Sex-specific Effects of Chaperone and Glial Defenses on Experimental Lewy Body Disease

Tarun Bhatia

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SEX-SPECIFIC EFFECTS OF CHAPERONE AND GLIAL DEFENSES ON
EXPERIMENTAL LEWY BODY DISEASE

A Dissertation

Submitted to the Graduate School of Pharmaceutical Sciences

School of Pharmacy

Duquesne University

In partial fulfillment of the requirements for
the degree of Doctor of Philosophy

By

Tarun N. Bhatia

May 2022
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Approved February 24, 2022
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May 2022

Dissertation supervised by Dr. Rehana K. Leak

Lewy body disorders are a group of neurodegenerative conditions characterized by the pathological misfolding and aggregation of the abundant protein, α-synuclein. The most common Lewy body disorders are Parkinson’s disease and dementia with Lewy bodies. Apart from ageing, male sex is a major risk factor for Lewy body disorders, as men are at ~1.5-fold higher risk for these diseases than women. Yet, preclinical studies on Lewy body disorders rarely examine sex as a biological variable, and the mechanisms underlying sex-skewedness in disease risk remain undetermined.

Here, we developed a sex-stratified model of Lewy body disorders by exposing primary neurons harvested from male versus female rat pups to aggregated, fibrillar α-synuclein in vitro. In Aim 1, we tested if the sex-skewedness in the ensuing α-synucleinopathy was mediated by sex differences in intrinsic defenses that regulate protein
homeostasis, such as the Hsp70 disaggregation machinery. Using primary rodent cultures as well as postmortem age- and sex-matched human tissue from subjects with Lewy body disorders, data from Aim 1 suggest that male cells may be more dependent on Hsp70 defenses under α-synucleinopathic disease conditions than female cells.

In Aim 2, we tested the therapeutic potential of Hsp70 via the intranasal route of drug delivery. Exogenously delivered Hsp70 (eHsp70) entered the aged male mouse brain within 3 hours, but, unexpectedly, eHsp70 did not penetrate the aged female mouse brain. Consistent with its nose-to-brain uptake, eHsp70 also mitigated pathology and behavior deficits in aged male mice injected with fibrillar α-synuclein. In contrast to its inability to permeate the female brain after intranasal delivery, eHsp70 was taken up by and reduced α-synuclein pathology in primary male and female neurons, via a mechanism that was partly dependent on its chaperone activities.

In Aim 3, we observed that eHsp70 increased the ability of primary microglia to engulf particles, but pretreatment of microglia with eHsp70 did not improve their ability to protect neighboring neurons from α-synucleinopathy. Rather, the mere presence of microglia in neuron/microglia co-cultures was protective against α-synucleinopathy—at least in male cells. In a final series of in vivo studies, we noted that partial depletion of microglia in aged male mice injected with fibrillar α-synuclein led to hyperactive and anxiety-like behaviors—effects that were not reversed post-microglial repopulation. In contrast, microglial repopulation improved spatial reference memory only in aged female mice injected with fibrillar α-synuclein, due perhaps to reversal of the ageing and diseased microglial phenotype. Thus, repopulating microglia may induce nootropic effects in aged female, but not male mice injected with fibrillar α-synuclein.
Collectively, this body of work reveals previously unrecognized sex differences in endogenous defenses and in the sex-skewed therapeutic benefits afforded by Hsp70 and microglia in α-synucleinopathic disease.
DEDICATION

For my mum, Jaya (Geeta) Bhatia, for always believing.
ACKNOWLEDGEMENT

I begin by acknowledging all the hard work, support, and encouragement offered by members of the Leak lab, including Rachel, Liz, Kristin, Anuj, Brett, Yaqin, Deepti, Hutch, and Dan. I am also grateful to Jess and Amanda for all our initial conversations before I joined Dr. Leak’s lab. Over the years, I have relied not only on data and samples generated by members of the Leak lab (present and past), but also on their generosity, for which I am enormously grateful. I’d like to thank Dan, Negin, Kristin, Deepti, Hutch, and Justin for serving as incredible mentors and for patiently teaching me techniques during my initial years. I thank Deepti and Hutch for being wonderful friends and colleagues and for always being warm and welcoming in the lab when I first started my PhD. Although our time together was limited, I enjoyed all the laughter and banter that we shared.

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I have been deeply fortunate to be guided by incredible scientists, as a part of my PhD committee and within the wider research community. I’m grateful to Dr. Lauren O’Donnell, Dr. David Johnson, Dr. Paula Witt-Enderby, and Dr. Jeffrey Brodsky for their valuable insights and encouragement during all my committee meetings. I’d like to thank Dr. Jeffrey Brodsky’s team, particularly Dr. Patrick Needham and Dr. Zhihao Sun, for their expertise. This project would also not have been completed without the generosity of Dr. Peter Wipf and his group, for which I am grateful, and I also thank Dr. Kelvin Luk for sharing α-synuclein fibrils and for his expertise and feedback. During my PhD, I have appreciated all the learning that I have acquired from Dr. Jun Chen, Dr. Xiaoming Hu, Dr. Edward Calabrese, Dr. Amy Wagner, Dr. Devika Manickam, and Dr. Aleem Gangjee, and I thank them for giving me the opportunity to contribute to projects, and for their advice and interactions. I’d like to thank the Center for Protein Conformational Diseases (University of Pittsburgh) for awarding me with a Graduate Student Fellowship. I am immensely grateful to Dr. Jeffrey Brodsky, Dr. Peter Wipf, Dr. Kelvin Luk, and Dr. Xiaoming Hu for their feedback on my manuscripts and experiments.

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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iv</td>
</tr>
<tr>
<td>Dedication</td>
<td>vii</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xviii</td>
</tr>
<tr>
<td><strong>Chapter 1: Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td>1. Lewy body disorders: Misfolding of α-synuclein as a shared mechanism</td>
<td>1</td>
</tr>
<tr>
<td>2. Lewy body disorders: Subtypes and Impact</td>
<td>7</td>
</tr>
<tr>
<td>3. Pattern of progression of pathology in Lewy body disorders</td>
<td>8</td>
</tr>
<tr>
<td>3.1. Braak staging hypothesis</td>
<td>8</td>
</tr>
<tr>
<td>3.2. DLB consortium staging</td>
<td>11</td>
</tr>
<tr>
<td>3.3. Unified staging system</td>
<td>12</td>
</tr>
<tr>
<td>3.4. Selective vulnerability hypothesis</td>
<td>16</td>
</tr>
<tr>
<td>4. Lewy body disorders: Risk factors</td>
<td>20</td>
</tr>
<tr>
<td>4.1. The impact of ageing on Lewy body disease</td>
<td>23</td>
</tr>
<tr>
<td>4.2. The impact of biological sex on Lewy body disease</td>
<td>26</td>
</tr>
<tr>
<td>4.2.1. Contribution of genetic factors to sex bias in Lewy body disorders</td>
<td>30</td>
</tr>
<tr>
<td>4.2.2. Contribution of sex hormones to sex bias in Lewy body disorders</td>
<td>33</td>
</tr>
<tr>
<td>5. Preclinical models of Lewy body disorders</td>
<td>38</td>
</tr>
<tr>
<td>5.1. The preformed α-synuclein fibril (PFF) model of Lewy body disorders</td>
<td>40</td>
</tr>
<tr>
<td>6. Chaperone proteins as modifiers of α-synucleinopathy</td>
<td>43</td>
</tr>
</tbody>
</table>
7. Glia as modifiers of α-synucleinopathy .................................................................51
8. Research objectives & Specific aims ......................................................................55

Chapter 2: Materials and Methods ...........................................................................58
1. Primary glial cultures ............................................................................................58
2. Primary neuron cultures ......................................................................................59
3. In vitro treatments of primary cultures ..................................................................60
4. Immunocytochemistry and In-Cell Western assays .............................................64
5. Stereotaxic surgeries ............................................................................................66
6. Intranasal infusions ...............................................................................................67
7. Behavior assays and analyses ..............................................................................69
8. Microglia depletion and repopulation ....................................................................72
9. Flow cytometry ....................................................................................................73
10. Perfusions and sacrifice .......................................................................................74
11. Immunohistochemistry and histological assays ..................................................75
12. Western immunoblotting .....................................................................................76
13. Image analyses ....................................................................................................77
14. Postmortem human samples ................................................................................79
15. RNA-sequencing ................................................................................................80
16. Statistical analyses .............................................................................................80

Chapter 3: Specific Aim 1: To determine if males versus females differ in their
dependence on endogenous Hsp70 defenses in α-synucleinopathic disease ...............82
   Rationale ..................................................................................................................82
   Results ......................................................................................................................85
Discussion .................................................................................................................. 104

Chapter 4: Specific Aim 2: To determine if exogenous delivery of Hsp70 (eHsp70) mitigates pathology and behavior deficits in the PFF model of α-synucleinopathic disease .............................................................................................................................. 111

Rationale .................................................................................................................... 111

Results ........................................................................................................................ 115

Discussion .................................................................................................................. 136

Chapter 5: Specific Aim 3: To investigate if microglia mitigate α-synucleinopathy and behavior deficits in the PFF model and if this effect is modified by biological sex . 146

Rationale .................................................................................................................... 146

Results ........................................................................................................................ 157

Discussion .................................................................................................................. 184

Chapter 6: Conclusions & Impact .............................................................................. 198

Chapter 7: References ............................................................................................... 203

Chapter 8: Appendices ............................................................................................... 257

Table 1: List of primary antibodies ........................................................................... 257

Table 2: List of secondary antibodies ........................................................................ 258

Table 3: List of antibodies for Flow Cytometry ....................................................... 260

Table 4: Demographic information of human postmortem samples ......................... 260

Copyright statement .................................................................................................. 261
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Structure of α-Synuclein</td>
<td>4</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Staging of pathology in Lewy body disorders</td>
<td>14</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Schematic of specific aims</td>
<td>57</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Women show higher Hsp70 in their amygdala than age-matched men</td>
<td>86</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Age at death is associated with amygdalar Hsp70 levels in males with disease, but not in females or unaffected control subjects</td>
<td>87</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Diseased subjects express high levels of detergent-insoluble phosphorylated α-synuclein in their amygdalae</td>
<td>88</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Biological sex or disease status does not alter the levels of Hsc70 in the OB or amygdala</td>
<td>89</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Biological sex or disease status does not alter the levels of Hsp40 in the OB or amygdala</td>
<td>90</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Exposure to PFFs for up to two weeks induces robust pSer129+ inclusion formation, but not cell loss in mixed-sex, primary hippocampal neuron cultures</td>
<td>93</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Primary hippocampal neuron cultures from male rat pups develop greater PFF-induced detergent-insoluble pathology than their female counterparts</td>
<td>95</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Patterns of uptake of ATTO647-α-synuclein fibrils by primary hippocampal cultures</td>
<td>97</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Biological sex does not impact the uptake of ATTO647-α-synuclein fibrils by primary hippocampal neuron cultures</td>
<td>99</td>
</tr>
</tbody>
</table>
Figure 13. Pharmacological inhibition of Hsp70 function exacerbates α-synucleinopathy more robustly in male neuronal cultures.................................................................102

Figure 14. Pharmacological inhibition of Hsp70 function with MAL3-101 leads to an increase in Hsc70 and Hsp40 in fibril-treated female neuronal cultures only...............103

Figure 15. Exogenous Hsp70 (eHsp70) reduces pSer129+ inclusions in fibril-treated, mixed-sex primary hippocampal neuron cultures.................................................116

Figure 16. Exogenous Hsp70 (eHsp70) decreases pSer129+ inclusion numbers in fibril-treated, mixed-sex primary hippocampal neuron cultures, but does not reduce detergent-insoluble pSer129/α-synuclein levels...............................................................119

Figure 17. Uptake of eHsp70 protein by male and female primary neuron cultures.....121

Figure 18. eHsp70 reduces α-synucleinopathy in fibril-treated male and female primary neuron cultures...........................................................................................................124

Figure 19. eHsp70 enters the male mouse brain within 3 h and gets cleared from the male brain within 72 h of its intranasal delivery .........................................................128

Figure 20. eHsp70 is not taken up by the aged female mouse brain after its intranasal delivery ................................................................................................................129

Figure 21. Daily intranasal eHsp70 delivery for 28 d reduces pSer129+ inclusions in the AON of PFF-injected aged male mice.................................................................132

Figure 22. Impact of transient intranasal infusions with eHsp70 on behavior deficits associated with PFF injections in young, 5-month-old male mice.........................135

Figure 23. The effect of intranasal eHsp70 on DNA repair activities .........................142

Figure 24. Summary schematic for Aims 1 and 2: Impact of endogenous and exogenous Hsp70 on PFF-seeded α-synucleinopathy.................................................................145
Figure 25. Characterization of primary cortical and hippocampal microglia ..............158

Figure 26. Primary hippocampal microglia reduce the numbers of pSer129\(^+\) inclusions in co-cultured primary hippocampal neurons .................................................................................................................................160

Figure 27. Primary astrocytes reduce the numbers of pSer129\(^+\) inclusions in co-cultured primary neurons ........................................................................................................................................................................162

Figure 28. \(\alpha\)-Synuclein fibrils stimulate microglial phagocytosis capacities ..........164

Figure 29. Male microglia reduce pSer129\(^+\) inclusions in co-cultured male neurons seeded with \(\alpha\)-synuclein fibrils ....................................................................................................................................................................167

Figure 30. Biological sex does not impact fluorescence signal of ATTO\(_{647}\)-\(\alpha\)-synuclein fibrils in primary hippocampal microglia at 6, 16, 24, 72, and 96 h post-exposure ........168

Figure 31. \(\alpha\)-synuclein fibril injections in the hippocampal CA2/CA3 region lead to an increase in Iba1 expression in some, but not all brain regions .................................................................171

Figure 32. PLX5622 leads to equivalent microglial depletion in male and female CD1 mice, but cessation of PLX5622 leads to greater microglial repopulation in females ....173

Figure 33. Depletion of microglia induces hyperactive and anxiety-like behaviors, which were greater in PFF-injected aged male mice, compared to their female counterparts...176

Figure 34. Repletion of microglia does not reduce the hyperactive behaviors induced on depleting microglia in PFF-injected aged male mice .................................................................177

Figure 35. PLX5622 abolishes sex differences in body weight in PFF-injected aged male and female CD1 mice ........................................................................................................................................................................178

