α-syn fibrils, fibrils-65, and fibrils-91 significantly induced vesicle rupture compared to unexposed cells (2.5-4 fold increase, p<0.0001 for fibrils and fibrils-65; p=0.0047 for fibrils-91), whereas α-syn ribbons were unable to demonstrate a similar significant induction of vesicle rupture (p=0.2646).
Figure 15. Vesicle rupture induced by WT α-syn polymorphs. SY5YchGal3 cells were subjected to 24 hr exposure to 660nM exogenous Atto488-labeled fragmented assemblies of WT α-syn fibrils, ribbons, fibrils-65, and fibrils-91, as well as α-syn monomers and oligomers in the culture media, followed by fixation and DAPI staining in PBS. α-Syn-induced vesicle rupture (arrowheads) identified by chGal3 puncta colocalizing with α-syn. Left panel inset box is enlarged and separated in right panels. Images representative of at least 20 images per treatment in 3 independent experiments. Scale bar=15 μm (left), 3 μm (right 3 panels).
Furthermore, α-syn fibrils were shown to be significantly more potent in their induction of vesicle rupture compared to α-syn monomers, on-fibrillar assembly pathway oligomers, and ribbons (p=0.0009 compared to monomers, p<0.0001 compared to oligomers, p=0.0004 compared to ribbons), and α-syn fibrils-65 were shown to be significantly more potent compared to α-syn oligomers (p<0.0001) (Figure 16). While α-syn ribbons failed to demonstrate a statistically significant increase in vesicle rupture induction compared to unexposed cells, we did observe instances where chGal3+ ruptured vesicles colocalized with α-syn ribbons (Figure 15). These data demonstrate that the fibrillar nature of α-syn assemblies is an essential but not sufficient factor for the induction of endocytic vesicle rupture by α-syn assemblies, and that the intrinsic structural conformation of α-syn fibrillar polymorphs dictates the potency with which this endocytic vesicle rupture occurs. Furthermore, given that distinct α-syn strains are associated with different synucleinopathies, strain-specific differences in disease phenotype may arise from varying levels of vesicle rupture potency induced by each α-syn strain. Overall, the potency of α-syn aggregate-induced vesicle rupture is determined by a unique combination of aggregate size and conformation that initiates membrane damage following endocytosis.

**Significance**

The above findings reveal new information regarding the ability of α-syn aggregates to induce vesicle rupture during the cell-to-cell propagation pathway. Transmission of α-syn aggregates from diseased cells and brain regions to unaffected
Figure 16. α-Syn strain conformation dictates vesicle rupture potency. Manual quantification of fold change in mean number of chGal3+ puncta per cell +/- SEM induced by each treatment type, with N>412 cells per type in total from 3 independent experiments. ANOVA p<0.0001, * denotes significance compared to unexposed cells (p<0.0001 for fibrils and fibrils-65, p=0.0047 for fibrils-91), # denotes significance compared to monomer (p=0.0009 for fibrils), + denotes significance compared to oligomer (p<0.0001), and ^ denotes significance (p=0.0004) compared to ribbons as determined by Tukey’s post-hoc multiple comparison test.

ones underlies the widespread pathological progression of PD and the onset of increasingly severe symptoms affecting an ever-growing number of brain functions [542, 642, 643]. Additionally, the infiltration of α-syn pathology into previously healthy brain regions may also contribute to the failure of striatal dopaminergic neuronal grafts as a potential therapeutic replacement for degenerating dopaminergic innervation in PD.
Insights into the mechanisms of cell-to-cell propagation have enormous potential to uncover new therapeutic targets that could be used to stem the tide of \(\alpha\)-syn deadly spread [448, 635, 645].

Prior to the work of our group, there was an unaddressed disconnect between the uptake of \(\alpha\)-syn amyloid aggregates via endocytosis and the seeding of further \(\alpha\)-syn aggregation in the cytosol [448, 465]. Since the endocytic pathways results in uptake of \(\alpha\)-syn aggregates sequestered within the vesicular compartment, the process by which exogenous \(\alpha\)-syn aggregates escape this compartment to contact cytosolic \(\alpha\)-syn and serve as misfolded templates was unclear. Our group was the first to directly address this question of \(\alpha\)-syn aggregate vesicular escape by employing the chGal3 relocalization assay, a novel approach to investigating \(\alpha\)-syn vesicular membrane damage having previously been used only in the investigation of cellular invasion by infectious pathogens [576-578, 606]. The application of this assay from the world of infectious diseases is particularly appropriate given the parallels between \(\alpha\)-syn aggregate cell-to-cell propagation and the infectious mechanism of prions in TSEs. Just as bacteria or viruses need to escape the vesicular compartment of entry to propagate their infection by hijacking host replication machinery in the cytosol, so too must an \(\alpha\)-syn aggregate leave the endocytic vesicle to contact its own “replication machinery”, the pool of naïve monomeric \(\alpha\)-syn expressed in the cytosol. The demonstration that \(\alpha\)-syn aggregates are capable of inducing lysosomal rupture following endocytosis explained the disconnect between the method of entry and the seeding of aggregation [4], although
many questions remained about which α-syn species is most capable of this entry mechanism and if various modifications to α-syn aggregates may modulate the potency of rupture.

The above results illuminate the α-syn species most capable of inducing endocytic vesicle rupture, short fragments of α-syn fibrils, and determined that S129 phosphorylation and strain conformation influence vesicle rupture potency. Using several variations of in vitro α-syn aggregates prepared from recombinant forms of WT or familial mutant α-syn, also with S129 phosphorylation in some cases, we have demonstrated that all forms of fibrillar α-syn aggregates were capable of inducing chGal3 relocalization to the site where they caused vesicle rupture. Consistent with our previous findings [4], this vesicle rupture phenomenon is not induced in response to treatment with α-syn monomers. We extend the nuances of vesicle rupture determinants by showing specific α-syn structures such as on-fibrillar assembly pathway oligomers that also do not induce vesicle rupture in target cells. Despite the early-onset of PD associated with familial missense mutations in α-syn, aggregates created from α-syn monomers carrying these mutations did not exhibit changes in vesicle rupture potency compared with WT α-syn aggregates, but instead were each capable of endocytic vesicle rupture to an equal extent. In contrast, aggregates created from α-syn monomers phosphorylated at S129 were shown to be more potent compared with non-pS129 forms, implicating this PTM in increasing α-syn aggregate pathogenicity in the cell-to-cell propagation pathway. That pS129 α-syn aggregates are more potent
inducers of endocytic vesicle rupture and the resulting neurotoxicity may underlie the enrichment of this PTM in pathological α-syn LB inclusions. Finally, the conformation with which α-syn monomers pack into a larger fibrillar assembly is a critical modifier of endocytic vesicle rupture potency, and highlights one mechanism for the variations in disease phenotype caused by distinct α-syn strains. These findings are significant for the new insights they bring into the damaging mechanism of cellular invasion by aggregates of α-syn in their cell-to-cell transmission life cycle.
CHAPTER FOUR

AMYLOID ASSEMBLIES OF TAU AND POLYGLUTAMINE-EXPANDED HUNTINGTIN INDUCE ENDOCYTIC VESICLE RUPTURE

Rationale

In addition to its relevance for α-syn in synucleinopathies, the prion-like cell-to-cell propagation of amyloid proteins has also been implicated in the pathogenesis and progression of numerous other neurodegenerative proteinopathies such as AD, CTE, HD, and ALS, where it has been blamed, as in PD, for contributing to worsening pathology and increasingly debilitating symptomatology [3, 42, 505, 522, 642, 646-650]. Although the monomeric protein building blocks differ, there are many similarities among the higher-order amyloid assemblies of each causative protein, and mechanistic insight regarding the cell-to-cell propagation of one amyloid protein form may be relevant for understanding the pathological behavior of others. Many aspects of the intracellular and intercellular life cycle of amyloid proteins are yet to be fully understood, but the prospect of translating insight from one proteinopathy to others holds enormous promise toward developing a potential therapeutic strategy to stem the progressive tide of numerous incurable neurodegenerative diseases.

In the case of tau in tauopathies and polyglutamine-expanded huntingtin in HD, these proteins also perform prion-like seeding and templating of additional monomeric constituents in the cytosol of naïve cells.
Faithful templating of misfolded structure and subsequent intracellular inclusion formation necessitates direct contact between exogenous tau or HTTExon1 seeds and soluble cytosolic monomers, and this direct contact has been demonstrated for exogenous and expressed versions of tau, using FRET, and HTTExon1, using BiFC [510, 534]. Endocytosis is again the predominant mechanism for cellular uptake of amyloid assemblies of tau and HTTExon1 [498, 506, 531], although like α-syn, its mechanism of vesicular escape remains an enigma [465, 522, 531]. Because all the aforementioned fibrillar α-syn polymorphs exhibited the ability to induce vesicle rupture in target cells, we explored whether this damaging mechanism of cellular invasion was a specific consequence of α-syn aggregate cellular entry, or if other neurodegenerative-related proteins could also induce vesicle rupture. Endocytic vesicle rupture induced by these amyloid aggregates would provide an explanation for contact between extracellular seeds and intracellular expressed monomers, and in a manner similar to α-syn, also contribute to degradative, mitochondrial, and oxidative stress in diseased cells.

With the aim of determining the conserved nature of amyloid cellular invasion through endocytic vesicle rupture, we sought to examine the cellular uptake of fibrillar assemblies composed of full-length WT tau isoforms 1N4R and 1N3R and HTTExon1-Q45, hypothesizing that short fibrillar forms of these prion-like amyloid proteins would also induce vesicle rupture following endocytosis. Investigation of this form of cellular entry by assemblies of HTTExon1 is entirely novel, and while a limited number of reports
have examined the vesicle rupture capability of tau aggregates [507, 560], no other study has thus far utilized multiple isoforms of WT full-length tau in a neuronal model, let alone employed rigorous assembly characterization to ensure specificity of effect. To measure vesicle rupture induced by tau and HTTExon1-Q45 amyloid assemblies, we again employed the chGal3 relocalization assay in SH-SYSY human neuroblastoma cells following exposure to well-defined exogenous fluorescently-labeled assemblies of these proteins. In the sections below, we note vesicle rupture induced by tau and HTTExon1-Q45 as indicated by colocalization between chGal3 puncta and fluorescently-labeled amyloid assemblies. Furthermore, we find a significant increase in vesicle rupture potency for assemblies composed of tau 1N4R compared with those composed of tau 1N3R, implicating differences in vesicle rupture potency as contributors to specific disease phenotypes for distinct tau isoform strains. These findings highlight the conserved nature of endocytic vesicle rupture in the cell-to-cell propagation pathway of amyloid proteins composed of multiple neurodegenerative disease-related proteins.