Figure 36. Microglial repopulation improves spatial reference memory in PFF-injected aged female, but not male mice .........................................................................................................................................................181
**Figure 37.** Microglial reactivity in the postmortem human amygdala may be correlated with age at death.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-AAG</td>
<td>17-N-allylamino-17-demethoxygeldanamycin</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADLB</td>
<td>Alzheimer’s disease with Lewy bodies</td>
</tr>
<tr>
<td>AIF1</td>
<td>Allograft inflammatory factor 1</td>
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<tr>
<td>AON</td>
<td>Anterior olfactory nucleus</td>
</tr>
<tr>
<td>AP</td>
<td>Anterior-Posterior (for stereotaxic coordinates)</td>
</tr>
<tr>
<td>APE1</td>
<td>Apurinic/apyrimidinic endonuclease 1</td>
</tr>
<tr>
<td>Apoe</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>Ara-C</td>
<td>Cytosine arabinofuranoside</td>
</tr>
<tr>
<td>ATP13A2</td>
<td>ATPase cation transporting 13A2</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
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<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu Ammonis (of the hippocampus)</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine receptor type 5</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CMA</td>
<td>Chaperone-mediated autophagy</td>
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<tr>
<td>CNTN1</td>
<td>Contactin 1</td>
</tr>
<tr>
<td>CSF1R</td>
<td>Colony stimulating factor 1 receptor</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DLB</td>
<td>Dementia with Lewy bodies</td>
</tr>
</tbody>
</table>
Dulbecco’s Modified Eagle Medium ................................. DMEM
Dorsal-Ventral (for stereotaxic coordinates) ...................... DV
Exogenous heat shock protein 70 ....................................... eHsp70
Enteric nervous system .................................................. ENS
Early-onset Parkinson’s disease ....................................... EOPD
Estrogen receptor alpha ................................................... Erα
Estrogen Receptor Ligand-Binding Domain ...................... ERLBD
Fetal Bovine Serum ....................................................... FBS
F-Box Protein 7 ............................................................... FBXO7
Fluorescence minus one .................................................. FMO
Glucocerebrosidase .......................................................... GBA
Glial fibrillary acidic protein ............................................ GFAP
Hank’s Balanced Salt Solution ......................................... HBSS
Heat shock cognate protein 70 .......................................... Hsc70
Heat shock factor 1 ........................................................... HSF1
Heat Shock Protein Family A (Hsp70) Member 9 ................... HSPA9
Heat shock protein .......................................................... Hsp (as in Hsp40, Hsp70, Hsp90, etc.)
Institutional Animal Care and Use Committee .................. IACUC
Ionized calcium binding adaptor molecule 1 ..................... Iba1
Interferon-gamma ......................................................... IFNγ
Insulin-like growth factor 1 .............................................. IGF-1
Interleukins ................................................................. IL (as in IL2, IL4, IL6, etc.)
Incidental Lewy body Disease ......................................... iLBD
Inducible nitric oxide synthase ................................................................. iNOS
Induced pluripotent stem cell ................................................................. iPSC
Institutional Review Board ................................................................. IRB
Lymphocyte-Activation Gene 3 ............................................................. LAG3
Late-onset Parkinson’s disease ............................................................ LOPD
Lectin-type oxidized low-density lipoprotein receptor 1 .................... LOX-1
Lipopolysaccharide ........................................................................... LPS
Leucine Rich Repeat Kinase 2 ............................................................. LRRK2
Microtubule associated protein 2 ...................................................... MAP2
Major histocompatibility complex .................................................. MHC (as in MHC-I, MHC-II, etc.)
Medial-Lateral (for stereotaxic coordinates) ..................................... ML
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine ..................................... MPTP
Non-Amyloid-β Component ............................................................. NAC
Neuronal nuclei ................................................................................ NeuN
Natural killer (cells) ........................................................................... NK
Neuropeptide Y .................................................................................. NPY
Olfactory bulb .................................................................................. OB
Parkinsonism Associated Deglycase ................................................ PARK7
Parkinson’s disease .......................................................................... PD
Parkinson’s disease dementia ........................................................... PDD
Preformed α-synuclein fibrils ............................................................. PFFs
PTEN-induced kinase 1 ..................................................................... PINK1
Phospholipase A2 Group VI ............................................................ PLA2G6
<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylmethylsulfonyl fluoride</td>
<td>PMSF</td>
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<tr>
<td>Parkinson’s Progression Markers Initiative</td>
<td>PPMI</td>
</tr>
<tr>
<td>Parkin E3 Ubiquitin Protein Ligase</td>
<td>PRKN</td>
</tr>
<tr>
<td>Prospero Homeobox 1</td>
<td>Prox1</td>
</tr>
<tr>
<td>Post-traumatic stress disorder</td>
<td>PTSD</td>
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<tr>
<td>Polyvinylidene fluoride</td>
<td>PVDF</td>
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<tr>
<td>Region of interest</td>
<td>ROI</td>
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<tr>
<td>Rat serum albumin</td>
<td>RSA</td>
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<td>Spinocerebellar ataxia type 1</td>
<td>SCA1</td>
</tr>
<tr>
<td>Substantia nigra</td>
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</tr>
<tr>
<td>Scavenger receptor expressed by endothelial cells 1</td>
<td>SREC-I</td>
</tr>
<tr>
<td>Sex determining region Y</td>
<td>Sry</td>
</tr>
<tr>
<td>Traumatic brain injury</td>
<td>TBI</td>
</tr>
<tr>
<td>Tris-buffered saline</td>
<td>TBS</td>
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<tr>
<td>Tyrosine hydroxylase</td>
<td>TH</td>
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<td>Toll-like receptor</td>
<td>TLR (as in TLR2, TLR4, etc.)</td>
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<td>Transmembrane protein 119</td>
<td>TMEM119</td>
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<tr>
<td>Tumor necrosis factor alpha</td>
<td>TNFα</td>
</tr>
<tr>
<td>Transcripts per million</td>
<td>TPM</td>
</tr>
<tr>
<td>Ubiquitin C-Terminal Hydrolase L1</td>
<td>UCHL1</td>
</tr>
<tr>
<td>Vesicular glutamate transporter</td>
<td>VGLUT</td>
</tr>
<tr>
<td>Vacuolar protein sorting ortholog 35</td>
<td>VPS35</td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>VSV</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1. Lewy body disorders: Misfolding of α-synuclein as a shared mechanism

Lewy body disorders refer to a group of neurodegenerative diseases, including Parkinson’s disease (PD), Parkinson’s disease dementia (PDD), dementia with Lewy bodies (DLB), and Alzheimer’s disease with Lewy body (ADLB) pathology. Although these disease subtypes may differ in their prevalence and timing of symptom onset, the aggregation and spread of misfolded α-synuclein in these diseases suggests a common pathophysiological mechanism. Indeed, these disease subtypes are all characterized by hallmark inclusion-like structures, comprised of proteins (including α-synuclein), lipids, fragments of mitochondria and vesicular membranes, etc. (Shahmoradian et al., 2019). These inclusions were originally described by Frederic Lewy in 1912, but were termed as Lewy inclusions by Konstantin Nikolaevich Tretiakoff in 1919 (Goedert, Spillantini, Del Tredici, & Braak, 2013). Lewy bodies (LBs) are the inclusions formed within the cell somas and Lewy neurites (LNs) are the inclusions present within neuronal projections. In addition, some studies have reported pale bodies and pale neurites in early disease stages and that these structures may change into mature or classic LBs and LNs, as they begin to disappear once LBs and LNs are detected (Dale et al., 1992; Wakabayashi et al., 2013).

A major component of Lewy inclusions is the protein, α-synuclein, which can misfold and aggregate; for this reason, Lewy body disorders are classified as α-synucleinopathies (Spillantini et al., 1997). The first link between α-synuclein and Lewy body disorders was established in 1997 (Polymeropoulos et al., 1997) when the G209A point mutation in SNCA (gene encoding α-synuclein) was discovered in an Italian kindred.
and in some Greek families, resulting in the A53T amino acid change. The pattern of inheritance of PD was autosomal-dominant and, over the next few years, several other point mutations were identified, along with duplication and triplication variants of SNCA. The α-synuclein protein is 140 amino acids in length and was first identified in 1988 in the electric eel Torpedo californica (Maroteaux, Campanelli, & Scheller, 1988). At the time, its specific function was unclear, but based on its localization at the presynaptic terminal and at the nuclear envelope, it was named “synuclein” (portmanteau of synapse and nucleus) (Maroteaux et al., 1988). It is known that under neutral pH and in the absence of interacting partners, α-synuclein is unstructured and present as a natively unfolded monomer of ~14 kDa (Eliezer, Kutluay, Bussell, & Browne, 2001; Weinreb, Zhen, Poon, Conway, & Lansbury, 1996). A study by Bartels et al. found that when isolated under non-denaturing conditions, α-synuclein may instead exist as a folded tetramer (~58 kDa) and stabilizing this tetramer prevents α-synuclein from aggregating (Bartels, Choi, & Selkoe, 2011; Dettmer et al., 2015; Pochapsky, 2015; Selkoe et al., 2014). The current consensus is that the oligomeric (i.e., tetrameric) form of α-synuclein exists in an equilibrium with the monomeric form (Lucas & Fernandez, 2020; Meade, Fairlie, & Mason, 2019).

There are three primary domains on α-synuclein (Fig. 1):

1. The apolipoprotein lipid-binding domain at the N-terminus facilitates the interaction of α-synuclein with macromolecules (e.g., membrane phospholipids) to form α-helical structures (Stefanis, 2012). Apart from membrane lipids, several interaction partners for α-synuclein have been identified, all of which assist in the folding of α-
synuclein into helices (Benskey, Perez, & Manfredsson, 2016). Within the amphipathic N-terminus region are also located all six of the major mutations of SNCA (Stefanis, 2012).

(2) When misfolded, α-synuclein may form β-sheet rich structures due to the hydrophobicity in its central domain—the non-amyloid-β component (NAC)—which confers an aggregation-potential onto α-synuclein (Stefanis, 2012; Ueda et al., 1993). These β-pleated sheets participate in the assembly of α-synuclein into higher-ordered fibrillar species (Vilar et al., 2008). This conversion of the soluble α-synuclein into the more insoluble fibrillar form is sequential, resulting in the formation of oligomers, protofibrils, and eventually fibrils, in a feed-forward process (Benskey et al., 2016; Uversky & Eliezer, 2009; Uversky, Li, & Fink, 2001). Notably, the intermediate species may also act as “seeds”, templating and recruiting additional soluble α-synuclein to form aggregates (Danzer, Krebs, Wolff, Birk, & Hengerer, 2009; Luk et al., 2009; Wood et al., 1999) (described in detail in the section on the preformed fibril model).

(3) At its C-terminal domain, α-synuclein is negatively charged, which assists in stabilization, but, notably, C-terminal truncated products of α-synuclein are also common within Lewy inclusions and up to 15-20% of α-synuclein within inclusions is C-terminally truncated (Hong, Xiong, Chang, & Jiang, 2011; W. Li et al., 2005; Sorrentino & Giasson, 2020; Stefanis, 2012).
Figure 1: Structure of α-Synuclein. (A) α-Synuclein is a 140 amino acid long protein, consisting of 3 distinct domains. N-terminal domain contains an amphipathic region where all the known mutations of α-synuclein are present. The central core region contains a hydrophobic non-amyloid-β component (NAC), which is the aggregation-prone region on α-synuclein. The C-terminal region is acidic and modulates the functions of α-synuclein. (B) Misfolding of α-synuclein results in the formation of higher ordered species, such as oligomers and fibrils, eventually resulting in the formation of insoluble aggregates, termed Lewy bodies or Lewy neurites.

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The physiological functions of neuronal α-synuclein continue to be debated, but there is considerable evidence demonstrating its role in synaptic vesicle trafficking (Cabin et al., 2002), DNA repair activities (Schaser et al., 2019), mitostasis (Ellis et al., 2005) membrane remodeling (Ouberai et al., 2013), synaptoplasticity (Cheng, Vivacqua, & Yu, 2011), lipid metabolism (Ellis et al., 2005), in sensing lipid packing defects (Ouberai et al., 2013), and in chaperoning activities (T. D. Kim, Paik, & Yang, 2002). In addition, α-synuclein is also be expressed by glia, albeit at much lower levels than in neurons (Booms & Coetzee, 2021; Saunders et al., 2018; Y. Zhang et al., 2014; Y. Zhang et al., 2016), and the physiological functions of glial α-synuclein are less clear. Some groups showed that endogenous α-synuclein in microglia may have anti-inflammatory roles and may assist in microglial phagocytic functions (S. A. Austin, Floden, Murphy, & Combs, 2006; S. A. Austin, Rojanathammanee, Golovko, Murphy, & Combs, 2011). It has also been suggested that SNCA is highly expressed in hematopoietic stem cells—the precursors of microglia—and, so, it was hypothesized that α-synuclein may have roles in microglial differentiation, although this has not been tested (Booms & Coetzee, 2021).

Notably, both gain-of-toxic function and loss-of-normal function of α-synuclein have been implicated in the toxicity of α-synucleinopathies (Winkelhofer, Tatzelt, & Haass, 2008). The finding that SNCA mutations, specifically gene multiplications, are linked to PD support the gain-of-function hypothesis due to a gene/dosage effect and a correlation between α-synuclein load and disease pathology (Benskey et al., 2016; Chartier-Harlin et al., 2004; Singleton et al., 2003). Additionally, overexpression of α-synuclein may result in a parkinsonian phenotype in mouse models (Visanji et al., 2016) and the microglia-specific overexpression of α-synuclein is known to lead to oxidative stress and impairments
in phagocytosis, which may ultimately kill nearby neurons (Bido et al., 2021). One must note that a correlation between α-synuclein load and disease is confounded by findings that the levels of soluble α-synuclein may be lower in the cerebrospinal fluid (CSF) of patients (L. Gao et al., 2015; Tokuda et al., 2006). This may be due to a shift of the soluble form of α-synuclein into more insoluble oligomers, which are increased in the disease, thereby supporting a loss-of-normal function hypothesis (D. W. Miller et al., 2004; J. G. Quinn et al., 2012).

Indeed, knockdown of α-synuclein in the nigra results in ~50% loss of dopamine-producing neurons and a rapid increase in neuroinflammation, which is characteristic of PD (Benskey et al., 2018). Furthermore, due to its sequestration within inclusion bodies, α-synuclein may be unable to perform its normal, physiological roles, which can also lead to toxic neuropathologies due to a loss-of-function mechanism (Benskey et al., 2016). In primary rodent neurons exposed to fibrillar α-synuclein, there was a >80% loss of soluble (endogenous) α-synuclein, due perhaps to its recruitment within inclusions (Benskey et al., 2016; Volpicelli-Daley et al., 2011). Apart from robust inclusion pathology, exposure to fibrillar α-synuclein led to ~40% neuron loss, loss in synaptic proteins, and changes in neural activities (Volpicelli-Daley et al., 2011). Consistent with these findings, an in vivo study showed that as inclusions mature, there is loss of soluble α-synuclein, which may ultimately lead to ~60% loss of inclusion-laden neurons (Benskey et al., 2016; Osterberg et al., 2015). Thus, preserving the homeostatic balance of α-synuclein is critical as small changes in α-synuclein levels or solubility exert neurotoxic (and potentially gliotoxic) effects, by influencing its aggregation potential (Benskey et al., 2016).
2. **Lewy body disorders: Subtypes and Impact**

Perhaps the most widely studied subtype of Lewy body disorders is Parkinson’s disease (PD) and is characterized by the presence of pathological inclusions in (and loss of) dopaminergic neurons of the nigrostriatal pathway. This may result in a spectrum of motor deficits including tremors, bradykinesia (slowness of movement), impairment of reflexes, and rigidity (R. Xia & Mao, 2012). Apart from these motor symptoms, loss of neurons in some other brain regions may also contribute to a range of non-motor deficits, including depression, autonomic dysfunction, cognitive impairments, and anosmia (i.e., the loss of smell) (Haehner, Hummel, & Reichmann, 2011; Poewe, 2008). It is estimated that there were ~1 million people living with PD in the US in 2017 and the financial burden was estimated at ~$51.9 billion (W. Yang et al., 2020). In the same study, Yang et al. projected that by 2037, PD will be prevalent in ~1.6 million people and that the financial impact will be upwards of ~$79 billion (W. Yang et al., 2020).

A disorder that is distinct from PD is Parkinson’s disease dementia (PDD), wherein patients begin to show dementia-like symptoms after the appearance of motor deficits (Sezgin, Bilgic, Tinaz, & Emre, 2019). Among PD patients, the prevalence of dementia is believed to be ~31.3% and its presence is known to increase among PD patients that survive longer than a decade or two after their clinical diagnoses (Y. Xu, Yang, & Shang, 2016). If dementia-like symptoms appear before or within one year of motor symptoms, patients are diagnosed with dementia with Lewy bodies (DLB) (Sezgin et al., 2019). After Alzheimer’s disease (AD), DLB is the second most common dementia, accounting for 0.3 to 24.4% of all cases in prevalence studies, and like PDD, ageing is also a natural risk factor for DLB (Donaghy & McKeith, 2014; Hogan et al., 2016). Another subtype of Lewy body
disorders is Alzheimer’s disease with Lewy bodies (ADLB), which is less studied but is worth mentioning, given that almost 50% of all sporadic AD cases show Lewy pathology (E. J. Chung et al., 2015; Hamilton, 2000). Similarly, amyloid plaques and tau tangles (neuropathological hallmarks characteristic of AD) have been observed among PD, PDD, and DLB patients suggesting that co-occurrence of AD and Lewy body diseases is possible (Apaydin, Ahlskog, Parisi, Boeve, & Dickson, 2002; Irwin & Hurtig, 2018). Apart from its presence among patients clinically diagnosed with disease, pathology is also detected postmortem in otherwise neurologically healthy individuals (Markesbery, Jicha, Liu, & Schmitt, 2009). This is termed incidental Lewy body disease (iLBD). For example, Markesbury et al. found Lewy pathology in brains of ~23% of autopsied healthy individuals (in 33 out of 139 cases examined) (Markesbery et al., 2009). These findings support the hypothesis that iLBD is a preclinical stage of Lewy body disorders (Dickson et al., 2008).

3. Pattern of progression of pathology in Lewy body disorders

3.1. Braak staging hypothesis

Spread of Lewy pathology may be dependent on neuroanatomical circuitry and may follow a stereotypic, predictable pattern based on brain connectivity. Thus, a well-defined staging of pathology was described by Heiko Braak and colleagues in 2003 (Braak et al., 2003). Braak’s hypothesis states that exposure of an individual to an as-yet undetermined trigger (e.g., pathogen, environmental toxicant, etc.) via the nasal cavity underlies α-synuclein aggregation in the olfactory bulb complex, from where pathology may spread into deeper regions within the central nervous system (CNS) (Braak et al., 2003). Patients
may also be exposed to the “trigger” via the gut, which may lead to α-synuclein aggregations within the gastrointestinal system (Braak et al., 2003). The vagus nerve may then serve as a conduit to transmit the aggregates from the enteric nervous system (ENS) to the CNS, and, consistent with this theory, it is known that truncal vagotomies reduce PD risk (B. Liu et al., 2017; Svensson et al., 2015). Recent work by Kim et al. also showed that vagotomies prevent the gut-to-brain spread of α-synuclein in mice inoculated with α-synuclein in the gastrointestinal system (S. Kim et al., 2019).

As per Braak’s staging of Lewy pathology, inclusions containing α-synuclein first emerge in dorsal motor nuclei of the glosopharyngeal and vagus nerves, in projection cells of the intermediate reticular zone, as well as in the anterior olfactory nucleus (AON) (Braak et al., 2003). The AON lies caudal to the olfactory bulb (OB) and, as per previous work, shows greater pathology than the OB, although the AON is often included as a part of the OB (Beach, White, et al., 2009; Pearce, Hawkes, & Daniel, 1995; Ubeda-Banon et al., 2010). In stage 2, pathology in lower brainstem nuclei becomes denser, appearing within the caudal raphe nuclei, the gigantocellular reticular nucleus, and the coeruleus-subcoeruleus complex (Braak et al., 2003). Notably, the substantia nigra (SN) is not involved in these early stages 1-2, wherein pathology is restricted to the brainstem and olfactory areas (Braak et al., 2003). Consistent with these histopathological observations, it is well established that in PD patients, nonmotor symptoms (e.g., loss of smell) predate motor symptoms by several years (Adler, 2011; Doty, 2012; Doty, Deems, & Stellar, 1988; Haehner et al., 2011; G. W. Ross et al., 2006). The initiation of motor symptoms in patients may be attributed to early involvement of non-nigral regions responsible for movement (e.g., brainstem nuclei), since nigral involvement is not seen until latter stages.
The burden of pathology in the specific brain regions involved in Braak stages 1-2 intensifies in stage 3. Furthermore, pathology begins to impact melanized neurons of the SN, neurons in the forebrain magnocellular nuclei, and the tuberomammillary nucleus of the hypothalamus (Braak et al., 2003). At stage 3, hippocampal cornu Ammonis subfield CA2 and the cortical areas projecting to the AON may also be involved (Braak et al., 2003). In contrast, the anteromedial temporal mesocortical areas and neocortical areas are unaffected at this stage (Braak et al., 2003).

Considerable damage to neurons in the SN is evident at Braak stage 4. Apart from the SN, extensive neurodegeneration is also evident in the AON (Braak et al., 2003). The pathology in the hippocampal CA2 subfield is exacerbated and the anteromedial temporal mesocortical areas also display pathology (Braak et al., 2003). Pathological inclusions form within those neurons of the raphe nuclei that are specifically filled with lipofuscin—an age-related by-product of material that fails to be degraded by lysosomal clearance machinery—but not within those neurons that do not accumulate lipofuscin (Braak et al., 2003). This may be indicative of selective vulnerability (detailed further below). Further, pathology develops and intensifies in forebrain magnocellular nuclei, in thalamic nuclei, in the tuberomammillary nucleus of the hypothalamus, in the stria terminalis, in the amygdalar central and accessory cortical nuclei, and in the claustrum (Braak et al., 2003).

It is only in the latter stages 5-6 that neocortical involvement is evident. Further, the nigral neurons show extensive loss of pigmentation (due to degeneration of melanized neurons in this area) leading to macroscopic “pallor” that is characteristic of PD (Braak et al., 2003). Apart from the SN, loss of melanized neurons is also evident in the brainstem. There is severe involvement of olfactory areas and pathology from the hippocampal CA2
subfield extends into the CA1 and CA3 regions (Braak et al., 2003). Higher-order sensory association areas, prefrontal areas, cingulate cortical areas, and insular cortical fields begin to show pathology at stage 5, and by stage 6, there is near-complete involvement of neocortical areas (Braak et al., 2003). In these latter stages, substantial damage accrues in limbic areas, such as the amygdala and the hippocampus, which may underlie cognitive impairments in patients at end-stages (Braak et al., 2003).

3.2. DLB Consortium staging

A caveat of the Braak staging system was that brains of patients with DLB were not analyzed. The DLB consortium proposed grading pathology in the brainstem (dorsal motor nuclei, coeruleus complex, and nigral regions), limbic regions (nucleus basalis, amygdala, transentorhinal and cingulate cortex), and neocortical regions (temporal, frontal, and parietal) by a semi-quantitative method that would assign the pathology to brainstem-predominant, limbic, or diffuse neocortical subtypes (McKeith et al., 2017; McKeith et al., 2005). Their recommendations focused on assessments of the severity of pathology, ranging from 0 (no pathology) to 1 (few LBs and LNs) to 2 (>1 LB and few LNs) to 3 (>4 LBs and scattered LNs) to 4 (several LBs and LNs) (McKeith et al., 2005). Leverenz et al. applied these criteria to a large autopsy sample of dementia patients and proposed adding an amygdala predominant group, which would increase the inclusion of cases that were unclassifiable by the original DLB consortium guidelines (Leverenz et al., 2008). In 2017, the DLB consortium added guidelines on assessing if the clinical dementia is likely to arise from DLB or AD (McKeith et al., 2017; McKeith et al., 2005). This further improved the grouping of cases, including those with amygdala-predominance or those
involving olfactory bulb only (McKeith et al., 2017). Lewy pathology is believed to spread from initial sites to first-order connections based on neuroanatomical connectivity (Braak et al., 2003). Examining several brain regions for pathology may also be important to correlate the topographical patterns to various behavior deficits.

3.3. Unified staging system

In 2009, Beach et al. proposed an alternate staging system that would categorize those cases that were unaccounted for in the Braak and DLB consortium staging systems (Beach, Adler, et al., 2009). Specifically, in their studies, it was suggested that up to ~50% of all cases remain unclassifiable if the Braak or DLB consortium systems were strictly followed (Beach, Adler, et al., 2009). Some of these cases could include patients with pathology that is limited to the OB or pathology that passes through the limbic pathways, without an initial involvement of brainstem structures (Beach, Adler, et al., 2009). Thus, Beach et al. proposed a single, unified staging system that classifies patients with PD, DLB, iLBD, as well as ADLB (Beach, Adler, et al., 2009).

As mentioned previously, pathological aggregates in Lewy body disorders contain α-synuclein. It is well-established that α-synuclein undergoes several posttranslational modifications, of which phosphorylation at Serine 129 (pSer129) is predominantly found within LBs (J. P. Anderson et al., 2006). Early studies showed that ~90% of α-synuclein within LBs is phosphorylated at this position (Fujiwara et al., 2002). Within the brains of subjects without LBs, only 4% of α-synuclein is phosphorylated at Ser129 (Fujiwara et al., 2002) and, thus, pSer129 may be used as a marker of disease as it may be present at only low-to-negligible levels among controls. Thus, another advantage of the Beach et al.
system is that they used this more sensitive immunostain to build on the work by Braak and by the DLB Consortium, and classified subjects with Lewy body disorders based on the extent of pathological phosphorylated α-synuclein (Beach, Adler, et al., 2009).

In their staging system, Beach et al. proposed that Stage I include only those cases that show the OB as the sole region displaying pathology. In the rare case that the OB is not involved or in those cases that do not show OB-first pathology, this system allows the classification of those cases to higher stages (Beach, Adler, et al., 2009). After the OB, the pathology may follow two distinct pathways based on the specific regions involved: Stage IIa may include cases with brainstem involvement and Stage IIb may include cases with limbic-predominant pathology (Beach, Adler, et al., 2009). Stage III may include cases with involvement of the brainstem and the limbic regions and Stage IV may include those cases with an appreciable involvement of any single neocortical area (Beach, Adler, et al., 2009). Based on this staging system, the authors found that a majority of iLBD cases are Stage IIa, PD and DLB cases are Stage III or Stage IV, and most ADLB cases are Stage I or Stage IIb (Beach, Adler, et al., 2009).

A summary of the different staging systems has been described in Fig. 2, which was reprinted from prior work (Outeiro et al., 2019).
**Figure 2:** Staging of pathology in Lewy body disorders. (A) Classification of DLB subjects based on the DLB consortium guidelines into olfactory only, amygdala predominant, brainstem predominant, limbic, and diffuse neocortical subtypes. (B) The Braak staging for PD patients suggested a progressive deposition of pathology, with the olfactory bulb and lower brainstem nuclei serving as initiation sites in the brain, from where pathology progresses to deeper regions. (C) Modification to the original DLB consortium guidelines to include amygdala-predominant cases. (D) The Unified staging system allows the classification of subjects with PD, DLB, iLBD, as well as ADLB, wherein pathology from the OB diverges into brainstem (IIa; see inset) or limbic-predominant (IIb; note amygdala in IIb) subtypes, followed by neocortical areas in Stage IV.

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Notably, the study by Beach et al. provided evidence of the unique involvement of the OB in virtually all cases that were examined, and the OB may either be the only affected region or the first affected region in many cases (Beach, Adler, et al., 2009). It is known that ~90% of PD patients show impairments in olfaction, before motor symptoms are manifested, indicative of a prodromal phase of the disease (Doty, 2012; Siderowf et al., 2012). A pioneering study by Pinto et al. has shown that loss in smell (i.e., anosmia) predicts mortality better than other risk factors such as cancer, heart attack, stroke, and diabetes (Pinto, Wroblewski, Kern, Schumm, & McClintock, 2014). Subjects were classified as anosmic, hyposmic, or normosmic based on number of odorants that were inaccurately identified (Pinto et al., 2014). As per their work, ~39% of seniors with anosmia (4-5 odors incorrectly identified out of total 5 odors presented) and ~19% with hyposmia (2-3 odors incorrectly identified) were dead within 5-years (Pinto et al., 2014). In contrast, only 10% of normosmic (0-1 odors incorrectly identified) seniors died within 5-years (Pinto et al., 2014). Findings from all of the Lewy body disease staging systems suggest that pathology in the OB may not always show widespread expansion into deeper areas but is a shared feature of all disease subtypes and, so, may be a useful marker of the disease, with a sensitivity range of ~67-97% and specificity range of ~81-91% (Beach, White, et al., 2009). The selective vulnerability of the OB may be due to its anatomical location as it is often the first site of the brain to receive environmental exposures (Beach, Adler, et al., 2009), and, based on prior work (Pinto et al., 2014), OB pathology may thus be implicated in the earlier mortality observed in patients with clinically diagnosed α-synucleinopathic disease.
3.4. Selective vulnerability hypothesis

Although there is considerable evidence supporting the sequential, predictable spread of pathology in Lewy body disorders, there is also an alternative theory that suggests that certain individual cells, cell types, or brain regions may be more vulnerable to the development of α-synucleinopathic aggregates. This theory was suggested because not all regions that show first-order connections to an affected region develop inclusions. In mice injected with fibrillar α-synuclein in the AON, Mason et al. found an absence of pathological inclusions in the horizontal limb of the diagonal band of Broca (Mason et al., 2016), despite this area being known to project to the AON (Brunjes, Illig, & Meyer, 2005). Furthermore, although dorsal motor nuclei neurons were substantially affected in Braak’s work, it is known that neurons in the nearby nucleus tractus solitarius are unaffected, despite axonal projections between the two areas (Braak et al., 2003; Rietdijk, Perez-Pardo, Garssen, van Wezel, & Kraneveld, 2017; Rinaman, Card, Schwaber, & Miselis, 1989; Shapiro & Miselis, 1985; Surmeier & Sulzer, 2013). These observations support the selective vulnerability hypothesis. It was theorized that poorly myelinated neurons with thin and long axonal arbors and extensive neuro- or synaptoplasticity exert a strong metabolic demand and may be more prone to pathology, as their mitochondria may be unable to sustain the energetic burden, leading to greater oxidative stress (Beach, Adler, et al., 2009; Braak & Del Tredici, 2004; Braak et al., 2003; Braak, Ghebremedhin, Rub, Bratzke, & Del Tredici, 2004; Rietdijk et al., 2017). In contrast, heavily myelinated neurons have lower energy expenditure (for neurotransmission) and a lesser propensity to undergo axonal sprouting (due to interactions with oligodendroglia), which increases their stability (Braak et al., 2004). Thus, in contrast to poorly myelinated neurons, heavily myelinated
neurons may be able to preserve bioenergetic resources for degrading aggregates via ATP-dependent chaperone functions, and this may contribute to their relative resilience against proteinopathic aggregates. Susceptible neurons also often possess large numbers of neurotransmitter-releasing vesicles, fire strong action potentials (even in the absence of an input) and have low levels of calcium-buffering (Surmeier, Obeso, & Halliday, 2017; Surmeier & Sulzer, 2013). If intracellular calcium is left unbuffered, it leads to oxidative stress, additional α-synuclein aggregation, impairment of protein clearance machinery, and apoptosis. Nigral dopaminergic neurons that are vulnerable in Lewy body disorders are known to typify these specific phenotypes (Pacelli et al., 2015).