**Multiple Isoforms of Wild-Type Full-Length Tau Induce Endocytic Vesicle Rupture**

In the diverse set of diseases characterized by tau inclusions, some diseases like AD, CTE, and TD are characterized by inclusions of both 3R and 4R tau isoforms, whereas inclusions of 3R isoforms predominate in PiD and 4R isoforms predominate in PSP, CBD, and AGD [488]. Because various isoform compositions can result in unique strain conformations that induce distinct disease phenotypes upon cell-to-cell propagation, we hypothesized that assemblies of tau1N4R and tau1N3R would both induce vesicle
rupture, but that differences in the potency of vesicle rupture induction may underlie strain-specific disease phenotypes as they do for α-syn strains in PD. To test this hypothesis, we generated in vitro fibrillar assemblies of full-length WT tau 1N4R and tau 1N3R from recombinant purified protein in a fashion similar to α-syn fibrillar polymorph production, including prolonged assembly reactions, fluorophore labeling, and fragmentation by sonication to produce homogenous preparations of tau fibrils of a smaller size distribution to be taken up by endocytosis in target cells. This species of tau assembly was chosen not only because of the efficacy of this amyloid assembly form for α-syn-induced vesicle rupture, but also to allow a direct comparison with vesicle rupture induced by α-syn assemblies of a similar size distribution, so that only protein composition and conformation differ. TEM analysis revealed that the assemblies were of fibrillar nature following aggregation, and maintained this fibrillar architecture following fragmentation despite the decrease in fibril size (Figure 17). SYSYchGal3 cells were then exposed for 24 hr to these fibrillar tau assemblies, followed by an assessment of chGal3 relocalization to indicate endocytic vesicle rupture as for αsyn assemblies. We noted chGal3 relocalization to a punctate phenotype upon exposure of cells to fibrillar assemblies of full-length WT tau 1N4R and 1N3R isoforms, whereas chGal3 remained diffuse in unexposed cells (Figure 18). We observed robust colocalization between chGal3+ ruptured vesicles and both isoforms of fluorescently-labeled fibrillar tau (Figure 18, arrowheads). This observation strongly suggests that fibrils composed of multiple isoforms of WT full-length tau cause vesicular membrane damage upon cellular entry.
Figure 17. Structural characterization of WT full-length tau polymorphs. Morphologies of tau 1N4R and tau 1N3R assemblies/strains. Representative negatively stained TEM of tau 1N4R and tau 1N3R polymorphs, shown before and after fragmentation. Fibrillar assemblies were fragmented for 20 min at a constant temperature (20 °C) in 2 mL Eppendorf tubes in a VialTweeter powered by an ultrasonic processor UIS250v (250 watts, 24 kHz, Hielscher Ultrasonic, Teltow, Germany) set at 75% amplitude, 0.5 s pulses every 1 s. Scale bar, 200 nm.

Quantification of punctate chGal3 clearly indicates that both isoforms of fibrillar full-length WT tau rupture endocytic vesicles to a significant extent compared to unexposed cells, with a further significant increase in rupture potency induced by fibrillar tau 1N4R
Figure 18. Vesicle rupture induced by multiple isoforms of wild-type full-length tau.
SY5YchGal3 cells were subjected to 24 hr exposure to 660nM exogenous Atto488-labeled fragmented assemblies of tau 1N3R or tau 1N4R in the culture media, followed by fixation and DAPI staining in PBS. Tau-induced vesicle rupture (arrowheads) identified by chGal3 puncta colocalizing with tau. Left panel inset box is enlarged and separated in right panels. Images representative of at least 20 images per treatment in 3 independent experiments. Scale bar=10 µm (left), 1 µm (right 3 panels).

compared to 1N3R (Figure 19). These findings provide the first demonstration that fibrils composed of multiple full-length wild-type tau isoforms 1N4R and 1N3R are capable of inducing vesicle rupture following endocytosis, and like α-syn, that the intrinsic isoform composition and structural conformation of tau fibrillar polymorphs dictates endocytic vesicle rupture potency. We have again implicated variations in vesicle rupture potency
Figure 19. Tau isoform composition and strain conformation dictate vesicle rupture potency. Manual quantification of fold change in mean number of chGal3+ puncta per cell +/- SEM induced by assemblies of tau isoforms, with N>135 cells per type in total from 3 independent experiments. ANOVA p <0.0001, * denotes significance compared to unexposed cells (p<0.0001) and # denotes significance compared to tau 1N3R (p<0.0001) as determined by Tukey’s post-hoc multiple comparison test.

in strain-specific differences in pathology. This observation underscores the importance of this conserved endocytic vesicle rupture event as essential in the cell-to-cell propagation life cycle of tau amyloid assemblies in diverse tauopathies.

Assemblies of Polyglutamine-Expanded Huntingtin Induce Vesicle Rupture

CAG repeat expansion in HTTExon1 yields a polyglutamine-rich huntingtin protein with an increased propensity to spontaneously aggregate into a fibrillar amyloid morphology when the polyglutamine tract expands to 37 glutamine residues or beyond (HTTExon1-Q\(\geq37\)) [524], causing HD in patients with this dominantly-inherited genetic
mutation [523]. Because HTTExon1 amyloid assemblies also propagate from cell-to-cell in a prion-like manner and serve as seeds and templates for further aggregation, we hypothesized that assemblies of HTTExon1 containing a polyglutamine tract of pathologic length (Q45) would induce vesicle rupture following endocytosis. To test this hypothesis, we again generated in vitro fibrillar assemblies of HTTExon1-Q45 from recombinant purified protein, just as for α-syn and tau fibrillar polymorphs, using prolonged assembly reactions combined with subsequent fragmentation to produce homogenous small fibrillar preparations of HTTExon1 fibrils of an equivalent size distribution to fragmented polymorphs of α-syn and tau. Because of this preparation scheme, the size distribution of HTTExon1 assemblies is standardized with that of α-syn and tau fibrillar polymorphs, allowing comparisons of vesicle rupture ability based only on protein composition and conformation in the preparation form most potent for inducing vesicle rupture, short amyloid fibrils. TEM analysis revealed that HTTExon1-Q45 assemblies are composed of amyloid fibrils just as assemblies of α-syn and tau, and short fibrillar preparations are induced by sonication (Figure 20). As for other amyloid preparations, we also utilized amine-reactive fluorophore labeling of HTTExon1 fibrils for fluorescent microscopic detection, but despite the presence of four reactive primary amine groups within the HTTExon1 amino acid sequence, we were unable to achieve sufficient fluorescent labeling perhaps due to the limited accessibility of these amine groups within the fibrillar aggregate.
Figure 20. Structural characterization of HTTExon1-Q45 polymorph. Morphology of HTTExon1-Q45 assemblies/strain. Representative negatively stained TEM of HTTExon1-Q45 polymorph, shown before and after fragmentation. Fibrillar assemblies were fragmented for 20 min at a constant temperature (20 °C) in 2 mL Eppendorf tubes in a VialTweeter powered by an ultrasonic processor UIS250v (250 watts, 24 kHz, Hielscher Ultrasonic, Teltow, Germany) set at 75% amplitude, 0.5 s pulses every 1 s. Scale bar, 200 nm.

SYSYchGal3 cells were exposed for 24 hr to these fibrillar HTTExon1-Q45 assemblies, and chGal3 relocalization was assessed as an indicator of endocytic vesicle rupture in target cells following their uptake of amyloid fibrils. We noted chGal3 relocation to a punctate phenotype upon cellular exposure to HTTExon1-Q45 fibrillar assemblies, whereas chGal3 again remained diffuse in unexposed cells (Figure 21). This observation strongly implies that amyloid fibrils composed of HTTExon1 with a pathologic stretch of polyglutamine residues also cause endocytic vesicular rupture just like amyloid fibrils of αsyn and tau. Due to the insufficient labeling of fibrillar HTTExon1-Q45, we were unable to visualize colocalization of assemblies with chGal3+ ruptured
Figure 21. Vesicle rupture induced by HTTExon1-Q45 polymorph. SY5YchGal3 cells were subjected to 24 hr exposure to 660nM exogenous Atto488-labeled fragmented assemblies of HTTExon1-Q45 in the culture media, followed by fixation and DAPI staining in PBS. HTTExon1-Q45-induced vesicle rupture identified by chGal3 puncta. Left panel inset box is enlarged and separated in right panel. Images representative of at least 20 images per treatment in 5 independent experiments. Scale bar=15 µm (left), 3 µm (right).

vesicles. However, quantification of punctate chGal3 clearly indicates that fibrillar HTTExon1-Q45 induces significant vesicle rupture as compared to unexposed cells
(Figure 22). These findings provide the first demonstration that fibrils composed of HTTExon1 with pathologic polyglutamine repeats are capable of inducing vesicle rupture following endocytosis as a means of propagating their misfolded amyloid structure from the extracellular to the intracellular environment. This observation is of critical importance for understanding HD progression, and the conserved nature of vesicle rupture induced following endocytosis of α-syn, tau, and HTTExon1 amyloid assemblies raises the possibility that therapeutic strategies targeting this pathologic cellular entry mechanism may be applied to reverse or prevent numerous proteinopathies.

Significance

The above results have uncovered a conserved role for endocytic vesicle rupture as a mechanism of cellular invasion by amyloid assemblies of multiple neurodegenerative disease-related proteins. Just as for α-syn in PD, amyloid assemblies of tau and HTTExon1 are known to propagate from one cell to another, templating their misfolded conformation onto soluble constituent monomers and inducing neurotoxicity through a variety of mechanisms in the cells encountered along the transmission pathway [465, 488, 522]. Much remains to be determined regarding the mechanisms employed by amyloid protein assemblies during this pathological progression, especially to understand if any mechanisms are shared between these prion-like amyloid proteins or if each transmission process is as distinct as the respective etiological protein. Given that the propagation of amyloid proteins underlies pathological disease progression,
Figure 22. HTTExon1-Q45 assemblies induce significant increase in vesicle rupture compared to unexposed cells. Manual quantification of fold change in mean number of chGal3+ puncta per cell +/- SEM induced by assemblies of HTTExon1-Q45, with N>441 cells per type in total from 5 independent experiments. * denotes significance compared to unexposed cells (p<0.0001) by unpaired student’s t test.

Insights into their intracellular and intercellular life cycle are incredibly important for a more complete picture of disease pathogenesis.