Cells or brain areas that harbor naturally higher levels of endogenous α-synuclein may also be more vulnerable, as there may be additional substrate available for the seeding of pathology (Courte et al., 2020; Hijaz & Volpicelli-Daley, 2020; Luna et al., 2018; Rietdijk et al., 2017; Taguchi, Watanabe, Tsujimura, & Tanaka, 2016, 2019; Vasili et al., 2022). Mutations (in the \textit{SNCA} gene) are known to contribute to PD due to an overexpression of the gene and its product (α-synuclein) (Nussbaum, 2017). Apart from gene-induced upregulation of α-synuclein, brain-region dependent differential levels of α-synuclein may also contribute to the theory on selective vulnerability (Taguchi et al., 2016). Erskine \textit{et al.} systematically compared levels of α-synuclein in control individuals to the regional spread of pathology in diseased subjects and found that there was a near-absence of pathology in the visual and cerebellar cortices—due perhaps to the low-to-negligible expression of α-synuclein in these regions under baseline conditions (Erskine et al., 2018). However, areas with the greatest propensity for pathology did not show the greatest α-synuclein levels (Erskine et al., 2018), suggesting that a multitude of factors work in
tandem to determine the region-specific susceptibility to pathology. Apart from regional differences, individual cell types express α-synuclein at different levels and, thus, some cells may be more vulnerable than others to pathology formation. Neurons show higher levels of α-synuclein than astroglia and microglia (Y. Zhang et al., 2014; Y. Zhang et al., 2016) and so it is unsurprising that these neuroglia may be less prone to intrinsically forming Lewy pathology, unless the aggregates are extruded by neurons and taken up by the neuroglia from the extracellular space (Choi et al., 2020; H. J. Lee et al., 2010; Loria et al., 2017; Sacino et al., 2013; Sorrentino, Giasson, & Chakrabarty, 2019).

A study (see Fig. 3b in (Stevenson et al., 2020)) examining the numbers of inclusions within neurons versus non-neuronal cell types in the AON of PD patients found that there were greater numbers of neurons with pSer129\(^+\) inclusions (~32/mm\(^2\)), compared to astroglia (~5/mm\(^2\) harbored inclusions) and microglia (~14/mm\(^2\) harbored inclusions). A greater proportion of microglia may be engulfing inclusions as these phagocytes are better at degrading the aggregates than their astroglial counterparts (H. J. Lee, Suk, Bae, & Lee, 2008). Among neurons, it is known that glutamatergic (and not GABAergic) neurons are more amenable to development of inclusions, perhaps because glutamatergic neurons are laden with α-synuclein at their synapses (Hijaz & Volpicelli-Daley, 2020; Taguchi et al., 2016; Taguchi et al., 2014). Among glutamatergic neurons, vulnerability to pathology further differs based on expression of the transcription factor, Math2, with CA2/3 hippocampal glutamatergic neurons showing high levels of Math2 and α-synuclein, and so higher levels of pathology, compared to other glutamatergic neurons of the hippocampus (such as those expressing Prox1) (Luna et al., 2018).
Cells with faulty or insufficient endogenous defenses may also be less able to stave pathology. Lewy body disorders are proteinopathic in origin and so the first line of defense against misfolded α-synuclein aggregates may be members of protein refolding or protein degradative machinery, such as proteasomes, lysosomes, chaperone proteins, or autophagic proteins. Lower levels of these proteins or impairments in these clearance systems may increase the risk of accumulating toxic aggregates of α-synuclein. In mice overexpressing α-synuclein, Malkus et al. showed that the brainstem develops greater α-synuclein+ pathology than other areas, due to a relatively inactive chaperone-mediated autophagic (CMA) system (K. A. Malkus & Ischiropoulos, 2012). In her dissertation, Malkus showed that, apart from lower CMA activities, lysosomal and autophagosomal markers were also expressed at lower levels in areas that showed the greatest propensity to develop inclusions (Kristen A. Malkus, 2011). Similar findings have been reported in brain extracts from autopsied PD patients (Murphy et al., 2015). In addition, antioxidants, such as melatonin, may also impact the development of proteinopathic aggregates and the development of motor or non-motor symptoms in Lewy body disorders (L. Li et al., 2020; Ono et al., 2012). For example, melatonin inhibits the fibrillization of α-synuclein and decreased the toxicity of α-synuclein (Ono et al., 2012). Although there is little known about the effects of melatonin on α-synuclein levels in the clinical condition, recent work revealed that there was a negative correlation between melatonin levels in the plasma of PD patients and non-motor symptoms, such as cardiovascular symptoms, gastrointestinal disturbances, etc. (L. Li et al., 2020) These reports suggest that melatonin may have neuroprotective effects in Lewy body disorders.
It is unlikely that the topography of pathology can be fully explained by a single triggering event, intrinsic risk factor, or neuroanatomical circuitry. Instead, intrinsic vulnerabilities combined with regional spread may be the best way to predict the pattern of propagation of Lewy pathology, as the two scenarios are not mutually exclusive. For example, Henderson et al. found that in α-synuclein-injected mice, regions that are most vulnerable to pathology can be predicted not only by retrograde connectivity, but also by expression of Snca as the vulnerability estimate of a particular region correlated well with its Snca expression (Henderson et al., 2019). Apart from the abovementioned structural, biochemical, and genetic traits of susceptible cells, ageing (Collier, Kanaan, & Kordower, 2011; Hindle, 2010; Hou et al., 2019) and the male sex (Cerri, Mus, & Blandini, 2019; Gillies, Pienaar, Vohra, & Qamhawi, 2014) are the two major organismal determinants of vulnerability to pathology in α-synucleinopathies. Future work incorporating these risk factors in the model by Henderson et al. are warranted to better elucidate the interplay between age, biological sex, and the regional spread of α-synuclein pathology.

4. Lewy body disorders: Risk factors

Several genetic and environmental factors that increase the risk of Lewy body disorders have been identified. It is estimated that about 10% of all PD cases may be of the familial type (Thomas & Beal, 2007) and, conversely, the cause of ~90% of all PD cases is unknown, termed “sporadic” (Cannon & Greenamyre, 2013). The association between PD risk and mutations in genes such as SNCA are well known (Polymeropoulos et al., 1997). Apart from SNCA, mutations in genes involved in autophagy (LRRK2, VPS35), maintenance of lysosomal (GBA, LRRK2, ATP13A2), lipid (PLA2G6), and mitochondrial
(PINK1, HSPA9) homeostasis, chaperone activities (DNAJC6, PARK7), and the ubiquitin-proteasome system (UCHL1, PRKN, FBXO7) have all been implicated in PD (Bonifati et al., 2003; Conedera et al., 2016; De Mena et al., 2009; Franco, Rivas-Santisteban, Navarro, Pinna, & Reyes-Resina, 2021; Kumari & Tan, 2009; Maraganore et al., 2004; Oczkowska, Kozubski, Lianeri, & Dorszewska, 2013; Olgiati et al., 2016; J. S. Park, Blair, & Sue, 2015; Pickrell & Youle, 2015; Shen et al., 2019; Sidransky & Lopez, 2012; Vilarino-Guell et al., 2011). Genes implicated in familial PD may also be linked to sporadic cases of PD (Ahn et al., 2008), in the form of gene polymorphs or variants (Campelo & Silva, 2017). However, even when examined collectively, only about 5% of sporadic PD may be linked to polymorphisms in genes that are otherwise implicated in familial PD (C. Klein & Westenberger, 2012). Thus, most of the PD cases may be traced to environmental risk factors, although the involvement of gene-environment interactions cannot be ruled out (Cannon & Greenamyre, 2013). The genetics of DLB are less clear, but familial cases of DLB are known to exist (Tsuang, DiGiacomo, & Bird, 2004). For example, there is a high likelihood (odds ratio ~2.3) that siblings of patients clinically diagnosed with DLB will also develop the disease (Nervi et al., 2011). Many of the genes responsible for PD also play a role in familial DLB, but there is a paucity of studies into the frequency of gene mutations in DLB (Outeiro et al., 2019). A recent genome-wide association study in DLB identified a new candidate locus CNTN1 that is a cell-adhesion molecule and maintains axonal function but may be implicated in DLB (Guerreiro et al., 2018). Future work examining the genetics of DLB is warranted.

There is a wide range of studies examining the role of environmental risk factors in Lewy body disorders. Exposure to insecticides and herbicides, such as paraquat or
rotenone, is known to increase the risk of PD (odds ratio ~2.5) (Tanner et al., 2011) and, accordingly, these chemicals are used to model PD in laboratory settings (T. Anderson et al., 2021; Betarbet et al., 2000; Cristovao et al., 2020; Uversky, 2004). In addition, drinking well water that is contaminated with these pesticides or with chemicals, such as methomyl, chlorpyrifos, propargite, and organophosphates, may increase the risk of PD by almost 70-90% (Gatto, Cockburn, Bronstein, Manthripragada, & Ritz, 2009). Paraquat and rotenone produce neurotoxicity by inhibiting the mitochondrial electron transport chain and by increasing the levels of reactive oxygen species (Bove, Prou, Perier, & Przedborski, 2005). Exposure to heavy metals and organic solvents increases α-synuclein aggregation, activates apoptotic pathways, and is also known to induce oxidative stress and neuroinflammation, thereby increasing the risk of developing Lewy body disorders (Chin-Chan, Navarro-Yepes, & Quintanilla-Vega, 2015; Lock, Zhang, & Checkoway, 2013; Moons et al., 2020). Lifestyle factors may also influence the risk of disease. For example, some studies suggest that coffee (G. W. Ross et al., 2000) or alcohol (D. Zhang, Jiang, & Xie, 2014) consumption, tobacco smoking (Mappin-Kasirer et al., 2020), and physical activity (H. Chen, Zhang, Schwarzschild, Hernan, & Ascherio, 2005) may be inversely associated with Lewy body disease risk. Comorbidities, such as a history of stroke (Boot et al., 2013), diabetes (Chohan et al., 2021), and obesity (H. Chen et al., 2004) are all risk factors for Lewy body disorders. In addition, patients with Lewy body disorders suffer from a history of psychosocial stress, anxiety, and depression (K. W. Austin, Ameringer, & Cloud, 2016; Boot et al., 2013; Nilsson, Kessing, & Bolwig, 2001; Shiba et al., 2000), all of which may be linked with the limbic-predominant pathology seen in many Lewy body disease subtypes.
Given the focus on ageing and biological sex in this dissertation, descriptions on the roles of these risk factors in Lewy body disorders are provided separately, below.

4.1. The impact of ageing on Lewy body disease

Ageing is an evolutionarily conserved process, typified by changes in genomic and proteomic integrity, due to impairments in pathways that regulate oxidative and proteostatic balance, neuroinflammation, immune competence, mitochondrial health, and cellular homeostasis. Pathological changes associated with normal ageing are often implicated in neurodegenerative diseases, including Lewy body disorders. Thus, ageing is a primary risk factor for disease and has been described by some as a “pre-parkinsonian state” (Pang et al., 2019). A meta-analysis of worldwide PD studies found an increase in the prevalence of the disease with age (Pringsheim, Jette, Frolikis, & Steeves, 2014). Per 100,000 individuals, the prevalence rose from 41 in the 40-49 years age group to 173 in the 55-64 years age group to 1087 in the 70-79 years age group to 1903 in those that were older than 80 years of age (Pringsheim et al., 2014). At any given time, it is estimated that ~1% of the population that is above 60 years of age suffers from PD (Tysnes & Storstein, 2017)—a proportion that rises to ~5% for individuals surviving to the age of 85 (Dashtipour, 2014). Apart from PD, ageing also contributes to the development of DLB and PDD. The incidence rate of DLB is known to rise from 10.3 per 100,000 subjects (age 60-69 years) to 30.1 per 100,000 among subjects older than 80 years (Savica et al., 2013). Similarly, the incidence of PDD also rises from 3.4 per 100,000 in individuals aged 60-69 to 47 per 100,000 among individuals over 80 years (Savica et al., 2013). Thus, there is a direct impact of age on the risk of developing Lewy body disorders.
About 5-7% of all PD cases are early-onset (EOPD) (Golbe, 1991) and although there is no clear demarcation in the age at onset of EOPD versus late-onset PD (LOPD), some reports suggest that if onset of motor symptoms occurs before the age of 50, it is considered EOPD (Schrag & Schott, 2006). Using data from the Parkinson’s Progression Markers Initiative (PPMI), Pagano et al. found that patients with an older age at onset suffer from worse symptoms, show lower striatal dopamine binding capacities, and show a greater reduction in soluble α-synuclein, compared to patients with a younger age at onset but same number of years of disease (Pagano, Ferrara, Brooks, & Pavese, 2016). In contrast, quality of life, social adjustment, and mental health are all more severely impacted in EOPD patients, compared to LOPD patients (Schrag & Schott, 2006). Age-related impairments in neurological function often overlap with the prodromal phase of neurodegenerative diseases, such as PD. For example, olfactory dysfunction rises with age in neurologically normal individuals. Per Schubert et al., ~11% of healthy individuals at the age of 60 show olfactory dysfunction and its prevalence rises to 35% in individuals above the age of 78 (Dan et al., 2021; Schubert et al., 2012; Seubert et al., 2017). It is known that ~90% of all PD patients report loss in sense of smell in the prodromal phase (Doty, 2012; Siderowf et al., 2012) and, per Ross et al., impairments in olfaction precede a clinical diagnosis of PD by at least 4 years (G. W. Ross et al., 2008). In their study, the average age at PD diagnosis was in the range of 76-93 years—a timeframe in which, coincidentally, prevalence of olfactory dysfunction among healthy individuals is known to also exponentially rise, as mentioned above for the study by Schubert et al. (Dan et al., 2021; G. W. Ross et al., 2008; Schubert et al., 2012; Seubert et al., 2017). One may therefore conclude that age-related loss in sense of smell may be a harbinger for the later
diagnosis of PD. Thus, several studies support the use of olfactory dysfunction as an early biomarker of PD, but it is confounded by the difficulty in establishing if the source of olfactory impairment is normal ageing or neurodegenerative disease (Dan et al., 2021).