Critically, the incongruity between the cellular uptake of exogenous aggregates into the endocytic vesicle and the seeding of further aggregation in the cytosol is shared among the propagation pathways of α-syn, tau, and HTTExon1 amyloid aggregates [448, 465, 522, 531]. Given the common ability of α-syn aggregates to rupture endocytic vesicles as a means of escape, and the utility of the chGal3 relocalization assay to provide a reliable indication of this vesicular damage [4, 280, 619, 620], we examined the vesicle rupture capability of tau and HTTExon1 amyloid assemblies. This process of
cellular invasion has never been investigated for HTTExon1 amyloids, and even for amyloid aggregates of tau, the studies examining its vesicle rupture capability have significant limitations to the scope of their conclusions. One study found an ability of P301L mutant tau aggregates to induce vesicle rupture in rat primary neurons expressing chGal3, although the assembly form of exogenous tau was poorly characterized since fluorescently-labeled whole cell lysates from HEK293 cells expressing P301L mutant tau were used as a treatment [507]. Another study utilized full-length WT 2N4R tau assemblies generated in vitro from recombinant purified protein, although these preparations were heterogeneous and of high molecular weight given that fragmentation was not performed, observing that these preparations induce vesicle rupture as seen by chGal3 relocalization in a non-neuronal HeLa cell model [560]. Both studies are limited in that the exogenous tau assemblies used to demonstrate vesicle rupture induction are not characterized to an extent that allows determination of a specific effect of distinct tau assembly species. We employed in vitro generation of tau assemblies composed of multiple isoforms of WT full-length tau, followed by sonication to homogenize the assembly population size distribution by fragmenting larger fibrillar assemblies into smaller amyloid fibrils more readily taken up through endocytosis into target cells. The structurally well-defined nature of these WT full-length tau assemblies guarantees the specific measurement of vesicle rupture induced by short fibrillar tau assemblies.
In demonstrating that fibrillar assemblies of tau 1N4R, tau 1N3R, and HTTExon1-Q45 rupture endocytic vesicles following cellular uptake, we have provided an explanation for the invasion mechanism by which exogenous fibrils contact intracellular monomers. The shared ability of α-syn, tau, and HTTExon1 assemblies to induce endocytic vesicle rupture implies that this pathological activity is a result of the fibrillar amyloid architecture common to many aggregation-prone proteins. Endocytic vesicle rupture is a shared mechanism of cellular invasion by these diverse prion-like amyloid proteins, despite differences in conformations and compositions, because of the short fibrillar amyloid structure standardized among α-syn, tau, and HTTExon1 preparations, enabling endocytic uptake, vesicle rupture, seeding, and cell-to-cell propagation. We have further demonstrated that assemblies of tau isoform 1N4R are significantly more potent in their induction of vesicle rupture compared with assemblies of tau isoform 1N3R, indicating that unique combinations of tau composition and conformation dictate vesicle rupture potency just as for α-syn-induced rupture. These variations in vesicle rupture potency between different tau isoform strains may contribute to the variety of disease phenotypes displayed by tauopathies. These findings are significant because of the determination that endocytic vesicle rupture, conserved for fibrillar assemblies of amyloid proteins in multiple neurodegenerative diseases, is a key mechanism during the cellular invasion phase of the cell-to-cell propagation life cycle.
CHAPTER FIVE

ENDOCYTIC VESICLE RUPTURE CONTRIBUTES TO INCLUSION FORMATION AND CELL-TO-CELL PROPAGATION IN PARKINSON’S DISEASE PATHOLOGY

**Rationale**

With the clear understanding that endocytic vesicle rupture is a conserved mechanism of cellular invasion by fibrillar assemblies of amyloid proteins, we next sought to determine the relevance of this damaging mechanism of cellular invasion in PD, investigating the contribution of α-syn-mediated endocytic vesicle rupture and its consequences to established aspects of PD pathology and propagation. Despite the highly reproducible nature of our findings demonstrating the induction of vesicle rupture by multiple fibrillar amyloid assemblies in recipient SY5YchGal3 cells, this immortalized human neuroblastoma cell line is not the model system most representative of neuronal physiology, and thus conclusions drawn from the use of this cell line leave doubt as to the occurrence of α-syn mediated endocytic vesicle rupture in authentic disease. SH-SY5Y cells, widely used as a neuron-like cell line, are comprised of a mixed phenotypic culture derived from a metastatic bone tumor biopsy, and despite the expression of tyrosine hydroxylase (TH) characteristic of dopaminergic neurons, numerous caveats exist when using cells derived from malignant tumors [651]. Alternatively, human midbrain dopaminergic neurons differentiated from hiPSCs are post-mitotic and display typical physiological characteristics and responses of...
dopaminergic neurons. These cells are >90% neurons (MAP2+/nestin-) of a
dopaminergic identity (>85% FoxA2+, >75% TH+), and exhibit spontaneous and evoked
action potentials and excitatory post-synaptic currents. As such, hiPSC-derived
dopaminergic neurons expressing the chGal3 marker of vesicle damage represent a
model system representative of human PD wherein to examine the process of α-syn-
mediated endocytic vesicle rupture. With the aim of determining the occurrence of
endocytic vesicle rupture in a more physiologically-relevant model system, we sought to
examine chGal3 relocalization in hiPSC-derived dopaminergic neurons following α-syn
aggregate exposure, hypothesizing that α-syn assemblies would also induce endocytic
vesicle rupture in this model system.

Among the known consequences of endocytic vesicle rupture are the production
of damaged vesicular debris needing to be cleared through autophagic degradation and
the dysfunctional lysosomal degradation resulting from LMP. Given that α-syn
aggregates invade target cells that may already be experiencing some degree of
degradative dysfunction for a variety of reasons in PD [340-343], the effect of endocytic
vesicle rupture on normal intracellular vesicle trafficking and degradation processes is
relevant for disease pathogenesis. Vesicles damaged by pathogen invasion are targeted
for autophagic degradation [560, 570, 576, 579, 597-600, 602, 603, 615], significantly
increasing the burden of autophagic substrates in need of clearance. Furthermore, LMP
itself directly depletes the pool of lysosomes competent to ultimately perform the
clearance of autophagic substrates, resulting in accumulation of autophagosomes and
eventual neuronal degeneration because this degradative dysfunction [130, 615]. This dual effect whereby the mechanism of cellular invasion both increases the substrate burden for degradative processes while at the same time inducing degradative dysfunction is expected to be at work with α-syn cellular invasion, and represents a significant challenge to functional proteostasis. With the aim of defining the impact of α-syn-mediated endocytic vesicle rupture on normal vesicular trafficking and degradation, we sought to examine the phenotype of ruptured vesicles as well as their recruitment to the ALP, hypothesizing that ruptured lysosomes and their α-syn cargo become targets for autophagic degradation.

As the proposed mechanism for the vesicular escape of exogenous fibrillar amyloid seeds to interact with cytosolic expressed monomeric constituents and template further aggregation, the relationship between amyloid-induced endocytic vesicle rupture and further seeding and inclusion formation needs more clarity. Interestingly, the very same small fragmented amyloid fibrils that are most potent for inducing endocytic vesicle rupture in our studies are known to also be most potent for seeding and templating of further aggregation [209-211]. Theories on the formation of cytosolic proteinaceous inclusions such as LBs center around the idea of aggresomes representing a protective response of cells overwhelmed by the burden of misfolded proteins and their inability to effectively degrade them [118, 124]. Condensing misfolded protein aggregates into a larger inclusion may be a cellular effort to increase efficiency of autophagic clearance, although failure of autophagy results in the recycling
locus becoming a junkyard instead [118]. Consistent with this idea, LBs are known to contain α-syn-associated vesicles as well as dysfunctional lysosomes and autophagosomes [128, 131, 134]. While the merging of misfolded α-syn aggregates and associated degradative vesicles may be originally intended to facilitate degradation, dysfunction in degradative processes instead results in agglomerations becoming pathological LB inclusions. With the aim of determining the influence of α-syn-induced endocytic vesicle rupture on pathologic inclusion formation, we sought to examine α-syn-containing LB inclusions in cultured cells and in PD brain, hypothesizing that Gal3 localization would indicate involvement of endocytic vesicle rupture in the formation of these intracellular proteinaceous inclusions.

Finally, because the same cellular stressors induced by endocytic vesicle rupture, such as ALP dysfunction and oxidative stress, are also well-known causes of α-syn release and intercellular propagation, the influence of endocytic vesicle rupture on α-syn cell-to-cell spreading is an important unanswered question. Diverse cellular stressors prompt α-syn release from cells affected by its aggregation and accumulation [294, 468-471, 473, 479, 484, 485], raising the possibility that consequences of the endocytic vesicle rupture entry mechanism may contribute to mechanisms of release. Many of the non-classical α-syn release mechanisms involve an association between α-syn and vesicles; within intracellular vesicles or autophagosomes that fuse with the plasma membrane to release their contents [468-471], on the internal or external leaflet of exosomes [473, 478, 479], or within lysosomes that traffic to neighboring cells inside
TNTs [445]. Since Gal3 is also released from cells through a non-classical mechanism [591-593], some α-syn release could be in association with Gal3+ ruptured vesicles it induces during its cellular invasion. With the aim of elucidating the contribution of endocytic vesicle rupture to α-syn cell-to-cell propagation, we sought to examine the extracellular localization and intercellular movement of α-syn aggregates following invasion via rupture, hypothesizing that the damaging consequences of endocytic vesicle rupture would promote pathologic α-syn release in association with ruptured vesicles.

In the sections below, we utilize a combination of approaches to demonstrate that α-syn-induced ruptured lysosomes are targeted for autophagic degradation, that merging of ruptured vesicles containing α-syn promotes LB inclusion formation, and that ruptured vesicles may act as both a stimulus and a vector for α-syn cell-to-cell propagation. These findings illustrate the impact of endocytic vesicle rupture in PD pathology and raise new possibilities for therapeutic targets based on its involvement and consequences.

**Alpha-Synuclein Induces Vesicle Rupture in Human Dopaminergic Neurons Derived From Induced Pluripotent Stem Cells**

Given the consistency of our observation that fibrillar assemblies of amyloid proteins induce endocytic vesicle rupture in recipient cells, we sought to extend our findings by performing the chGal3 relocalization assay in an authentic human dopaminergic neuron to determine the occurrence of endocytic vesicle rupture induced
by α-syn following endocytosis in this model. We hypothesized that α-syn induced vesicle rupture would be a conserved phenotype in multiple cellular model systems. To confirm our observations in a setting more representative of that in human PD, we utilized hiPSC-derived dopaminergic neurons as a model of neuronal physiology. hiPSC-derived dopaminergic neurons stably overexpressed the chGal3 marker of vesicle rupture using lentiviral transduction, and we exposed these cells for 48hr to fragmented WT α-syn PFFs. We observed chGal3 relocalization to distinct puncta, and many chGal3+ ruptured vesicles colocalized with α-syn fibrils fluorescence (Figure 23). This observation demonstrates that α-syn assemblies induce endocytic vesicle rupture in hiPSC-derived dopaminergic neurons, and validates our earlier results obtained in the SY5YchGal3 model of vesicle rupture.

**Lysosomes Ruptured by Alpha-Synuclein are Targeted for Autophagic Degradation**

It is known that recognition of ruptured vesicles by cytosolic galectins targets these vesicles for autophagic degradation [560, 570, 576, 579, 597-600, 602, 603, 615]. However, it remains unclear to what degree α-syn can prevent these ruptured vesicles from re-establishing a low pH during autophagic degradation, and how lysosomal rupture by α-syn affects normal vesicular trafficking in the ALP. We hypothesized that ruptured lysosomes would also become autophagic substrates just like their aggregated α-syn cargo. To more deeply understand the effect of lysosomal rupture on vesicular trafficking and degradation pathways, we first sought to determine the degree to which ruptured lysosomes become incorporated into autophagosomes. In agreement with our
Figure 23. α-Syn induces vesicle rupture in human dopaminergic neurons derived from induced pluripotent stem cells. hiPSC-derived chGal3 dopaminergic neurons were subjected to 48 hr treatment with 200 nM exogenous Dylight488-labeled WT α-syn PFFs in the culture media, followed by live cell imaging to identify vesicle rupture. Treatment with α-syn induced vesicle rupture and subsequent chGal3 relocalization to discrete puncta that colocalized with α-syn fluorescence (arrowheads). Inset box in top left image is enlarged and separated in other panels into Overlay, α-syn fluorescence, or chGal3 fluorescence. Images are representative of at least 10 images from 3 independent experiments. Scale bar = 10 µm for left panel and 3 µm for inset-enlarged panels.
previously published data [4], we observed that chGal3+ ruptured vesicles containing α-syn fibrils colocalized with the lysosomal marker lysosome-associated membrane protein 1 (LAMP1) upon immunofluorescent labeling (Figure 24A). We generated SH-SY5Y cells expressing both the chGal3 marker of ruptured vesicles and a YFP-LC3 construct to identify autophagosomes. Upon exposure of these cells to Dylight650-labeled WT α-syn PFFs for 24 hr in the culture media, we observed that many chGal3+ ruptured vesicles containing α-syn colocalized with YFP-LC3+ autophagosomes, although α-syn-containing ruptured vesicles and autophagosomes existed independently as well (Figure 24B). While these findings indicate that α-syn-induced ruptured lysosomes become targeted to autophagic degradation, they do not allow a real-time monitoring of vesicular pH and membrane integrity throughout the duration of this pathway.

To follow the fate of vesicles ruptured by α-syn in real-time, we performed live cell imaging using the LysoTracker Deep Red dye to label and track acidic organelles in live cells. This fluorescent acidotropic probe selectively accumulates in cellular compartments with low internal pH. Whereas the presence of chGal3 demonstrates that an individual vesicle has been previously ruptured, the accumulation of LysoTracker in ruptured vesicles to mark a low pH indicates re-establishment of a pH gradient through the autophagic degradation system. At single time points, individual Z-stack images were acquired to determine the degree of colocalization of Dylight488-labeled WT α-syn assemblies, chGal3, and LysoTracker. These images revealed that a subset of α-syn+,
Figure 24. Lysosomes ruptured by α-syn are targeted for autophagic degradation. Vesicular identity and pH status of α-syn-induced chGal3+ ruptured vesicles identified by (A) 24 hr exposure of SY5YchGal3 cells to 660nM exogenous Atto488-labeled WT α-syn fibrils followed by fixation and staining for LAMP1, (B) 24 hr exposure of SY5YchGal3 YFP-LC3 cells to 660nM exogenous Dylight650-labeled WT α-syn PFFs followed by live cell imaging, and (C) 24 hr exposure of SY5YchGal3 cells to 200nM exogenous Dylight488-labeled WT α-syn assemblies followed by loading of Lysotracker Deep Red dye and live cell imaging. Inset box from top image is enlarged and separated in below images. Colocalization between all three channels shown with arrowheads. Images are representative of at least 10 images from 3 independent experiments. Scale bar = 15 µm for top panel and 3 µm for inset-enlarged lower panels.
chGal3+ ruptured vesicles were additionally LysoTracker+, indicating a re-establishment of the pH gradient during autophagic degradation of ruptured vesicles. In these cells, instances of α-syn+, chGal3+ vesicles were also observed, as were α-syn puncta that did not colocalize with either chGal3 or LysoTracker (Figure 24C). Additionally, live cell imaging of chGal3-expressing N27 rat dopaminergic neuronal cells exposed to DyLight488-labeled WT α-syn assemblies in the presence of Lysotracker indicated the accumulation of multiple smaller α-syn-containing ruptured vesicles within a larger low pH compartment (Figure 25, arrowheads). This observation indicates ongoing autophagic recruitment of ruptured vesicles induced by α-syn endocytic uptake. These findings demonstrate that lysosomes ruptured by fibrillar amyloid assemblies of α-syn are targeted for autophagic degradation and thus re-establish a low pH gradient. The damaged vesicles and vesicular debris created by lysosomal rupture following α-syn endocytosis therefore add to the cellular burden of substrates needing to be degraded through the ALP.

**Fusion of Ruptured Vesicles Leads to the Formation of Large Cytoplasmic Inclusions of Alpha-Synuclein**

We next used live cell imaging to monitor the intracellular trafficking of vesicles ruptured by α-syn assemblies to determine their relationship with the subsequent formation of cytoplasmic α-syn-containing LB inclusions. Because endocytic vesicle rupture is the damaging mechanism whereby exogenous α-syn seeds come into contact with expressed monomeric α-syn in the cytosol and can subsequently template further
Figure 25. Ruptured vesicles containing α-syn present in large low pH compartment.
N27chGal3 cells were subjected to 24 hr treatment with 200nM exogenous Dylight488-labeled WT α-syn assemblies in the culture media, followed by live cell imaging to identify vesicle rupture. chGal3+ ruptured vesicles (red) can be observed colocalizing with α-syn assemblies (green) and low pH compartments (Lysotracker, blue) in this snapshot of the live cell movie. Additionally, large low pH compartments are seen containing multiple smaller ruptured vesicles that colocalize with α-syn (arrowheads). Scale bar is 10 µm.

aggregation and LB formation [200, 202, 209-213, 347], we hypothesized that endocytic vesicle rupture would be involved in the formation of α-syn LB inclusions. Time lapse
imaging revealed that colocalization between α-syn assemblies and the chGal3 marker of vesicle rupture was maintained as the ruptured vesicles and α-syn cargo trafficked considerable distances throughout the cell. In many instances, colocalizing vesicles could be observed trafficking throughout both the soma and cellular projections of chGal3-expressing hiPSC-derived dopaminergic neurons, SH-SY5Y cells, and N27 cells. We also observed evidence of vesicle fusion in which multiple smaller vesicles containing both α-syn assemblies and chGal3 appear to merge together into a larger inclusion during the acquisition period. In N27chGal3 cells exposed to Dylight488-labeled WT α-syn assemblies and loaded with Lysotracker, multiple smaller low pH compartments each containing α-syn and the chGal3 marker of previous membrane rupture merge together into a larger compartment (Figure 26). This merging of α-syn-containing ruptured vesicles also occurred in SY5YchGal3 cells exposed to Dylight488-labeled WT α-syn assemblies (Figure 27). This merging of ruptured vesicles containing chGal3 and α-syn assemblies produced larger structures that substantially exceeded the size of the individual ruptured vesicles from which they were derived. Such structures were also observed in fixed cell images following exposure of SY5YchGal3 cells to exogenous Dylight-488 labeled α-syn assemblies, and in some cases these larger structures assumed the appearance of a LB in which a large perinuclear α-syn inclusion was surrounded by a peripheral border of chGal3 (Figure 28). This phenotype of chGal3 enrichment at the periphery of α-syn LB inclusions indicates previous membrane rupture in the α-syn-containing endocytic vesicles that subsequently merged together
Figure 26. Fusion of ruptured vesicles containing α-syn in N27chGal3 cells. N27chGal3 cells (red) were incubated with Dylight488-conjugated WT α-syn assemblies (green) for 24 hr. Prior to live cell imaging, cells were incubated with 20 nM Lysotracker (blue) for 30 minutes. Colocalization between these three channels is seen in white. Inset box at top left is enlarged in other panels, and timed snapshots of this region are shown with the time stamp in the bottom left of each frame. Z-stack images were acquired at ~28 second intervals for 12 minutes. Scale bars are 20 µm in top left and 3 µm in panels enlarged from inset box.

into a larger inclusion. Despite the degradative intention behind the merging of vesicles and α-syn aggregates into aggresomes, this process ultimately facilitates LB inclusion
Figure 27. Fusion of ruptured vesicles containing α-syn in SY5YchGal3 cells. SY5YchGal3 cells (red) were incubated with Dylight488-conjugated WT α-syn assemblies (green) for 24 hr. Timed snapshots are shown with the time stamp in the bottom left of each frame. Z-stack images were acquired at ~13 second intervals for 10 minutes. Scale bar = 5 μm.
Figure 28. α-Syn LB inclusion surrounded by corona of chGal3 in SY5YchGal3 cells. SY5YchGal3 cells (red) were subjected to 24h exposure to 200nM exogenous Dylight488-labeled A53T α-syn assemblies (green), followed by fixation and DAPI staining (blue) in PBS. Image separated into overlay, chGal3, and α-syn fluorescence. Scale bar = 10 µm.
formation when degradation becomes compromised [118]. Gal3 presence around the LB is an indicator of the accumulation of ruptured vesicles unable to be degraded in aggresomes. These findings highlight the close relationship between ruptured vesicles and α-syn LB inclusions, where fusion of α-syn-containing ruptured vesicles and the specific enrichment of chGal3 surrounding a larger LB inclusion implicate endocytic vesicle rupture in the formation of pathologic intracellular α-syn inclusions.