Dysregulation of protein clearance machinery with age has been reported (Cuervo et al., 2005; Cuervo & Dice, 2000; Ebrahimi-Fakhari et al., 2011; Keller et al., 2004; W. Peng, Minakaki, Nguyen, & Krainc, 2019), which may result in a greater accrual of α-synuclein with age even under baseline conditions, predisposing seniors to proteotoxic stress associated with excess α-synuclein. Consistent with this theory, Jellinger reported α-synuclein pathology in as many as 30% of otherwise cognitively normal aged subjects examined postmortem (Jellinger, 2004). Ageing is known to contribute to the selective vulnerability hypothesis (Mattson & Magnus, 2006). In 2003, Braak had suggested that age-related decline in lysosomal capabilities and the ensuing buildup of lipofuscin (“the age pigment”) results in the selective development of pathology in lipofuscin-burdened neurons, but not in lipofuscin-negative neighbors (Braak et al., 2003). In a comprehensive study, Chu and Kordower assessed α-synuclein in postmortem nigra of 18 neurologically normal humans (age range 18-102 years) and 24 rhesus monkeys (age range 2-34 years) (Chu & Kordower, 2007). Compared to younger (~31 years of age) humans, there was: 1) a ~270-640% increase in the number of α-synuclein+ neurons in the nigra of middle-aged (~55 years of age) and aged (~84 years of age) subjects, 2) a ~57% increase in the optical density of α-synuclein in neurons of aged subjects, and 3) mislocalization of α-synuclein from the synaptic terminals to the cell soma in aged subjects (Chu & Kordower, 2007). Similar patterns were noted in the nigra of rhesus monkeys (Chu & Kordower, 2007). Notably, the age-related increase in α-synuclein in the human and monkey brain was
coupled with a reduction in the levels of tyrosine hydroxylase (TH)—an enzyme critical for dopamine synthesis (Chu & Kordower, 2007). These findings were corroborated in another study by Xuan et al. (Xuan et al., 2011) and add credence to the theory that ageing may be a “pre-parkinsonian state”.

Collectively, these findings indicate that, 1) several neuropathological features of PD and natural ageing overlap, 2) ageing directly hastens the decline in PD patients, and 3) PD patients may be less able to compensate for loss of their faculties if they are older (Ferguson, Rajput, & Rajput, 2016). One must also note that despite the close association of ageing with the risk of disease, most seniors do not develop PD, as it affects only ~5% of individuals that survive up to 85 years, suggesting that ageing is not the sole determinant of disease. Instead, it is more likely that exposures to environmental stressors generate a more robust accrual and transmission of pathology among aged neurons that are already depleted of endogenous defenses and so may be “hotspots” for pathology.

4.2. The impact of biological sex on Lewy body disease

Apart from advanced age, male sex is the other major risk factor for Lewy body disorders (Baldereschi et al., 2000; Cerri et al., 2019; P. T. Nelson et al., 2010; Savica et al., 2013). In the earliest known descriptions of PD by James Parkinson (in 1817), all patients were men and a two-fold higher risk of disease in males was first posited by Sir William Richard Gowers, back in 1886 (Gowers, 1886; Parkinson, 2002). In Beach’s Unified staging system study, 61.5% of all iLBD subjects, 69.7% of PD subjects, and 65% of DLB subjects were male (Beach, Adler, et al., 2009). Both sexes were equally represented in AD subjects with or without Lewy bodies (Beach, Adler, et al., 2009).
because AD is more predominant in women (Podcasy & Epperson, 2016), as in a report by Mouton et al. wherein 70% of all AD subjects were women (A. Mouton et al., 2018).

At least for PD, the ~1.5-2 fold higher risk in men compared to women has been continually reported by several groups, suggesting that Gowers’ original descriptions in the late 1800s have held true, even after ~130 years (Baldereschi et al., 2000; Cerri et al., 2019; de Lau et al., 2004; Elbaz et al., 2002; Gillies et al., 2014; Gowers, 1886; Taylor, Cook, & Counsell, 2007; Van Den Eeden et al., 2003; Wooten, Currie, Bovbjerg, Lee, & Patrie, 2004). In addition, it is now suggested that the age at onset is also ~2.1 years later in women compared to men (Haaxma et al., 2007; Twelves, Perkins, & Counsell, 2003). Some studies have suggested that the male-skewedness in PD risk shifts in old age, but only among some ethnicities (J. H. Park et al., 2019; Van Den Eeden et al., 2003). In a study by Van Den Eeden et al., the male/female ratio in the incidence of PD shifted from 1.7 in those aged 60-69 years to 0.4 in those above 80 years, but only among Asians (Van Den Eeden et al., 2003). For other ethnicities, the male/female ratio in the incidence of PD was either unchanged or was even increased to ~3-3.1 for individuals above 80 years of age (Van Den Eeden et al., 2003). Consistent with this work, it was recently reported that for every 10 years increase in survival, the male/female ratio in the incidence of PD increases by 0.14 (Moisan et al., 2016). Thus, males are at a heightened risk for PD and may show an earlier age at disease onset.

In contrast, it is not inevitable that male Lewy body disease patients will also have higher rates of mortality compared to their female counterparts. For example, it has been suggested that, at least in PDD and DLB, female patients suffer from excess mortality (excess hazard ratio 1.45), but this may be due to the longer life expectancy of females in
the general population (Larsson, Torisson, & Londos, 2018). Similarly, levels of α-synuclein and the pathological load may also show sex differences based on the region analyzed and the specific subtype of Lewy body disorders. Female PD patients may show greater severity of pathology in their frontal lobes, amygdalae, and locus coeruleus (Iannuzzelli et al., 2020). In the plasma, a decrease in the soluble form of α-synuclein is seen only among male PD subjects, which may hint toward a greater load of intracellular, insoluble α-synuclein in their brains (Caranci et al., 2013). Consistent with these findings, unmedicated female PD patients showed ~38% slower decline and ~16% higher striatal dopamine, both of which contributed to a milder disease phenotype in women (Haaxma et al., 2007). It is important to note that women are more likely to suffer from an initial presentation of a tremor, and that this is a benign disease phenotype, suggestive of a milder progression of motor degeneration (Haaxma et al., 2007). In contrast, male PD patients may be more likely to present with bradykinesia and rigidity, which may indicate a more progressive decline (Cerri et al., 2019; Gottgens et al., 2020). In a neuroimaging study, de novo male PD patients showed greater brain degeneration and damage to white matter connectivity (Tremblay et al., 2020). In addition, microgliosis and neuron loss was more apparent in OB of men, but not women with PD (Flores-Cuadrado et al., 2021), indicative of sex dimorphisms in the mechanisms underlying PD pathology. In contrast to PD, female DLB patients tend to have lower levels of soluble α-synuclein in their CSF and worse outcomes, suggesting that the directionality of sex-specific effects in DLB may be different from PD (van de Beek et al., 2020).

Studies on sex differences in disease outcomes often show conflicting results as early-stage, drug naïve patients are seldom examined. Female PD patients on drugs such
as levodopa (precursor of dopamine) are more prone to drug-induced dyskinesias and motor and nonmotor fluctuations due to long-term therapy (Bjornestad et al., 2016; Eusebi et al., 2018; Hassin-Baer et al., 2011; Picillo et al., 2016; Zappia et al., 2005). Thus, early-stage, unmedicated patients of both sexes must be examined and as far as is feasible, longitudinal studies must be performed. In addition, many studies do not include age- and sex-matched healthy controls, and sex-biased baseline differences in behavior or endogenous defenses are often not taken into consideration. For example, female sex may be associated with better olfactory and cognitive outcomes, but worse depression and anxiety-like symptoms in PD (Khedr, Abdelrahman, Elserogy, Zaki, & Gamea, 2020; R. Liu et al., 2015). However, these sex differences in disease symptoms may be shaped by the findings that women, in general, outperform men on cognitive and olfactory tests, but are twice as likely to show depression-like symptoms (Georgiev, Hamberg, Hariz, Forsgren, & Hariz, 2017; Salk, Hyde, & Abramson, 2017; Sorokowski et al., 2019). Thus, many of the sex differences noted by authors in disease outcomes may not be specific to Lewy body disorders (Georgiev et al., 2017). In addition, women have up to 50% greater numbers of neurons and glia in their OB (Oliveira-Pinto et al., 2014) and greater dopamine transporters in their striata than men (Lavalaye, Booij, Reneman, Habraken, & van Royen, 2000; Mozley, Gur, Mozley, & Gur, 2001; Staley et al., 2001). Female (versus male) rodents may also show ~3-4 times greater dopaminergic neurons in some areas (Simerly, Swanson, & Gorski, 1985) and recent work showed that high levels of the vesicular glutamate transporter, (VGLUT), in dopaminergic neurons of the female sex may mediate sex differences in age-related neuronal vulnerability (Buck et al., 2021). In the nigra, men may show basally higher α-synuclein (Cantuti-Castelvetri et al., 2007), suggestive of
greater substrate available for seeding of pathology. These baseline sex differences are important to consider when assessing disease outcomes, pathological load, and extent of neurodegeneration in Lewy body disorders.

Additional factors confounding our understanding of sex differences in Lewy body disorders are that, 1) men may show more cumulative occupational exposures to environmental or industrial toxicants over their lifetime, men suffer from head traumas more than women, and the response to stress differs widely between men and women, all of which influence the risk of disease (Baldi et al., 2003; Bruns & Hauser, 2003; Gillies et al., 2014; Tanner, Goldman, Ross, & Grate, 2014), 2) men show, on average, a shorter lifespan by ~4.85 years (Crimmins, Shim, Zhang, & Kim, 2019) that must be accounted for in survival/mortality studies, 3) sex may influence the rate at which patients are “undercalled” or “overcalled” in the clinic and clinical diagnoses often do not match the neuropathological diagnoses at autopsy (P. T. Nelson et al., 2010), 4) female PD patients show worse self-reported disabilities, are not as likely to consult with specialists, and are less prone to hire formal caregivers (Abraham et al., 2019; Dahodwala et al., 2018), and, 5) women are generally underrepresented in neurology clinical trials (Steinberg et al., 2021; Tosserams et al., 2018), which likely contributes to the findings that women tend to be more prone to suffer from adverse drug reactions (e.g., levodopa-induced dyskinesias), as, data from men has been extrapolated to women (Zucker & Prendergast, 2020).

4.2.1. Contribution of genetic factors to sex bias in Lewy body disorders

Independent of sex hormones, genes on sex chromosomes may be responsible for male sex bias in Lewy body disorders, suggestive of intrinsic sex differences. This sex bias
may be due to specific genes present on the Y-chromosome (and thus only in males) or due to higher dose of those genes that are on the X-chromosome (producing greater X-linked effects in XX-chromosome carrying females) (Gillies et al., 2014). For example, it was shown that mice carrying the XX-chromosome had higher levels of prodynorphin in their striata, compared to XY-carrying males or XO-carrying female mice that had their second “X” chromosome experimentally deleted (X. Chen, Grisham, & Arnold, 2009). Prodynorphin was later shown to be linked to an increased risk of developing levodopa-induced dyskinesias—an adverse drug effect that, as mentioned above, is more likely to impact women (Hanrieder et al., 2011). Thus, some of the sex-biased effects in PD may be linked to X-linked genes that are not inactivated in females, producing a greater dosage of those genes (Smith & Dahodwala, 2014), however this idea is relatively under-researched. In contrast, a Y-linked gene, \textit{SRY}, was shown to be directly linked to PD in males, given its physiological function in regulating dopamine synthesis and motor behavior (Czech et al., 2012; Dewing et al., 2006). Early work on this gene suggested that its functions are limited to sex determination, as it is involved in forming the testes (Fechner, 1996). Later work revealed that \textit{SRY} is expressed in many tissues and organs, including within nigral dopaminergic neurons (Czech et al., 2012; Dewing et al., 2006). Apart from TH, \textit{SRY} also regulates enzymes responsible for dopamine synthesis and breakdown, such as DOPA decarboxylase, dopamine β-hydroxylase, and monoamine oxidase A (Czech et al., 2012; J. B. Wu, Chen, Li, Lau, & Shih, 2009).

It is possible, therefore, that high levels of \textit{Sry} stimulate dopamine breakdown, due to a \textit{Sry}-mediated increase in monoamine oxidase A (Smith & Dahodwala, 2014). Lee \textit{et al.} recently discovered that in response to parkinsonism-inducing neurotoxicants such as
6-OHDA, or to insecticides such as rotenone, male rodents show an abnormal increase in \textit{Sry} in the nigra, and that this upregulation precedes the loss of TH in the nigra (J. Lee et al., 2019). Silencing this upregulated \textit{Sry} lowered motor deficits and dopaminergic neuron loss by mitigating inflammation, DNA damage, apoptosis, and mitochondrial dyshomeostasis (J. Lee et al., 2019). Notably, the authors found that silencing \textit{Sry} in the healthy nigra induced motor defects and reduced TH (J. Lee et al., 2019), suggesting that levels of \textit{Sry} must be fine-tuned to prevent the toxicities related to its loss in the healthy male brain and its upregulation in the diseased male brain. No effects of silencing \textit{Sry} were seen in females, as they do not express this gene, suggesting that, at least in their PD model, the male sex-specific defects were being mediated by a male sex-specific expression of \textit{Sry} (J. Lee et al., 2019). Thus, the male-specific expression of the dopamine regulator, \textit{SRY} in the nigra (and not the gonads) may underlie the male sex bias in risk of PD. These studies strengthen prior work showing that sex differences in the brain are not inevitably mediated by sex steroids, since brain cells harvested before the \textit{in vivo} surge in secretion of sex hormones (\textit{i.e.}, at early embryonic stages) maintain their sex differences when grown in culture (Carruth, Reisert, & Arnold, 2002). In this regard, the \textit{Sry} gene appears to be a leading candidate underlying brain sex differences, particularly of mesencephalic dopaminergic systems (Carruth et al., 2002).

Apart from differences in genes on sex chromosomes, there may also be baseline sex differences in genes present on autosomes. As mentioned above, Cantuti-Castelvetri \textit{et al.} discovered higher expression of the \textit{α}-synuclein gene in the male nigra (Cantuti-Castelvetri et al., 2007). In addition, the authors reported higher expression of the \textit{PINK1} gene in the male nigra, and mutations in \textit{PINK1} underlie a small proportion of PD cases.
Simunovic et al. evaluated dopamine neurons from brains of normal postmortem humans of both sexes (Simunovic, Yi, Wang, Stephens, & Sonntag, 2010) and an upregulation of genes that control metabolic processes, calcium homeostasis, and mitochondrial health was seen in males (Simunovic et al., 2010). The authors suggested that this may be linked to the propensity of dopamine neurons to harbor high metabolic burden, which ultimately leads to an increase in PD risk (Simunovic et al., 2010).

Despite some of this work, only a few studies have extensively examined the role of autosomal genes in determining the sex bias in PD risk, and recent work suggests that gene differences in the autosomes of males and females do not underlie sex differences in PD (Blauwendraat et al., 2021). A potential caveat of almost all studies examining the cause underlying sex-biased disease risk is that environmental factors and lifestyle may modify the epigenetic signature. It is a challenge to tease apart if sex differences arise due to the intrinsic sex of the organism or due to extrinsic cues that may be more strongly linked to one sex and so more likely to impact gene regulation in that sex (Blauwendraat et al., 2021; Gillies et al., 2014; Kaminsky, Wang, & Petronis, 2006; Oliva et al., 2020).