Vesicle Rupture Markers Surround Alpha-Synuclein in Lewy Bodies

The data above suggest that the failed degradation of α-syn fibrils within the autophagic compartment following vesicle rupture leads to the gradual fusion of these vesicles into structures resembling LBs. To determine the influence of endocytic vesicle rupture on LB inclusion formation in authentic PD, we examined α-syn-containing LBs in human post mortem SN tissue sections from PD patients, hypothesizing that endogenous Gal3 localization would also implicate endocytic vesicle rupture in LB formation in vivo. To determine if LBs present in PD patients exhibit a similar phenotype to that in SY5YchGal3 cells exposed to exogenous α-syn assemblies, we performed immunofluorescence microscopic analysis on SN sections, staining these sections with antibodies specific for pS129 α-syn as a marker of LBs and endogenous Gal3, detected by secondary antibodies conjugated to Cy2 and Cy5, respectively. In these sections, we observed numerous examples of pS129 α-syn+ inclusions that closely resembled LBs. Many of these LBs demonstrated a characteristic peripheral outward-radiating filamentous halo of pS129 α-syn and a dim center, consistent with other published
reports detailing the ultrastructure of LBs where α-syn staining comprises a peripheral halo surrounding a dense granular core with a high abundance of ubiquitin staining [117, 118]. Additionally, we observed that a majority of the LBs present in these sections exhibited a corona of Gal3 surrounding the aggregated pS129 α-syn within the LB (Figure 29). Of the 305 LBs identified in samples from 5 PD patients, 170 (~56%) of these LBs exhibited a discernable Gal3 corona. No similar fluorescent pattern was observed in the neighboring fluorescent channel (Cy3) under identical acquisition conditions or in tissue sections from the same patient stained with secondary antibodies alone, demonstrating that this observation is not due to signal bleedthrough or tissue autofluorescence. The presence of Gal3 at the periphery of LBs suggests that this region contains membrane components where the lectin ligands present on the luminal leaflet of vesicles had been previously exposed to the cytoplasm. Notably, normal autophagic degradative mechanisms do not result in the cytoplasmic exposure of these lectin ligands. Thus, the Gal3 corona around LBs is evidence of previous membrane perturbations having occurred in the lipid components which form the periphery of these structures. This observation implicates endocytic vesicle rupture in the formation of LBs in authentic PD, meaning that the cellular response of merging ruptured vesicles and α-syn cargo to facilitate degradation instead promotes pathologic inclusion formation when degradative mechanisms are dysfunctional.
**Figure 29. LB inclusions from PD brain surrounded by corona of Gal3.** Brain sections from H&Y5 PD patients were immunostained for pS129 α-syn (FITC, green) and Gal3 (Cy5, red), and additionally imaged in the Cy3 channel to detect tissue autofluorescence (blue). Six representative images of LBs are shown from three PD patients, and left Overlay panel is separated into single channels for α-syn fluorescence, Gal3 fluorescence, and autofluorescence to appreciate the degree of partial colocalization between Gal3 corona and radiating halo of pS129 α-syn within LBs. Scale bar = 5 µm.
Ruptured Vesicles Containing Alpha-Synuclein are Released and Propagate Between Cells

In addition to the intracellular consequence of promoting pathologic LB inclusion formation, endocytic vesicle rupture is also known to induce cellular stressors that can stimulate \( \alpha \)-syn release and cell-to-cell propagation [294, 468-471, 473, 479, 484, 485]. With this fact in mind, we aimed to clarify the contribution of \( \alpha \)-syn induced endocytic vesicle rupture to its cell-to-cell propagation. We hypothesized that \( \alpha \)-syn-mediated lysosomal rupture and its consequences would prompt \( \alpha \)-syn cellular release in association with ruptured vesicles. To test this hypothesis, we examined both the extracellular localization and the intercellular movement of exogenous \( \alpha \)-syn aggregates using a combination of fixed and live cell imaging. Under identical conditions to those that produce intracellular vesicle rupture following endocytosis of exogenous \( \alpha \)-syn aggregates, we observe instances where chGal3+ ruptured vesicles containing \( \alpha \)-syn are present in the extracellular environment (Figure 30). Given that this now extracellular \( \alpha \)-syn aggregate remains associated with a lipid vesicle exhibiting an enrichment of chGal3 derived from the cytosol of the original recipient cell, this finding indicates the existence of a process for the cellular release of \( \alpha \)-syn-containing ruptured vesicles. Using live cell imaging of hiPSC-derived chGal3 dopaminergic neurons exposed to Dylight488-labeled \( \alpha \)-syn PFFs, we have also observed the intercellular movement of \( \alpha \)-syn aggregates contained within ruptured vesicles (Figure 31), indicating that this process of \( \alpha \)-syn cellular release within ruptured vesicles can facilitate cell-to-cell propagation. While
Figure 30. Ruptured vesicles containing $\alpha$-syn observed in the extracellular environment. Annular chGal3+ ruptured vesicle containing $\alpha$-syn found in the extracellular environment adjacent to SY5YchGal3 cell affected by $\alpha$-syn-induced vesicle rupture. SY5Y chGal3 cells were treated for 24 hr with Dylight488-labeled $\alpha$-syn aggregates. Highlighted extracellular vesicle within inset box is not connected to any portion of adjacent cell or its processes. Inset box in merged panel (left) is enlarged and separated by channel (right) to emphasize co-localization. Blue DAPI-stained nucleus shown. Scale bar = 10 $\mu$m left, 1.5 $\mu$m right.

much remains to be understood regarding the specific factors that stimulate this release of ruptured vesicles and $\alpha$-syn cargo as well as its mechanistic underpinnings, these
Figure 31. Ruptured vesicles containing α-syn propagate between hiPSC-derived chGal3 dopaminergic neurons. hiPSC-derived chGal3 dopaminergic neurons (red) were subjected to 48 hr treatment with 200 nM exogenous Dylight488-labeled WT α-syn PFFs (green) in the culture media, followed by live cell imaging to identify vesicle rupture. Ruptured vesicles containing α-syn seen in yellow. Inset box enlarged at bottom right, with timed snapshots shown at the times indicated in the bottom right of each frame. Scale bar = 15 µm.

results indicate that endocytic vesicle rupture and its consequences can act as both a stimulus and a vector for pathologic α-syn cell-to-cell propagation.

Significance

The above results have established the contribution of endocytic vesicle rupture to dysfunctional degradative processes, intracellular inclusion formation, and intercellular aggregate transmission, making this damaging mechanism of cellular
invasion clearly relevant for PD pathology and progression. Although much remains to be determined regarding the life cycle of α-syn within and between diseased neurons, this examination of the intersection of endocytic vesicle rupture and established aspects of PD pathology brings the research endeavor one step closer to an understanding of pathogenesis necessary for effective therapeutic intervention. Beginning with the demonstration that α-syn aggregates induce endocytic vesicle rupture in hiPSC-derived chGal3 dopaminergic neurons just as they do in SH-SY5YchGal3 cells verifies the occurrence of this invasion mechanism in a model system more representative of neuronal physiology. This validation establishes the utility of the chGal3 relocalization assay for detecting this vesicle damage, and again underscores the conserved nature of endocytic vesicle rupture as a mechanism of amyloid protein escape from the vesicular compartment during the propagation pathway. Furthermore, these findings confirm that, just like those ruptured by invading pathogens or sterile damage [560, 570, 597, 601-603, 615], lysosomes ruptured by α-syn become targets of autophagy, adding to the cellular burden of substrates requiring clearance via the ALP. The combination of lysosomal dysfunction induced by α-syn mediated LMP [4] and the addition of damaged or destroyed lysosomal vesicles to the already overwhelming buildup of ALP substrates in PD [130] that now also include the invading misfolded α-syn aggregates themselves is a clear cause of dysregulated proteostasis.

Two of the most problematic aspects of proteinopathies include the prion-like seeding and templating of further amyloid misfolding upon cellular invasion and the cell-
to-cell propagation of amyloid aggregates [3, 465, 647, 648]. With our findings, we have established a role for endocytic vesicle rupture as contributing to both. We first examined the trafficking of ruptured vesicles and their α-syn cargo, and noted the merging or coalescence of multiple smaller ruptured vesicles into larger low pH compartments representing ongoing autophagic degradation. However, the presence of Gal3 at the periphery of LB inclusions in SY5YchGal3 cells and in PD SN brain tissue suggests that degradative mechanisms fail to cope with the accumulation of ruptured vesicles and misfolded protein, which instead results in LB inclusion formation. Moreover, understanding that the increased lysosomal, autophagic, and oxidative stress resulting from endocytic vesicle rupture is known to contribute to α-syn release and intercellular spreading [468, 469, 479, 485], we identified a process of α-syn cellular release within ruptured vesicles that is competent to shuttle α-syn aggregate cargo between cells. These observations implicate the mechanism of endocytic vesicle rupture as contributing to inclusion formation and cell-to-cell propagation in PD, providing both the substance and the stimulus for both pathological processes. We have provided an explanation for how the damaging mechanism of cellular invasion through endocytic vesicle rupture directly contributes to PD pathology and progression, and these findings are significant because the multifaceted nature of this invasion mechanism and its consequences could provide numerous new therapeutic targets for reversing or preventing disease.
CHAPTER SIX

CONCLUSIONS

Introduction

No disease modifying therapy exists for numerous neurodegenerative diseases characterized and defined by the accumulation of misfolded amyloid proteins. α-Synucleinopathies, tauopathies, and polyglutamine expansion diseases each encompass several diverse neurodegenerative diseases exhibiting the accumulation of insoluble fibrillar amyloid aggregates of α-syn, tau, and polyglutamine-containing proteins respectively. These diverse proteinopathies have also been referred to as prionopathies for their numerous similarities to prion diseases [42]. As prion-like proteins, amyloid assemblies of α-syn, tau, and HTTExon1 exhibit misfolding into an insoluble, fibrillar β-sheet-rich amyloid structure, induce seeding and templating of additional misfolding in their cytosolic monomeric constituent proteins, and undergo spreading from affected to naïve cells throughout the nervous system [488, 522, 634]. Given that the prion-like mechanisms for amyloid protein aggregation and propagation are remarkably conserved between neurodegenerative diseases, and even that the fibrillar amyloid structure itself occurring as a final product of constituent assembly is structurally very similar in these diseases despite being composed of different proteins, there is a possibility that understanding the mechanisms underlying a single disease may lead to insights about
many, forming the foundation for future disease-modifying therapeutic strategies [3].

To realize this potential, much remains to be learned about the mechanisms by which misfolded amyloid aggregates corrupt their soluble counterparts, disrupt cellular homeostasis, and transmit their pathologic protein misfolding from cell-to-cell.

Examining the specific steps in the intracellular and intercellular life cycle of amyloid proteins through the lens of host-pathogen interactions is beneficial for understanding the consequences of and cellular response to this prion-like process. Using the situation of α-syn in PD, our group was the first to demonstrate that fibrillar protein aggregates cause lysosomal rupture following their endocytic uptake, occurring in a fashion identical to infection by bacterial or viral pathogens [4, 280]. Despite the new insight gained from this finding, many key questions remained about its mechanism, its contribution to cellular dysfunction, and its relevance to the pathology of PD and other proteinopathies. The studies discussed in this dissertation aimed to address the above questions, with the goal of exploring endocytic vesicle rupture as a mechanism of cellular invasion for several amyloid proteins. Furthermore, focusing on α-syn in PD, we aimed to define specific protein modifications that influence vesicle rupture potency, as well as determine the effect of α-syn-mediated vesicle rupture on cellular homeostasis and aggregate propagation. Below we revisit key findings identified in each chapter and discuss how they contribute to an enhanced understanding of amyloid protein propagation in neurodegenerative disease. We also point out questions that remain unanswered and needing to be addressed in future work. With more insight
into the pathological life cycle of misfolded amyloid proteins, new therapeutic targets are identified that may allow future treatment targeted to disease pathogenesis.

**Endocytic Vesicle Rupture is a Conserved Mechanism of Cellular Invasion by Amyloid Proteins**

Building from our earlier work demonstrating that heterogeneous aggregates of α-syn rupture lysosomes following their endocytic uptake, we next sought to determine if this endocytic vesicle rupture phenomenon is a specific effect of α-syn aggregates, or if aggregates of other neurodegenerative disease-associated amyloid proteins were also capable of inducing this type of vesicular membrane damage following endocytosis. The rationale for this hypothesis was that misfolded higher-order aggregates of many aggregation-prone neurodegenerative disease-associated proteins adopt a stable, protease-resistant, detergent-insoluble fibrillar β-sheet rich architecture known as amyloid that is very similar despite these protein aggregates being composed of different proteins for each disease [2, 3, 465, 647, 652]. This characteristic amyloid structure also defines the disease-associated prion PrP
t{\textsuperscript{Sc}} [6, 11, 12], and it is this structure that imparts to prions the ability to corrupt like monomeric constituents through templating and seeding of further aggregation [17]. Thus, given the similarities in their structure, we hypothesized that amyloid assemblies of tau and HTTExon1 would also induce endocytic vesicle rupture following their cellular uptake by endocytosis.

Endocytosis is the predominant mechanism for the cellular uptake of fibrillar assemblies of α-syn, tau, and HTTExon1, being too large for direct translocation across
the plasma membrane [448-450, 498, 506-508, 531]. However, if these fibrillar amyloid proteins are very elongated and/or tightly bundled with many other fibrils due to ongoing aggregation, then these elongated or grouped fibrils are too large to enter cells via endocytosis. Instead, smaller fibrillar forms such as fragments of longer α-syn, tau, or prion fibrils are the assembly species most efficiently taken up by endocytosis [45, 200, 509]. Following cellular entry in this manner, the question of how amyloid aggregates escape from the vesicular compartment was unanswered for assemblies of tau and HTTExon1 just as it had been for α-syn prior to our demonstration of α-syn induced vesicle rupture [448, 465, 522, 531]. To properly assess this possibility of endocytic vesicle rupture representing a shared invasion mechanism for these various aggregated amyloid proteins, we employed an in vitro aggregation protocol of recombinant purified α-syn, tau, and HTTExon1 that relies on the combination of extended aggregation times to produce elongated fibrils and the subsequent fragmentation of these elongated fibrils by sonication to produce homogenous populations of smaller fibrillar assemblies that retain their original β-sheet-rich architecture that had been present in the elongated fibril after aggregation. Not only does this aggregation scheme allow for a homogenous, uniform population of similar size distribution between samples of α-syn, tau, and HTTExon1 (Figures 14, 17, 20), standardizing the variable of aggregate size for the comparison of vesicle rupture, the fragmentation of larger fibrils to smaller fibrillar forms matches the species of amyloid proteins known to best invade recipient cells through endocytosis.
When we incubated small fibrillar forms of α-syn, tau, and HTTExon1 with our SY5YchGal3 cell model of vesicle rupture, we observed a shared ability of these fibrillar amyloid proteins to induce chGal3 relocalization indicative of endocytic vesicle rupture (Figures 15, 18, 21). The specific enrichment of cytosolic chGal3 at the site of ruptured vesicles colocalized strikingly with the fluorescence from internalized amyloid assemblies of α-syn and tau, although we were unable to visualize this colocalization of ruptured vesicles with assemblies of HTTExon1 due to technical limitations of the fluorescent labeling approach. To extend and validate the observation of amyloid-induced endocytic vesicle rupture in a more physiologically-relevant model system, we utilized hiPSC-derived dopaminergic neurons expressing the chGal3 construct as more closely resembling authentic neuronal physiology. In this system, hiPSC-derived dopaminergic neurons that successfully expressed the chGal3 protein indicated clear relocalization of its expression to cytosolic punctate structures that colocalized with the fluorescence of exogenous α-syn PFFs, indicating the induction of endocytic vesicle rupture in these neurons just as in SY5YchGal3 cells (Figure 23). These findings allow us to confidently conclude that endocytic vesicle rupture is the mechanism by which fibrillar assemblies of amyloid proteins escape from the vesicular compartment following their uptake, and the recruitment of cytosolic chGal3 is a reliable indicator of this damage caused during invasion (Figure 32, middle).

The pattern of colocalization between exogenous fluorescently-labeled amyloid protein assemblies and the chGal3 indicator of vesicle rupture warrants further
Figure 32. Schematic of amyloid-induced vesicle rupture and its consequences. Fibrillar amyloid protein assembly (green) taken up into endocytic vesicle along with β-galactoside sugars (orange) from the outer leaflet of the plasma membrane now on the inner leaflet of the internalized vesicle. Endocytic vesicle rupture induced by amyloid protein aggregates is indicated by the enrichment of cytosolic Gal3 (red cytoplasm, red dots) at the site of membrane damage where it binds to β-galactoside sugars exposed upon vesicle rupture (middle). Colocalization (yellow) can be observed between amyloid assembly fluorescence and chGal3 in inset middle image. Cellular homeostasis depends on the balance between amyloid protein degradation (left) and amyloid protein accumulation (right). Degradation of ruptured vesicles and amyloid protein cargo occurs via autophagy (left), where ruptured vesicles containing α-syn can be observed in low pH compartments positive for the autophagic marker LC3 (inset left images). Accumulation of ruptured vesicles and amyloid protein cargo occurs when degradative mechanisms are dysfunctional or overwhelmed (right), prompting both inclusion formation, where the presence of Gal3 at the periphery indicates merging of previous ruptured vesicles into the larger cytoplasmic inclusion (inset right images), and cellular release of ruptured vesicles and amyloid protein cargo.
We often observe localization of fluorescent amyloid assemblies to curved or comma-shaped structures at the periphery of larger annular or ring-like intracellular ruptured vesicles, closely associated with the vesicular membrane rather than occupying the visible vesicular lumen (Figure 32, inset middle image). In addition, smaller punctate or dot-like structures are also observed, where more exact overlap between amyloid assembly and chGal3 fluorescence is observed (Figure 32, inset middle image). While both phenotypes of intracellular vesicles exhibit rupture as indicated by chGal3 enrichment, their dramatically different appearances suggest involvement of diverse intracellular vesicle phenotypes in the process of endocytic vesicle rupture. Our previous work demonstrated vesicle rupture of endosomes and lysosomes by α-syn aggregates, with a higher number of ruptured vesicles exhibiting a lysosomal phenotype [4]. The results presented here validate the observation of lysosomal rupture, and demonstrate that ruptured lysosomes are recruited to autophagosomes for degradation (Figure 24). The chGal3 relocalization assay does not allow us to distinguish if one vesicular phenotype is more predominantly affected by amyloid-mediated vesicle rupture, as the recruitment of cytosolic galectins is an all-or-none phenomenon that occurs immediately following rupture and the presence of this indicator would persist as vesicles move through intracellular trafficking and degradative pathways. Thus, additional study must be performed to more completely understand the stage within the endocytic pathway when endocytic vesicle rupture is induced by exogenous amyloid assemblies. This question has important implications for the consequences of amyloid-
mediated vesicle rupture, as early egress from the endocytic pathway during the endosomal stage is employed by pathogens such as adenovirus which have an evolutionary pressure to avoid lysosomal degradation [575, 606, 609]. Early endocytic vesicle rupture thus causes fewer LMP events and avoids the more severe proteolytic toxicity and oxidative stress resulting from cathepsin leakage. It is possible that various amyloid protein assemblies induce vesicle rupture at different endocytic stages, and thus would be expected to have very different consequences for the host cell.

With the finding that endocytic vesicle rupture is a conserved mechanism of cellular invasion by assemblies of numerous amyloid proteins, we have contributed an important advance in the understanding of the amyloid cell-to-cell propagation pathway. Although the existence of a mechanism to physically disrupt the endocytic vesicle compartment had been hypothesized, and always assumed to exist given the induction of intracellular aggregation by exogenous seeds, such a mechanism had never been specifically demonstrated for aggregates of α-syn, tau, and HTTExon1 prior to this work. Future studies will expand these observations and the understanding of the endocytic vesicle rupture entry mechanism by examining the cell-to-cell propagation pathway of additional amyloid proteins such as Aβ, SOD-1, and TDP-43. Probing this cellular invasion mechanism for specific factors that increase or decrease its potency may reveal mechanisms whereby it can be inhibited, and thus represent attractive future therapeutic targets to prevent or reverse disease progression.
Given the conserved nature of endocytic vesicle rupture in the cellular invasion of amyloid proteins during neurodegenerative disease, we next sought to determine specific protein modifications that dictate the potency of this cellular entry mechanism. We focused this effort on amyloid assemblies of α-syn in PD, examining familial missense mutation, pS129 phosphorylation, and strain conformation as three factors highly implicated in disease pathogenesis. An increased understanding of the vesicle rupture entry mechanism from the perspective of factors that increase or decrease its potency may generate new therapeutic targets to intervene on amyloid protein cell-to-cell propagation.