4.2.2. Contribution of sex hormones to sex bias in Lewy body disorders

The protective role of estrogen in Lewy body disorders has been widely studied, with an obvious focus on its protective effects among women. Recently, it was revealed that for every 1-year increase in a woman’s menopausal age, the risk of PD decreases (odds ratio: 0.84) (Kusters et al., 2021). Conversely, removal of the ovaries increases the risk of PD (Canonico et al., 2021; Rocca et al., 2008) and symptoms of PD are worse during the
menstrual phase, at which estrogen levels are lowest (N. P. Quinn & Marsden, 1986). Consistent with these observations, women with PD may go through menopause at a younger age (at ~46 years of age or less), and estrogen use among postmenopausal women with PD is associated with a lower risk of developing dementia (Benedetti et al., 2001; Marder et al., 1998; Nitkowska, Czyzyk, & Friedman, 2014). Apart from age at menopause and reproductive lifespan, parity—defined as the number of times a woman gives birth—was also positively correlated with age at onset, such that for every child that is born, the age at onset of PD for the mother increases by ~2.7 years (Haaxma et al., 2007). Women on oral contraceptive pills may also be at a lower risk of PD, but only if the use of the contraceptive medication was for more than 10 years (R. Liu et al., 2014). Even among male PD patients (versus control males), a significantly lower level of estrogen was noted in the plasma and, adding to the evidence on the beneficial effects of estrogen, a positive correlation was found between blood estrogen and mood/quality of life in men with PD (Nitkowska, Tomasiuk, Czyzyk, & Friedman, 2015). It is also known that loss of aromatases leads to impairment of the dopaminergic nigrostriatal pathway in response to exposure to parkinsonism-inducing dopaminergic neurotoxicants and single nucleotide polymorphisms in aromatases may also increase the susceptibility to PD (S. J. Chung et al., 2011; Morale et al., 2008). These findings with aromatases are consistent with the protective effects of estrogens in Lewy body disorders, as aromatases are the enzymes involved in estrogen synthesis.

Notably, not all studies show that estrogen use is protective in PD. For example, its postmenopausal use depends on the type of menopause, with a study showing that estrogen use after a surgically induced, but not naturally occurring menopause, increases the risk of
PD by ~2.6-fold (Popat et al., 2005). Our understanding of the role of estrogen in PD is confounded, perhaps because, 1) most of the studies are retrospective and not longitudinally performed pre- and post-menopause, 2) a wide-ranging dose of hormone replacement therapy has been used in clinical trials, 3) effects may be dependent on the type of estrogen used, as estriol, for example, can have greater anti-aggregation effects on α-synuclein compared to other estrogens, and synthetic estrogens also have differential effects on disease (Hirohata, Ono, Morinaga, Ikeda, & Yamada, 2009; Naciff et al., 2002), and, finally 4) the time at which the therapy is administered has shown divergent outcomes, as Haaxma et al. found no benefit of estrogen after PD reaches a clinical stage (Gillies et al., 2014; Haaxma et al., 2007; I. N. Miller & Cronin-Golomb, 2010). These latter findings are supported by work in parkinsonian animal models, wherein estrogen was only protective when initiated prior to, and not after nigrostriatal degeneration (Dluzen, 1997; Gajjar, Anderson, & Dluzen, 2003; X. Gao & Dluzen, 2001). It is noteworthy that the number of studies showing conflicting results are outweighed by those that support the protective effects of estrogen in PD. Estrogen is a pleiotropic hormone and its mechanism of action in PD remains unclear. Effects of estrogen in PD may be by its protective role in maintaining lipid and mitochondrial homeostasis, in facilitating dopamine synthesis, in mitigating neuroinflammation, as an antioxidant, in supporting calcium buffering capacities, in iron metabolism, etc. (Cerri et al., 2019).

Estrogens are also known to inhibit the aggregation of α-synuclein and to disrupt α-synuclein fibril formation, suggesting that estrogens may exert beneficial effects in α-synucleinopathies (Hirohata et al., 2009). Recently, Lee et al. discovered that the ligand binding domain of estrogen receptor α (ERLBD) prevents the amyloid fibril formation of
α-synuclein, by directly binding to α-synuclein, whether or not estradiol was present as an agonist of ERα (D. Lee et al., 2020). Conversely, antagonizing ERα with tamoxifen prevented ERLBD-mediated inhibition of α-synuclein fibrillization (D. Lee et al., 2020). Thus, apart from estradiol, some fibril destabilizing effects may also be mediated by the estrogen receptor and designing novel therapies that mimic the protein sequence and function of the ERLBD may be beneficial in Lewy body disorders. As mentioned previously, stabilizing the tetrameric form of α-synuclein, and maintaining a balance between the tetramers and monomers may be vital in preventing the aggregation of α-synuclein. In a recently described transgenic mouse model of PD (Nuber et al., 2018), female mice had a delayed age at onset of motor deficits compared to male mice (Rajsombath, Nam, Ericsson, & Nuber, 2019). It was suggested that this sex difference may be due to higher levels of tetrameric α-synuclein in female (versus) male mice and lesser loss of dopaminergic and cortical fiber integrities in females (Rajsombath et al., 2019). Finally, the authors tested if the sex differences were mediated by estrogen and found that estradiol exposure in these mice increases the proportion of the more stable tetramers, improves autophagic capacities, reduces motor deficits, and strengthens dopaminergic and cortical fibers (Rajsombath et al., 2019). This work suggests that the anti-aggregation properties of estrogen (Hirohata et al., 2009) may be due to its ability to stabilize α-synuclein as a tetramer (Rajsombath et al., 2019).

Although limited, there is evidence suggesting that metabolism of progesterone may be impaired in the nigra of PD patients—a region with basally high progesterone turnover due to reasons that remain unknown (Bixo, Andersson, Winblad, Purdy, & Backstrom, 1997; Cerri et al., 2019; Luchetti, Bossers, Frajese, & Swaab, 2010). In
addition, many of the metabolites of progesterone were found to be present at low levels in the CSF and blood of PD patients, due to a dysregulation in enzymes—such as 5α-reductase—responsible for the normal breakdown of progesterone to its active metabolites (Cerri et al., 2019; di Michele et al., 2003; Luchetti et al., 2010; Luchetti et al., 2006). Progesterone, and its metabolites, such as allopregnanolone, have been tested as a therapy in toxicant models of parkinsonism. These studies have shown that progesterone protects against loss of dopamine and dopaminergic neurons, improves dopamine binding, and mitigates motor and nonmotor deficits (Adeosun et al., 2012; Bourque et al., 2016; Casas et al., 2011; Grandbois, Morissette, Callier, & Di Paolo, 2000; Smith & Dahodwala, 2014).

If sex hormones in females are protective against PD-related toxicities, one may speculate that testosterone in males may be implicated in inducing PD-related toxicities, given the male sex bias in PD. Indeed, exposure to androgenic compounds does not exert neuroprotective effects in toxicant models and, in some studies, may even exacerbate the loss of dopamine (Ekue, Boulanger, Morissette, & Di Paolo, 2002; Gillies & McArthur, 2010; Lewis & Dluzen, 2008; Smith & Dahodwala, 2014). In contrast, testosterone may mitigate motor deficits in gonadectomized male mice exposed to a PD-inducing toxicant, but in those studies testosterone was administered after gonadectomy and prior to the neurotoxicant, so it is unknown if testosterone may be used as a therapeutic intervention in disease (Antzoulatos, Jakowec, Petzinger, & Wood, 2010). In a clinical trial examining testosterone supplements as a therapy in male PD subjects, no significant improvements were reported (Okun et al., 2006). Some studies suggest that the potential neurotoxicity of testosterone in males may be attributed to its local aromatization to estrogens. Thus, there may be sex dimorphisms in the effects of endogenous estrogens—producing
neuroprotective effects in females, but potential toxicity in males (Gillies & McArthur, 2010; Gillies et al., 2014; Thanky, Son, & Herbison, 2002). However, this idea might be overly simplistic, given that these studies examined the role of estrogen in adult males that had been castrated and only the nigrostriatal pathway or the locus coeruleus was investigated. Locally aromatized estradiol in the hypothalamus of male rodents at early development is known to play critical roles in masculinization, male sex behaviors, and in brain sexual differentiation, suggesting that the physiological function of estrogens in males must not be discounted (Lenz & McCarthy, 2015; Lenz, Nugent, Haliyur, & McCarthy, 2013; Schwarz, Sholar, & Bilbo, 2012). This latter concept is explained in detail in Aim 3. Irrespective of the nature of the sex steroid, the early and often perinatal exposure to sex hormones may prime brain cells, such that the cells maintain their sex-specific phenotypes, even when isolated at adult stages and grown in culture or even when transplanted into brains of the opposite sex, as was recently shown for microglia (Villa et al., 2018).

5. Preclinical models of Lewy body disorders.

Early tools to model Lewy body disorders included the use of neurotoxicants (e.g., 6-hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, etc.), pesticides or herbicides (e.g., rotenone, paraquat, etc.), and proteasome inhibitors (e.g., lactacystin, MG132, PSI, etc.) (Bentea, Verbruggen, & Massie, 2017; Bove et al., 2005; Chia, Tan, & Chao, 2020; Jagmag, Tripathi, Shukla, Maiti, & Khurana, 2015). However, these models do not recapitulate classic features of the disease, may produce toxicities unrelated to the underlying pathologies, do not mimic the slowly progressive form of neurodegeneration,
and, most notably, many of these toxicants do not reproducibly lead to hallmark Lewy inclusions containing phosphorylated α-synuclein. The discovery that α-synuclein is a major protein present in Lewy inclusions (Spillantini et al., 1997) led to the development of gene-based models using either wildtype or mutated Snca, in which α-synuclein was overexpressed at germline or was introduced stereotaxically using viral vectors (Duffy, Collier, Patterson, Kemp, Fischer, et al., 2018). Although these models were better than the toxicant models at mimicking human PD and provided several insights into the mechanisms of α-synuclein-mediated toxicity, many disadvantages of these models have also been reported. For example, promoters used to drive gene expression may lead to α-synuclein overexpression in areas that do not develop extensive pathology in the clinical condition (Visanji et al., 2016). Second, these models rely on increasing the levels of α-synuclein, sometimes by as much as ~30-fold (Duffy, Collier, Patterson, Kemp, Fischer, et al., 2018; Oliveras-Salva et al., 2013; Volpicelli-Daley, Kirik, Stoyka, Standaert, & Harms, 2016). Supraphysiological increase in α-synuclein concentrations may be a key feature of familial PD, but this only accounts for ~10% of all PD cases (Thomas & Beal, 2007). The remaining ~90% of cases are idiopathic/sporadic (Cannon & Greenamyre, 2013), in which high levels of α-synuclein are not a classic feature (Duffy, Collier, Patterson, Kemp, Fischer, et al., 2018; Volpicelli-Daley et al., 2016). Third, these models have only been used to target specific circuitry and the pathology that is formed is not widespread and does not mimic human LBs in terms of the morphology, biochemistry, and subcellular localization (Duffy, Collier, Patterson, Kemp, Fischer, et al., 2018; Gomez-Benito et al., 2020; Volpicelli-Daley et al., 2016).
5.1. The preformed α-synuclein fibril (PFF) model of Lewy body disorders

To mitigate some of the abovementioned caveats, the preformed α-synuclein fibril (PFF) model was developed, wherein the α-synuclein fibrils generate robust pathological inclusions containing phosphorylated α-synuclein and other known components of Lewy bodies (Luk et al., 2009; Volpicelli-Daley et al., 2011). Notably, the α-synuclein fibrils elicit pathology in wildtype cells, without the need to introduce any other cofactors or to overexpress α-synuclein above physiological levels (Volpicelli-Daley et al., 2011). In addition, prior work showed that the α-synuclein fibril-seeded pathology was present in many disease-relevant areas, led to progressive neurodegeneration, and behavior deficits (Luk, Kehm, Carroll, et al., 2012; Luk, Kehm, Zhang, et al., 2012).

α-Synuclein fibrils are generated by agitating recombinant α-synuclein monomers (Luk et al., 2009). Before use, the PFFs must be sonicated to produce shorter fibrils that should be, on average, less than 50 nm in size to elicit the formation of robust inclusion pathology (Volpicelli-Daley et al., 2016). This is a self-amplifying model; the PFFs act as “seeds”, leading to the recruitment, phosphorylation, and aggregation of endogenous α-synuclein, which is a prerequisite for this model, as the PFFs are not phosphorylated and do not induce cell loss or pathology in systems wherein α-synuclein is knocked out (Luk, Kehm, Zhang, et al., 2012; Volpicelli-Daley, Luk, & Lee, 2014; Volpicelli-Daley et al., 2011). Furthermore, the inclusions and PFFs can propagate and spread through neural circuitry in a staged manner and so this model has been used to empirically test many of the original hypotheses about the patterns of spread of pathology in Lewy body disorders.

Some of the earliest studies in this model involved intrastriatal injections of PFFs to mimic dopaminergic cell loss, motor deficits, and classic PD pathology (Luk, Kehm,
Carroll, et al., 2012). Over the past decade, several groups have independently verified this model in different in vitro and in vivo platforms and the findings have been largely consistent and reproducible. Apart from the PD-relevant nigrostriatal pathway, PFFs have been injected into the eye, gut, OB, AON, hippocampus, cortex, etc. and the outcomes from some of these studies were recently reviewed (Polinski, 2021). Although early work in this model was only performed in male animals, recent work has included sex as a biological variable, furthering the translational value of this model. For example, Mason et al. injected PFFs (in parallel) into the AON of male and female mice and found that PFF-injected males developed greater pathology, olfactory impairments, and cell loss as compared to age-matched, PFF-injected females (Mason et al., 2016; Mason et al., 2019). In addition, the fibril-seeded pathology in the AON remained confined to the limbic connectome (Mason et al., 2016; Mason et al., 2019). Thus, this model by Mason et al. was the first to mimic some of the sex differences that are seen in the clinical condition and recapitulated some aspects of Beach’s Stage IIb, limbic-predominant Lewy body disease (Beach, Adler, et al., 2009; Mason et al., 2016; Mason et al., 2019). Given that PFF-seeded pathology in the AON did not have major effects outside the limbic system and elicited only mild cell loss, the model by Mason et al. may be that of early disease and may thus be useful to test therapies in a sex-matched manner, as interventions may be more successful prior to massive cell loss that normally occurs at latter disease stages. Although other recently published studies in the PFF model have incorporated animals of both sexes (Johnson et al., 2020; M. E. Johnson et al., 2021), it is important to note that these studies did not test for statistical interactions with sex as one variable—a practice that may be critical for reporting of sex differences (Garcia-Sifuentes & Maney, 2021).
Limitations to this model that have recently been reported. First, experimental variations and a lack of validation of new stocks or sources of PFFs between and within labs may lead to conflicting results, although there are several guidelines that have been published with an aim to standardize practices to be employed while using PFFs (Duffy, Collier, Patterson, Kemp, Fischer, et al., 2018; Patterson et al., 2019; Polinski et al., 2018a; Volpicelli-Daley et al., 2014). Second, it is unclear if the PFFs are indeed the pathological “strain” of α-synuclein in the clinical condition (Oliveira et al., 2021) and if it is the fibrillar or non-fibrillar form of α-synuclein that is enriched within human LBs (Lashuel, 2020; Shahmoradian et al., 2019). Third, it is also not clear if the inflammatory response triggered by the exogenously added PFFs is an experimental artifact or if it also underlies human disease (Gomez-Benito et al., 2020; Oliveira et al., 2021). Fourth, it is a matter of contention if formation of the more mature inclusions via sequestration of aggregates in this model is a toxic or protective response, as it may be better to localize the aggregates to a part of an already diseased cell, thereby preventing their transmission to healthier cells (Beyer, Domingo-Sabat, & Ariza, 2009; Chartier & Duyckaerts, 2018; Mahul-Mellier et al., 2020; Olanow, Perl, DeMartino, & McNaught, 2004; M. Tanaka et al., 2004). Fifth, the exact mechanism by which PFFs are taken up by cells is uncertain, although studies show that it could be mediated by direct uptake, by clathrin- or adsorptive-mediated endocytosis, by binding to molecules such as heparan sulfate proteoglycans, or by receptors such as the lymphocyte-activation gene 3 (LAG3) receptor (Emmenegger et al., 2021; Hijaz & Volpicelli-Daley, 2020; Holmes et al., 2013; Ihse et al., 2017; Mao et al., 2016; S. H. Oh et al., 2016; Rodriguez, Marano, & Tandon, 2018; Volpicelli-Daley et al., 2011; Q. Zhang et al., 2020; S. Zhang et al., 2021; X. Zhang et al., 2020).
Despite these limitations, the findings from this model have provided insights into the mechanisms underlying the classic neuropathology and neurodegeneration in Lewy body disorders. Importantly, given that this model provides better spatiotemporal control and is more translatable than prior methods, it has been used to test novel, disease-modifying therapeutic approaches. Specifically, there is a need for therapies that facilitate the degradation of pathological inclusions once they form within neurons, in response to the triggering event—such as PFFs in this model. Furthermore, inclusion-laden neurons may extrude PFF seeds or aggregates into the extracellular space, thereby transmitting pathology to nearby, otherwise healthy neurons. For this cell-to-cell spread to occur, inclusions and/or fibrils would have to at least transiently be present extracellularly, wherein they may be more accessible to therapies that can destroy these aggregates while they are in transit (Visanji et al., 2016). These scenarios are also not mutually exclusive as impairments and cell loss mediated by the initially intrinsic formation of pathology within selected, vulnerable cells may be amplified due to the progressive involvement of cells or regions that were formerly unaffected. In this dissertation, we propose that chaperone proteins and neuroglial cells harbor defenses that may be exploited to mitigate the onslaught of α-synuclein pathology, at both, intracellular and extracellular levels, thereby reducing the deficits associated with Lewy body disorders.

6. Chaperone proteins as modifiers of α-synucleinopathy

To deal with protein misfolding stress, such as α-synuclein pathology in Lewy body disorders, cells have evolved multiple defenses. One such mechanism is a network of chaperones and co-chaperones that can refold misfolded proteins. Alternatively, if the
protein cannot be repaired, chaperones and co-chaperones assist in the degradation and clearance of the misfolded proteins. In other words, these molecules play a major role in protein “triage” and changes in their expression or function may exemplify ageing and neurological disease (Hohfeld, Cyr, & Patterson, 2001; Lanneau, Wettstein, Bonniaud, & Garrido, 2010). For example, it is known that pro-folding chaperones (ATP-dependent) decrease with age, but that ageing is associated with a rise in the pro-holding chaperones (ATP-independent) (Brehme et al., 2014; de Graff, Mosedale, Sharp, Dill, & Grainger, 2020). It is known that holding chaperones prevent protein aggregation, but do not actively refold misfolded proteins. Thus, ageing may be associated with a change in the balance of foldases vs. holdases, with holdases being predominant in older model systems perhaps as an energy-conserving adaptive process (Brehme et al., 2014; de Graff et al., 2020).

Heat shock proteins (Hsps) are the most commonly studied chaperones and they are evolutionarily conserved across diverse species, are highly expressed in long-lived species, and genes for Hsps are termed “vitagenes” as they are critical for survival and longevity (Brodsky & Chiosis, 2006; Calabrese et al., 2011; Calabrese, Cornelius, Dinkova-Kostova, & Calabrese, 2009; Leak, 2014; Salway, Gallagher, Page, & Stuart, 2011). Hsps may be induced as a cytoprotective response to cellular stress and, in humans, ~180 chaperones have been identified (Freilich, Arhar, Abrams, & Gestwicki, 2018), which are classified into multiple groups based on their molecular weights.

Small heat shock proteins, such as Hsp27 or αβ-crystallin, are known to bind and inhibit the aggregation and toxicity of α-synuclein fibrils (Cox & Ecroyd, 2017; Cox et al., 2018; Outeiro et al., 2006; Vicente Miranda et al., 2020; Zourlidou, Payne Smith, & Latchman, 2004). However, it is also known that small Hsps only transiently and weakly
interact with α-synuclein fibrils, and they may only be effective at inhibiting the fibrils if the aggregation-kinetics are slow (Cox, Selig, Griffin, Carver, & Ecroyd, 2016) or if they are applied with other chaperones (Jia et al., 2019). In the latter study, Hsp104 was one of the chaperones that synergized with Hsp27 to prevent α-synuclein from aggregating (Jia et al., 2019). Hsp104 reduces dopaminergic neurodegeneration in preclinical PD (Lo Bianco et al., 2008), and Mack et al. have developed substrate-specific Hsp104 variants that can reduce the toxicity associated with α-synuclein (Mack et al., 2020). Notably, Hsp104 may not work in isolation, and several studies suggest that it forms a complex with Hsp70 and Hsp40 to fulfill its normal functions (Lo Bianco et al., 2008; Shorter, 2011; Tittelmeier et al., 2020). In addition, Hsp104 does not appear to be present in metazoans (Lo Bianco et al., 2008) and some reports suggest that mammalian Hsp110 is analogous to Hsp104 (Shorter, 2011) and, like Hsp104, may be efficacious only when combined with Hsp70 and Hsp40 (Shorter, 2011; Wentink et al., 2020), perhaps because Hsp110 is a nucleotide exchange factor in the Hsp70 disaggregation machinery (Yakubu & Morano, 2021). Recent work showed that Hsp110 also reduces pathology in culture and in animal models of α-synucleinopathy (Y. V. Taguchi et al., 2019). Overall, there are many Hsps that cooperate to mitigate the toxicity linked with misfolded α-synuclein. However, many of these Hsps are also found within human LBs (Auluck, Chan, Trojanowski, Lee, & Bonini, 2002; Leverenz et al., 2007; McLean et al., 2002; Outeiro et al., 2006). It is unclear if this is an entrapment of Hsps within inclusions, but if this is the case, there may be a depletion of the soluble form of Hsps in the cytosol, which may lead to a loss in the normal function of Hsps. Thus, it is possible that Lewy pathology may be toxic because of a direct effect of the inclusions, or the toxicity may be due to an indirect effect of loss of protein quality.
control. Alternatively, presence of Hsps within inclusions may be a protective response to counter the proteotoxic stress of LBs, but this has been difficult to tease apart, given the functional redundancies and compensatory changes seen among some groups of Hsps. In fact, compensatory changes among Hsp families have even been exploited for therapeutic purposes. For example, loss of Hsp90 induces Hsp70 by freeing its precursor—the transcription factor, heat shock factor 1 (HSF1)—which is otherwise maintained in a tight complex with Hsp90 (Bharadwaj, Ali, & Ovsenek, 1999; Lackie et al., 2017). Pharmacological inhibitors of Hsp90 (e.g., Geldanamycin or its analogue, 17-AAG) are known to induce Hsp70 and have thus been shown to reduce α-synuclein aggregation in preclinical models (McLean, Klucken, Shin, & Hyman, 2004; Riedel, Goldbaum, Schwarz, Schmitt, & Richter-Landsberg, 2010). One must note that these (along with other) Hsp90 inhibitors suffer from low blood brain barrier (BBB) penetrability, off-target effects, low oral bioavailability, and hepatotoxicity, making them less likely to succeed in the clinic (Chiosis & Tao, 2006; Cysyk et al., 2006; Hay et al., 2004; Kalia, Kalia, & McLean, 2010; Supko, Hickman, Grever, & Malspeis, 1995). Thus, there is a need for new approaches to increase Hsp70 levels and function in disease.

Hsp70 is present in a constitutive (Hsc70) as well as stress inducible (Hsp70) form and is perhaps the most well-studied chaperone in neurodegeneration. It is known to work in concert with co-chaperones (e.g., Hsp40) and co-factors (e.g., ATP) in a tightly regulated cycle to refold misfolded proteins. Hsp70 is composed of two domains: a ~43 kDa N-terminal ATP binding domain and a ~25 kDa C-terminal substrate binding domain (Lackie et al., 2017; S. Wu et al., 2020). To fulfill its chaperone functions, ATP hydrolysis is a rate-limiting step—a process that can be stimulated by the co-chaperone, Hsp40 (Minami,
Hohfeld, Ohtsuka, & Hartl, 1996; Misselwitz, Staeck, & Rapoport, 1998). A misfolded protein is delivered by Hsp40 to Hsp70, which results in stimulation of the ATPase activities and hydrolysis of ATP to ADP (Lackie et al., 2017; Misselwitz et al., 1998). This ADP-bound state stabilizes the interaction between the client and Hsp70 (Lackie et al., 2017). Next, a nucleotide exchange factor exchanges ADP for ATP. Binding of ATP “opens” the substrate binding domain to release the client protein, but the protein can re-enter the chaperone cycle if it was not correctly folded (Lackie et al., 2017; Misselwitz et al., 1998; Schlecht, Erbse, Bukau, & Mayer, 2011).

In stressed cells, there is an early and strong induction of Hsp70, and studies show that Hsp70 is also anti-apoptotic (Daugaard, Rohde, & Jaattela, 2007; Evans, Chang, & Gestwicki, 2010; Jaattela, Wissing, Kokholm, Kallunki, & Egeblad, 1998; Radons, 2016; Ravagnan et al., 2001). Posimo et al. found that Hsp70 is upregulated in allocortical neurons exposed to the proteotoxicity of proteasome inhibition and that this may be a protective response as inhibition of Hsp70/Hsc70 activities synergistically worsened the toxicity of proteasome inhibitors (J. M. Posimo et al., 2015). Apart from the allocortex, neurons in the OB may also rely on Hsp70/Hsc70 defenses to maintain proteostasis after exposure to proteasome inhibitors (Crum et al., 2015). It is worthy to note that both the allocortex and OB develop pathology relatively early in PD and other Lewy body disorders (Braak et al., 2003; Braak, Rub, Schultz, & Del Tredici, 2006). These and other brain regions may show pathology in Lewy body disorders because Hsp70 defenses may not be maximally engaged if they are entrapped within inclusions (Auluck et al., 2002; Leverenz et al., 2007; McLean et al., 2002; Outeiro et al., 2006), or because the heat shock response may not be effectively induced by α-synuclein aggregates (San Gil et al., 2020). In addition,
the disease could induce a loss in Hsc70 levels, as is seen in some brain areas of PD patients (Alvarez-Erviti et al., 2010; Chu, Dodiya, Aebischer, Olanow, & Kordower, 2009; Mandel et al., 2005), or there may be a loss in Hsp70 with natural ageing, as is seen in olfactory neurons of otherwise healthy humans (Getchell, Krishna, Dhooper, Sparks, & Getchell, 1995). Finally, some Hsp70 gene variants that increase the risk of PD have also been identified (Y. R. Wu et al., 2004). Thus, all these studies shed light on the impact of faulty Hsp70/Hsc70 defenses on α-synucleinopathies.

There is a vast body of literature showing that Hsp70 binds and interacts with α-synuclein, inhibiting the associated toxicity and preventing the fibrillization, elongation, oligomerization, and aggregation of α-synuclein (Aprile, Arosio, et al., 2017; Auluck et al., 2002; Danzer et al., 2011; Dedmon, Christodoulou, Wilson, & Dobson, 2005; Kalia et al., 2010; Kilpatrick et al., 2013a; Klucken, Shin, Masliah, Hyman, & McLean, 2004; Luk, Mills, Trojanowski, & Lee, 2008; Muchowski & Wacker, 2005; Tao et al., 2021; Wentink et al., 2020). Notably, Hsp70 directly recognizes, inhibits, and interacts with an expanse of hydrophobic residues present at the center of α-synuclein—the NAC core region—and this is the region that participates in α-synuclein fibrillization (Giasson, Murray, Trojanowski, & Lee, 2001; Luk et al., 2008; Ueda et al., 1993). In line with this, studies show that inhibition of Hsp70 activity increases the aggregation of α-synuclein (Aprile, Kallstig, et al., 2017; Kilpatrick et al., 2013a) and, conversely, that agonists of Hsp70 function decrease the aggregation of α-synuclein (Chiang et al., 2019; Kilpatrick et al., 2013a), at least in cell lines and using pharmacological tools. In contrast to this work, Shimshek et al. showed that overexpression of Hsp70 in mice that co-overexpress the mutant form of α-synuclein produces no beneficial effects (Shimshek, Mueller, Wiessner, Schweizer, & van der Putten,
2010). The authors speculated that this may be because Hsp70 is inadequate to inhibit the toxic sequelae associated with α-synuclein, but one must also note that, as mentioned in previous sections, overexpression models are not always translatable and are thus not the best to test therapeutic potential of interventions.

As mentioned previously, an ideal therapeutic agent with clinical benefit would be one that can inhibit α-synuclein in the extracellular compartment (preventing its trans-synaptic spread), apart from inhibiting the pathology formed in situ. There is considerable evidence in the literature showing that Hsp70 can be released extracellularly, where it may be able to chaperone α-synuclein (Calderwood, Mambula, Gray, & Theriault, 2007; Danzer et al., 2011; De Maio, 2014; Dukay, Csobož, & Toth, 2019; Multhoff, 2007; Tytell, 2005). Extracellular Hsp70 may also be a “chaperokine” (Asea, 2008), serving immunoregulatory roles by improving the phagocytic capabilities of glia or macrophages, reducing proinflammatory gene expression, priming innate and adaptive immune cells, etc. (Dukay et al., 2019; Kakimura et al., 2002; Kovalchin et al., 2006; Multhoff, 2007; Yu et al., 2018). In the extracellular space, Hsp70 exists in diverse forms—in exosomes, as a freely soluble protein, bound to other molecules, etc. (Radons, 2016). Although there is not one putative “Hsp70 receptor”, there are several cell-surface receptors to which extracellular Hsp70 can bind, such as glucocorticoid receptors, toll like receptors (TLR2, TLR4), scavenger receptors (LOX-1, SREC-1), chemokine receptors (CCR5), as well as CD40, CD91, etc. (Alberti et al., 2021; Calderwood et al., 2007; Dukay et al., 2019; Petta et al., 2016; Vandevyver, Dejager, & Libert, 2012) and these are present on several cells, including neurons, microglia, and macrophages. It is known that extracellular Hsp70 activates microglia in a TLR4-dependent manner and can stimulate the internalization of amyloid-β.
by microglial cells (Kakimura et al., 2002). Thus, apart from its role as an intracellular chaperone, Hsp70 may also facilitate the engulfment of protein aggregates by neuroglia, which may be protective as it may help divert aggregates away from the more vulnerable and postmitotic neurons.