We began by investigating the influence of missense mutations in the α-syn protein associated with familial, early-onset PD, with the hypothesis that an increased potency of endocytic vesicle rupture induced by amyloid assemblies of these mutants may account for their earlier disease onset. Although only 10% of PD cases have a genetic cause and the remaining 90% are idiopathic cases [229, 230], examination of α-syn familial missense mutants is valuable because the onset of PD is directly attributable to alterations in the α-syn protein, and insights gained from examining these forms of α-syn may lead to new information about the behavior of WT α-syn becoming misfolded and causing toxicity in the vast majority of PD cases. When we produced aggregates of WT, E46K, A53T, A30P, and G51D α-syn in vitro from recombinant purified protein
(Figure 9), and exposed target SY5YchGal3 cells to these exogenous aggregates in the culture media, we noted clear vesicle rupture as indicated by the colocalization of \(\alpha\)-syn aggregates with distinct chGal3 puncta at the site of vesicle damage (Figure 10). Quantification of this phenomenon revealed an identical propensity to induce vesicle rupture between WT and familial mutant \(\alpha\)-syn (Figure 11), indicating that individual amino acid missense mutations do not alter the structural characteristics of the resulting amyloid aggregate enough to influence its vesicle rupture potency. Although this finding again emphasizes the conserved nature of endocytic vesicle rupture induced by a variety of amyloid protein assemblies, we were unable to observe a significant change in vesicle rupture potency with assemblies composed of any missense mutant form of \(\alpha\)-syn. These findings are limited, though, in the preparation scheme utilized to generate amyloid aggregates. The intermediate aggregation period and lack of fragmentation utilized in these studies results in a heterogeneous population of aggregates that may contain large, small, or even monomeric forms of \(\alpha\)-syn. Future studies will examine the impact of familial disease-associated \(\alpha\)-syn missense variants in the context of assembly populations of a homogenous size distribution.

Given the preferential and consistent upregulation of S129 phosphorylation in PD [260], and the fact that 90% of \(\alpha\)-syn in pathological LB inclusions is phosphorylated at S129 compared to less than 5% of soluble \(\alpha\)-syn exhibiting this PTM [119-122], we next investigated the influence of S129 phosphorylation on endocytic vesicle rupture potency alone or in combination with \(\alpha\)-syn familial missense mutations. Despite the
prevalence of this α-syn PTM, its influence on α-syn pathology is still poorly understood. Even the question of whether S129 phosphorylation is a cause or a consequence of α-syn toxicity is unanswered. We reasoned that S129 phosphorylation would affect aggregate structure, membrane binding, or both in such a way that increases the endocytic vesicle rupture potency of assemblies exhibiting this PTM. To address this hypothesis, we generated aggregates of recombinant purified WT, A30P, and A53T α-syn that had been expressed in *E. coli* with or without PLK2, which induces specific phosphorylation only at S129. Incubation of recipient SY5YchGal3 cells with these exogenous aggregates results in clear induction of endocytic vesicle rupture as shown by relocalization of chGal3 to the site of vesicle rupture and colocalization with α-syn fluorescence (Figure 12). Quantification of this experimental data, which had been acquired and analyzed in a blinded fashion, revealed a consistent 50% increase in vesicle rupture potency induced by S129 phosphorylation (Figure 13). Our observation that S129 phosphorylation increases the potency of endocytic vesicle rupture, combined with the specific enrichment of this PTM in pathologic LB inclusions, raises the possibility that these two events are linked, and that increases in endocytic vesicle rupture potency by pathologic pS129 α-syn increases the likelihood of LB inclusion formation. Whether S129 phosphorylation occurs to cause α-syn pathological behavior or simply results from already increased pathological characteristics, once α-syn aggregates become phosphorylated, before or after their assembly, S129 phosphorylation induces more toxicity because of increased vesicle rupture potency.
Finally, given the fact that distinct α-syn strain conformations underlie the tremendous heterogeneity of disease phenotypes in the α-synucleinopathy diseases PD, DLB, and MSA [304], we next examined the influence of α-syn strain conformation on endocytic vesicle rupture potency with the hypothesis that differences in rupture potency induced by distinct α-syn strains may result in strain-specific differences in disease phenotype. Distinct α-syn strains exhibiting different structures, levels of toxicity, and seeding and propagation properties have been generated in vitro by varying the conditions of aggregation [218, 305-310, 315], and the existence of distinct strains in vivo has been verified with PD and MSA samples [312-314]. Strain diversity is encoded by variable conformations of PrP, α-syn, tau, and HTTExon1 in their respective proteinopathies, and these differences may account for differences in disease pattern as even slight changes to aggregate conformation may have a big impact on biological behavior. We hypothesized that variable strain conformations exposing different surfaces and exhibiting distinct physical properties would dictate membrane association and subsequent endocytic vesicle rupture potency. To test this hypothesis, distinct α-syn strains generated in vitro from WT α-syn and fragmented to produce smaller sized fibrillar assemblies (Figure 14) were used as a treatment for recipient SY5YchGal3 cells, and their relative abilities to induce vesicle rupture were quantified. We observed vesicle rupture in response to endocytosis of all fibrillar strains of α-syn examined, although vesicle rupture was not observed in response to treatment with α-syn monomers or oligomers (Figure 15). Quantification revealed significant increases in
vesicle rupture induced by α-syn fibrils, fibrils-65, and fibrils-91, but α-syn ribbons were unable to induce a significant increase in vesicle rupture compared to unexposed cells (Figure 16) despite observing instances of α-syn ribbons fluorescence colocalizing with chGal3 puncta just as for other α-syn fibrillar strains. These findings indicate that the fibrillar nature of α-syn assemblies is necessary but not sufficient for the ability to induce vesicle rupture, and the intrinsic conformation of α-syn fibrillar polymorphs dictates endocytic vesicle rupture potency. Because α-syn fibrils have been shown to induce higher levels of toxicity in vitro [305] and in vivo [218] compared with α-syn ribbons, and these two polymorphs yield different synucleinopathies when injected into recipient animals [218], we believe that differences in endocytic vesicle rupture potency may account for strain-specific differences in disease pathology. Additionally, in the setting of endocytic vesicle rupture induced by multiple isoforms of full-length WT tau, we observed a significant increase in vesicle rupture potency for tau isoform 1N4R compared with tau isoform 1N3R (Figure 19), indicating that vesicle rupture induction may also underlie phenotypic variability among tauopathies just as for α-synucleinopathies. Finally, in an intriguing turn of events, since the completion of our work examining the effect of S129 phosphorylation on vesicle rupture potency, another study found that pS129 α-syn assembles into a unique strain conformation distinct from non-pS129 α-syn [316], offering an explanation for our observed increase in vesicle rupture potency with S129 phosphorylation based on the existence of a unique strain conformation that may be capable of more potent endocytic vesicle rupture induction.
Our work examining the mechanism of endocytic vesicle rupture induced by fibrillar amyloid protein assemblies forms the basis for the established induction of seeding and templating of expressed proteins by invading exogenous assemblies. In the life cycle of amyloid protein cell-to-cell propagation, exogenous amyloid assemblies need to break out of the endocytic vesicle to access the cytosolic “replication machinery”, additional monomeric constituent building blocks. Such a mechanism is deviously employed by intracellular bacteria and viruses, which escape the vesicular compartment and evade cellular defense responses, sometimes even using these responses to their advantage [572]. Bacteria and viruses have an evolutionary pressure to develop these characteristics or else face extinction. Aggregated amyloid proteins do not have this evolutionary pressure or agenda. These proteins are pulled away from their endogenous roles when they become misfolded and aggregated, and their accumulation results in highly-stable protein aggregates whose behavior is dictated only by the environmental conditions they experience. Seeding of additional monomeric misfolding is part of the definition of prion-like proteins, and this seeding process cannot occur unless the exogenous amyloid seed contacts intracellular naïve monomeric constituents to serve as a template for further misfolding. While many groups have separately established the importance of both uptake by endocytosis [448-450, 498, 506-508, 531] and induction of intracellular seeding [18, 19, 21, 197, 200-202, 212, 213, 347, 478, 490, 498, 510, 525, 526, 534], these two essential aspects of the amyloid protein propagation pathway have never been linked [448, 465, 522, 531]. No study,
until our prior work [4], had directly assessed the ability of amyloid proteins to escape
the endocytic compartment. Additionally, building on these previous findings, the
specific aggregate species involved and the molecular determinants of rupture potency
remained to be determined. We have established that short fragments of fibrillar
amyloid assemblies represent the aggregate species most potent for inducing vesicle
rupture, perfectly aligning with the prevailing view that these same species most easily
enter recipient cells via endocytosis [45, 200, 509], most effectively disrupt artificial
liposomes [557], most potently induce seeding [22-25, 209-211, 525, 527, 653] and
toxicity [129, 389-393], and most efficiently propagate from one cell to another [202,
203, 212, 213, 219, 439, 441, 442]. These findings add endocytic vesicle rupture to the
list of characteristics of prion-like amyloid proteins, with its amyloid structure imparting
this function just as it does for seeding/templating and propagation. Due to the
conserved nature of endocytic vesicle rupture for the cellular entry of amyloid protein
assemblies from multiple neurodegenerative diseases, there may be a shared
therapeutic approach that could have a broad impact.

**Affected Cells Target Ruptured Vesicles and Alpha-Synuclein Cargo to Autophagy**

Knowing that endocytic vesicle rupture represents the mechanism for the
cellular invasion of exogenous amyloid assemblies, we next sought to examine the
impact of this vesicle damage on vesicular trafficking and degradation processes.
Although we have exploited their properties as PRRs, recognizing exposed β-galactoside
sugars abnormally present in the cytosol [570, 571], for the fluorescent microscopic
detection of endocytic vesicle rupture events[619, 620], cytosolic galectins such as Gal3 and Gal8 play an integral role in degradative and innate immune defense responses within cells affected by endocytic vesicle rupture [570, 579, 587-589, 602, 603]. In the context of infectious pathogens or sterile damage that cause endocytic vesicle rupture in an identical fashion to that induced by invading amyloid protein assemblies, it is known that ruptured vesicles become recognized by the autophagic machinery for degradation [570, 576, 579, 597-600, 603], and as a result of recruitment to the ALP can subsequently recover a low pH despite their previous rupture [560, 615]. We examined the situation of α-syn-induced endocytic vesicle rupture to learn if this process of autophagic recruitment of ruptured vesicles also occurs in the context of α-syn invasion, or if ongoing membrane damage would prevent autophagic recruitment and re-establishment of a low pH environment. To do this, we utilized immunofluorescent detection of the lysosomal marker LAMP1 in addition to live cell imaging of YFP-labeled LC3 and the identification of low pH compartments labeled by Lysotracker in SY5YchGal3 cells exposed to α-syn assemblies. We confirmed our previous finding that lysosomes are ruptured by α-syn aggregates [4] by demonstrating colocalization of chGal3+ ruptured vesicles containing α-syn with LAMP1 (Figure 24). Furthermore, we also confirmed that, like ruptured vesicles caused by invading pathogens or sterile damage, lysosomes ruptured by α-syn aggregates are recruited to LC3+ autophagosomes and re-establish a low pH gradient (Figure 24, 25). This recruitment of damaged vesicles and α-syn cargo to the ALP counteracts accumulation of misfolded
proteins and dysfunctional organelles (Figure 32, left), but would also be expected to add to the overall burden of substrates needing to be cleared through this degradative mechanism, causing an increased workload for this system to maintain proteostasis. Future studies will examine the signaling cascades that mediate this targeting of ruptured vesicles to the ALP, and determine if enhancing their detection and recruitment and/or boosting the overall degradative capacity of the pathway may have therapeutic efficacy for tipping the scale back in the direction of equilibrium.

**Endocytic Vesicle Rupture Can Promote Inclusion Formation**

As the mechanism for exogenous pre-formed amyloid assemblies to trigger the aggregation and intracellular inclusion formation of expressed naïve constituents, endocytic vesicle rupture delivers the seed to the cytoplasm where it continues its prion-like cascade. Additionally, rupture of lysosomes by invading amyloid assemblies directly impairs lysosomal degradation by causing cathepsin leakage and produces oxidative stress [4]. Given these roles, endocytic vesicle rupture is uniquely positioned to provide both the substance, misfolded seeds and damaged vesicles, and the stimulus, degradative dysfunction, for pathologic proteinaceous inclusion formation. The prevailing hypothesis regarding the formation of LB inclusions of α-syn is that this process is related to the formation of aggresomes, agglomerations of degradative substrates occurring as a protective mechanism of the cell to more efficiently facilitate degradation [118, 124]. This observation is supported by numerous demonstration of LBs as clusters of vesicles containing lysosomal and autophagic markers and proteolytic
enzymes [128-135]. Additionally, activation of autophagy can facilitate the dissolution of larger inclusions, increasing the number of smaller cytoplasmic aggregates [327].

Alternatively, mature fibrillar inclusion bodies are known to be refractory to lysosomal or autophagic clearance [134, 334], exemplified by the buildup of dysfunctional lysosomes and autophagosomes [131, 339, 346]. When degradative mechanisms are dysfunctional, the merging of substrates into aggresome-like structures instead facilitates LB inclusion formation [118, 124] (Figure 32, right). Given the nature of vesicles ruptured by α-syn as targeted for autophagic degradation just like their misfolded protein aggregate cargo, and the contribution of α-syn-mediated LMP to degradative dysfunction, we reasoned that endocytic vesicle rupture may contribute to pathological inclusion formation. To test this hypothesis, we examined intracellular trafficking of ruptured vesicles for the presence and formation of intracellular inclusions, and investigated the localization of endogenous Gal3 in PD post-mortem SN tissue. Live-cell imaging of ruptured vesicles containing α-syn demonstrated instances where multiple smaller ruptured vesicles merged into larger low pH inclusions (Figure 25, 26, 27), and large perinuclear inclusions of exogenous α-syn identified by fixed cell imaging exhibited a corona of chGal3 fluorescence around the periphery of the inclusion, indicating rupture in the vesicles that merged to create the inclusion (Figure 28, 32 right inset image). Finally, immunofluorescent detection of pS129+ LBs and endogenous Gal3 in the post-mortem SN of PD patients also demonstrated Gal3 localization as a corona surrounding pathological LB inclusions, indicating a history of previous membrane
rupture as preceding LB formation (Figure 29, 32 right inset image). The observation that Gal3 is trapped within the majority of LBs (≥55%) in the brains of PD patients suggests that these proteinaceous inclusions do not originate solely from the aggregation of expressed, misfolded α-syn, but rather have as their origin the invasion of exogenous α-syn fibrils through endocytic vesicle rupture. Indeed, the trafficking of exogenous fibrils from affected to naïve cells, either naked or encapsulated within intracellular vesicles, would lead through vesicle rupture to their accumulation into LBs and the trapping of Gal3 within these LBs. While these findings demonstrate that endocytic vesicle contributes to pathological inclusion formation in authentic PD, we have, to date, only characterized LB inclusions in 5 separate patients, all of whom were in a very advanced H&Y5 stage of PD. Future studies will expand this work by including more patient-derived samples from various disease stages and brain regions, and focus on characterizing the involvement of vesicle rupture in the stages of LB development in various brain regions. Also, pathological examination of endocytic vesicle rupture in fetal dopaminergic graft tissue implanted into the brains of PD patients would provide an elegant way to focus our analysis exclusively on α-syn pathology arising from exogenous amyloid aggregates, as young fetal dopaminergic grafts would not be expected to develop LB pathology spontaneously. The finding that ~55% of SN LBs exhibit a discernable Gal3 corona that indicates vesicle rupture may increase if the source of LB pathology is exclusively exogenous, and would need to induce endocytic vesicle rupture to trigger inclusion formation in recipient cells.
Endocytic Vesicle Rupture Can Promote Alpha-Synuclein Cell-to-Cell Propagation

Many of the same stimuli prompting intracellular inclusion formation, such as lysosomal or autophagic dysfunction and oxidative stress, are also known to promote the pathological release and cell-to-cell transmission of amyloid protein aggregates [294, 468-471, 473, 479, 485, 518] (Figure 32, right). We aimed to determine if endocytic vesicle rupture could fulfill this criterion as well. Interestingly given the association of the invading amyloid aggregate with an, albeit ruptured, intracellular vesicle, there have been numerous mechanisms of non-classical secretion proposed for both α-syn and Gal3 that raise the possibility that α-syn aggregates may be released into the extracellular environment through fusion of intracellular vesicles with the plasma membrane or through release of vesicles as exosomes [468-473, 478, 480, 591-593]. These types of non-classical release mechanisms have also been demonstrated for amyloid assemblies of tau and HTTExon1 [444, 516-519]. Interestingly, prions and amyloid assemblies of α-syn, tau, and HTTExon1 have each been shown to traffick from affected to naïve cells within TNTs, and also to upregulate the cellular expression of TNTs to facilitate their own transmission [47, 48, 445, 520, 521, 535]. In the case of α-syn, TNT-mediated transmission was shown to occur within the lumen of lysosomes, raising the intriguing possibility that ruptured lysosomes containing α-syn may be involved in this process [445]. Finally, the same small fibrillar forms of amyloid assemblies most potent for inducing vesicle rupture are most often the species responsible for cell-to-cell propagation [202, 203, 212, 213, 219, 439, 441, 442],
suggesting that cellular invasion and release may be intertwined when induced by these pathogenic amyloid species. To assess the involvement of endocytic vesicle rupture in the cellular release and intercellular propagation of $\alpha$-syn aggregates, we examined the extracellular localization and the intercellular movement of $\alpha$-syn containing ruptured vesicles. We observed ruptured vesicles containing $\alpha$-syn present in the extracellular environment (Figure 30), and we furthermore captured an instance of $\alpha$-syn intercellular trafficking between chGal3-expressing hiPSC-derived dopaminergic neurons that revealing $\alpha$-syn propagation occurring within a ruptured vesicle (Figure 31). While these findings are preliminary and in need of additional verification, our observation that $\alpha$-syn release and cell-to-cell propagation can occur in association with ruptured vesicles has great importance for understanding disease pathogenesis. Future work will focus on characterizing the population of free and membrane-associated $\alpha$-syn, investigating the cellular factors that facilitate release, and examining the potential chaperone or facilitative role for Gal3 in recognizing ruptured vesicle damage and trafficking with $\alpha$-syn to the extracellular environment and to neighboring cells. We believe that the damaging mechanism of cellular invasion by $\alpha$-syn assemblies and other aggregated amyloid proteins through endocytic vesicle rupture sets into a motion a positive feedback loop whereby the cellular stressors induced by this damage lead to the inability of the damaged vesicle and its $\alpha$-syn cargo to be degraded via autophagy and instead result in pathological inclusion formation, amyloid protein release, and cell-to-cell propagation of misfolded protein pathology.
Concluding Remarks

The above results have uncovered a conserved role for endocytic vesicle rupture in the cell-to-cell propagation pathway of fibrillar amyloid protein assemblies. In the intracellular and intercellular life cycle of misfolded amyloid protein aggregates, thought to underlie the inexorable progression of numerous neurodegenerative diseases, the mechanism by which amyloid aggregates escape from the vesicular compartment following their endocytic uptake was unknown. We have characterized this process in multiple model systems, observing its occurrence as a common mechanism of amyloid aggregate invasion in immortalized cells and hiPSC-derived dopaminergic neurons. Furthermore, in a fashion resembling the cellular infection by bacterial and viral pathogens, lysosomes ruptured by α-syn are targeted for autophagic degradation, but not before their problematic permeabilization releases proteolytic cathepsins and mitochondrial ROS creating an environment of oxidative and degradative stress. Dysfunctional degradative processes may fail to cope with the burden of misfolded protein aggregates and damaged vesicles, instead causing pathological inclusion formation and cellular release of pathologic α-syn species. The localization of Gal3 to the periphery of LB inclusions in PD brain implicates amyloid assembly-mediated endocytic vesicle rupture as occurring in authentic human disease, and provides a unique opportunity for further assessment of this phenomenon as a therapeutic target to prevent or reverse neurodegenerative proteinopathies.
APPENDIX

ADDITIONAL FIGURES FOR REFERENCE
Figure 33. **chGal3+ ruptured vesicles containing α-syn observed in undeconvolved and deconvolved fluorescent microscopic images.** Image of SY5YchGal3 cells exposed to WT α-syn fibrils from Figure 15, shown as undeconvolved and deconvolved images. As in Figure 15, Overlay images are separated into separate channels for either α-syn fluorescence or chGal3 fluorescence, and inset box in top images is enlarged in bottom images. Arrowheads indicate colocalization between chGal3+ ruptured vesicles appearing as discrete puncta and α-syn assemblies, observed in both undeconvolved and deconvolved image sets.

Figure 34. **Three-dimensional surfaces encapsulating α-syn-containing chGal3+ ruptured vesicles superimposed onto deconvolved fluorescent microscopic images.** Image of SY5YchGal3 cells exposed to A53T α-syn assemblies from Figure 12, analyzed for puncta formation by use of the Surpass Mode of the Imaris software package (Bitplane). A three-dimensional surface was created around chGal3 puncta by designing an algorithm for each experiment that specifically detected punctate events that increased in intensity sufficiently above background fluorescence. Algorithm-detected semi-spherical chGal3+ puncta are seen in gray, either superimposed on fluorescent image in left panel or alone in right panel.
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