Exogenous, recombinant Hsp70 (eHsp70) has been supplied to cells and animals. Several groups have reported beneficial effects of eHsp70 in models of diabetes, stroke, epilepsy, AD, parkinsonism, dementia, and ageing (Bobkova et al., 2015; Bobkova et al., 2014; Demyanenko et al., 2020; Ekimova et al., 2010; M. Evgen'ev et al., 2019; M. B. Evgen'ev et al., 2017; Pastukhov, Plaksina, Lapshina, Guzhova, & Ekimova, 2014; Tiefensee Ribeiro et al., 2021; Tytell et al., 2018). In some of this work, eHsp70 not only reduced cell loss, neuropathology, and deficits associated with disease, but also extended survival (Bobkova et al., 2015). Notably, sequencing studies suggest that in a mouse model of AD, eHsp70 upregulates genes related to the adaptive immune response, antigen presentation and processing, activation of the immunoproteasome, leukocytosis, etc. (M. Evgen'ev et al., 2019; M. B. Evgen'ev et al., 2017). Other studies have shown that eHsp70 increases synaptic proteins and reduces markers of ageing (e.g., lipofuscin), inflammation, and senescence (Bobkova et al., 2015; Ekimova et al., 2010; Tiefensee Ribeiro et al., 2021). These studies thus indicate that Hsp70 is a pleiotropic protein, and it exerts protective effects in both, the intracellular and extracellular compartments.

Thus, the therapeutic potential of Hsp70 in α-synucleinopathies is expected to be two-pronged: 1) It may reduce α-synuclein\(^+\) inclusions in situ, and 2) it may reduce the transcellular spread of α-synuclein or its aggregates. We focused on both these aspects of Hsp70 in Aims 1-2 of this dissertation.
7. **Glia as modifiers of α-synucleinopathy**

Originally described as supportive cells of the nervous system, the diverse roles of neuroglia (Greek: glue) or *nervenkitt* (German: nerve glue/nerve putty) are now being increasingly acknowledged (Allen & Barres, 2009; Barres, 2008; Somjen, 1988). Glia make up ~90% of all human brain cells and have multifaceted roles, which include providing trophic support, maintaining metabolic function, assisting in synaptic pruning and plasticity, developing and preserving neural circuitry, as well as phagocytosis and clearance of pathogens, *etc.* (Jakel & Dimou, 2017). In neurodegenerative diseases, glia are important players, serving protective or detrimental roles (F. C. Bennett & Sloan, 2021; Gleichman & Carmichael, 2020; Sheeler et al., 2020). For example, in Lewy body disorders, astroglia are known to harbor α-synuclein+ inclusions in a staged manner that resembles the pathology seen in nearby neurons (Braak, Sastre, & Del Tredici, 2007; Wakabayashi, Hayashi, Yoshimoto, Kudo, & Takahashi, 2000). Astroglia are not major producers of α-synuclein (Kam, Hinkle, Dawson, & Dawson, 2020; H. J. Lee et al., 2010; Y. Zhang et al., 2014; Y. Zhang et al., 2016) and so one might assume that presence of pathology within these glia might be a protective response as astrocytes may engulf these aggregates from the extracellular space after their extrusion from diseased neurons (Loria et al., 2017). In contrast, some studies suggest that astroglia may be “carriers” of α-synuclein pathology, potentially worsening disease (H. J. Lee et al., 2010; Sorrentino et al., 2019). In either case, it is likely that the complete absence of astrocytes from the diseased brain may actually *worsen* neuronal injury given their largely neuroprotective roles (Bhatia et al., 2019), and harnessing their supportive functions may hold therapeutic value. Apart
from astroglia, oligodendroglia are the other “macroglia” in the brain and are primarily
tasked with axonal myelination (Simons & Nave, 2015). In multiple system atrophy, α-
synuclein+ inclusions form within oligodendroglia and not neurons—unlike in Lewy body
disorders (Norris, Giasson, & Lee, 2004; C. Peng et al., 2018). However, even in Lewy
body disorders, Braak has suggested that inadequately myelinated neurons may show a
greater propensity to develop Lewy inclusions (Braak & Del Tredici, 2004). Thus, although
macroglia express low levels of α-synuclein, their presence or absence may influence the
response to pathology underlying Lewy body disorders.

The focus of this dissertation is on microglia, which belong to the mesodermal
lineage, in contrast to astroglia and oligodendroglia, which are neuroectodermal in origin
(Chan, Kohsaka, & Rezaie, 2007). Microglia enter and colonize the brain during early
developmental stages (between the 4th and 24th week of human gestation), prior to BBB
closure (Menassa & Gomez-Nicola, 2018). As innate immune cells, microglia may be the
first line of defense against brain diseases. Microglia also have surveillance properties as
they can detect and engulf pathogens or debris, and they may release cytokines for
maintaining brain homeostasis (Jakel & Dimou, 2017; Kreutzberg, 1995). Recent work
examining the extent of α-synuclein pathology within brain cells in the AON of PD subjects
found that, *almost as many microglia (7.78%) as neurons (8.6%) harbor α-synuclein+*
*inclusions* (Stevenson et al., 2020). Notably, in that study, pericytes and astroglia were
minimally affected, whereas oligodendroglia showed no involvement (Stevenson et al.,
2020). The participation of non-neuronal cells in Lewy body disorders may be skewed
toward microglia since microglia may be most efficacious at degrading α-synuclein
aggregates (H. J. Lee et al., 2008). This suggests that microglia play potentially as important a role in α-synucleinopathies as the neurons they seek to protect.

The earliest known link between microglia and Lewy body disorders was in a study by McGeer et al., published in 1988 (McGeer, Itagaki, Boyes, & McGeer, 1988). In the postmortem nigra of PD patients and in the hippocampus of PDD patients, large numbers of reactive microglia were noted, and the authors also found that, in some cases, the reactive microglia were endocytosing neuromelanin and dopaminergic neurons (McGeer et al., 1988). Apart from PD and PDD, some cases with Alzheimer’s dementia also showed reactive microglia and the authors posited that microglial reactivity may be the underlying link between the parkinsonian diseases and AD (McGeer et al., 1988). Ever since this report, microglial reactivity has also been described in several preclinical studies using PD neurotoxicants (Dai, Tan, Wu, Warner, & Gustafsson, 2012; Klusa et al., 2010; Swarnkar et al., 2013; W. Zhang et al., 2021) and α-synuclein fibrils (Duffy, Collier, Patterson, Kemp, Luk, et al., 2018; Harms et al., 2017; Thakur et al., 2017; Verma et al., 2021). Some studies have shown that PFF-induced microgliosis precedes neuronal loss (Duffy, Collier, Patterson, Kemp, Luk, et al., 2018) and that a surplus of α-synuclein—especially in its aggregated form—impedes phagocytic capacities (Bido et al., 2021; Haenseler et al., 2017; J. Y. Park, Paik, Jou, & Park, 2008). Microglia may thus become carriers of α-synuclein, spreading it by exosomes (Guo et al., 2020; Y. Xia et al., 2019) and, in this manner, PFF-triggered microglial reactivity states and neuronal α-synucleinopathy may propel each other, especially if microglia are unable to degrade and clear the α-synuclein. Together with McGeer’s theory that microglial reactivity is “the strongest premonitory indication of an impending disease” (McGeer et al., 1988), one might thus assume that microglia are
detrimental in Lewy body disorders. However, an ~80% loss of microglia exacerbates the cell-to-cell transfer of α-synuclein (George et al., 2019), suggesting that a near-complete loss of microglia is not beneficial at mitigating disease and may not even be desirable. Instead, diseased microglia may have evolved to tolerate intense proteotoxic stress. For example, when microglia are overwhelmed with α-synuclein, they may form tunneling nanotubes with healthy microglia from where the α-synuclein-overloaded microglia can accept additional mitochondria (Scheiblich et al., 2021). Thus, this may be an adaptive process, by which microglia can share the burden of degrading α-synuclein with other healthier microglia (Scheiblich et al., 2021), potentially by a process of selective autophagy, termed “synucleinphagy” (Choi et al., 2020).

Despite these findings, it is also true that microglia undergo dystrophic changes with ageing and disease. For example, it is known that with age, microglia become more inflamed, less motile, may produce more free radical stress, may show impairments in phagocytosis, etc. (Angelova & Brown, 2019). In addition, microglia are exceptionally long-lived cells. Some microglia survive for ~20 years in humans and, in mice, about half of the original microglial population survives for the entire lifespan of the mouse (~26-28 months) (Fuger et al., 2017; Reu et al., 2017). Thus, with natural ageing, microglia may be prone to senescence and some groups have attempted to reverse this ageing microglial phenotype by depletion and subsequent repopulation, as this strategy may replace aged microglia with newer cells that may be better able to fulfill normal microglial functions. These studies have shown that microglial replacement improves cognition and long-term potentiation and restores expression of genes involved in cytoskeletal remodeling, axonal growth, synaptic function, microtubule transport, neurotransmitter release, etc. (M. R. P.
Apart from ageing, this strategy has also shown beneficial effects in traumatic brain injury (Willis et al., 2020) and AD (N. R. Johnson et al., 2021).

In this manner, it may be possible to reset microglial phenotypes in Lewy body disorders, such that the newly replaced microglia may be less burdened and better able to clear α-synuclein aggregates, preventing their cell-to-cell transmission. This strategy may thus mitigate pathology and behavior deficits associated with α-synucleinopathic disease. However, this has not been tested in the PFF-seeded model of α-synucleinopathy—the goal of Aim 3 of this dissertation.

8. Research objectives & Specific aims

As mentioned above, there is a vast body of literature suggesting that men may be at a higher risk of Lewy body disorders than women, with the earliest observations in this regard dating back to Gowers’ report from 1886. Despite the clinical differences among male and female patients, the mechanisms underlying sexual dimorphisms in Lewy body disorders have not been widely studied and, consequently, it is unknown if sex-specific mechanisms can be leveraged for therapeutic intervention. An ideal therapeutic candidate would mitigate α-synuclein aggregates when they are formed in situ as well as when they are in transit to nearby, recipient cells. In response to the intracellular formation of α-synuclein aggregates, the Hsp70 chaperone network may be induced as the first line of defense against proteotoxicity. In response to extrusion of aggregates into the extracellular space, brain phagocytes, such as microglia, may be activated as the first line of defense to clear these proteinaceous seeds, which may be recognized as foreign. It is not known if the stress-mediated induction of Hsp70 or microglial activities differs based on the biological
sex of the organism. It is also unclear if these defenses can be replenished with the exogenous delivery of additional Hsp70 or by the replacement of diseased microglia with a fresh pool of newly colonized cells.

Thus, using α-synuclein fibrils as a testbed in sex-stratified primary neuronal and microglial cultures from rats, and using stereotaxic injections of α-synuclein fibrils in mice, followed by behavioral, biochemical, and neuropathological analyses, we examined the function of Hsp70 and microglia in α-synucleinopathic disease. Assessments of Hsp70 levels and microglial reactivity in postmortem brain tissue from age- and sex-matched unaffected controls or patients diagnosed with Lewy body disorders were also performed and were used to inform the preclinical work and vice versa. Specifically, our objectives for this dissertation funnel into the following 3 aims (schematic in Fig. 3):

**Specific Aim 1:** To determine if males versus females differ in their dependence on endogenous Hsp70 defenses in α-synucleinopathic disease

**Specific Aim 2:** To determine if exogenous delivery of Hsp70 (eHsp70) mitigates pathology and behavior deficits in the PFF model of α-synucleinopathic disease

**Specific Aim 3:** To investigate if microglia mitigate α-synucleinopathy and behavior deficits in the PFF model and if this effect is modified by biological sex
Exposure of cells or mice to preformed α-synuclein fibrils (PFFs) elicits a proteostatic challenge, inducing the formation of inclusions containing pathologically phosphorylated α-synuclein (pSer129), which can ultimately lead to behavior deficits in both males and females. As the first line of defense against an intracellular proteotoxic challenge, the Hsp70 chaperone network may be induced. In **Aim 1**, we tested, a) if endogenous Hsp70 defenses mitigate inclusions, b) if inclusion formation and disease *per se* leads to loss of Hsp70, and c) if endogenous Hsp70 defenses underlie sex differences in inclusion formation. It is known that Hsp70 can also be released extracellularly, and prior work has tested the therapeutic potential of exogenous Hsp70 (eHsp70) in multiple disease models. In **Aim 2**, we tested, a) if eHsp70 also reduces inclusions in culture and *in vivo*, and b) if eHsp70 mitigates behavior deficits in response to PFF infusions in mice. eHsp70 is a pleiotropic protein, with multiple mechanisms of action. It may function as a chaperone, but it may also have pro-phagocytic effects in microglia. In **Aim 3**, we tested, a) if eHsp70 stimulates microglial phagocytic activities and if eHsp70-pre-treatment of microglia reduces inclusions in co-cultured neurons seeded with PFFs, b) if microglia *per se* display sex differences in their abilities to protect co-cultured neurons from PFF-seeded pathology, and c) if reversing the ageing microglial phenotypes with a fresh pool of younger, healthier microglia using the established depleting agent, PLX5622 in the diet, mitigates the behavior deficits associated with PFF-infusions in aged mice of both sexes. Note that studies from all Aims were performed in cells or mice of both sexes and we tested if biological sex modifies the impact of Hsp70/microglia defenses in α-synucleinopathies.
Chapter 2

Materials and Methods

All animal work was performed with prior approval by the Duquesne University IACUC and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. CD1 mice and Sprague Dawley rats were used for the animal work and our breeding colonies were occasionally repopulated by acquiring animals from external sources. Breeders were always obtained from external sources to avoid genetic drifting.

1. Primary glial cultures

As indicated in the Results, studies were performed in mixed-sex cultures or in sex-stratified cultures, for which male and female Sprague Dawley rat pups (breeders obtained from Hilltop Lab Animals, Scottdale, PA) were sexed based on their anogenital distances. This technique was confirmed by PCR for the Y-linked Sry gene (Mouse Genotype, Escondido, CA). Glia from the cortex or hippocampus of postnatal day 0-4 Sprague Dawley rat pups were harvested, as described in prior work (Bhatia et al., 2019; Gleixner et al., 2017; Gleixner, Posimo, Pant, Henderson, & Leak, 2016; Titler, Posimo, & Leak, 2013). Briefly, tissue was dissected in Dulbecco’s Modified Eagle Medium (DMEM; 12100061, Gibco; Life Technologies, Grand Island, NY), supplemented with 10% Fetal Clone III (SH30109.03, HyClone; Thermo Scientific; Pittsburgh, PA) and 1% penicillin-streptomycin (15070, Gibco; Life Technologies). After a seven-min incubation in 0.25% Trypsin-EDTA (25200, Invitrogen; Life Technologies, Waltham, MA) at 37 °C and 5% carbon dioxide, tissue was mechanically triturated in DMEM. Cells were plated on T175 flasks (12-556-011, Thermo Scientific) coated with poly-D-lysine (10 µg/ml; P0899,
Sigma-Aldrich, St. Louis, MO), at a cell density of $24 \times 10^6$ cells/flask or on poly-D-lysine coated T75 flasks (12-556-010, Thermo Scientific) at $15 \times 10^6$ cells/flask. Media were refreshed every 2-3 days and once confluence was achieved (within 10-12 d), glia were shaken at 260 rpm and 37 °C.

For microglia, the supernatant was collected after 3 h and centrifuged (5810R, Eppendorf; Hamburg, Germany) at 190 $x$ g and 21 °C for 8 mins (Xuqin Chen, Zhang, Sadadcharam, Cui, & Wang, 2013). The cell pellet containing microglia was resuspended in fresh, warm 3 mL DMEM and plated at 20,000 cells per well in a 96-well plate (3595, Corning, Corning, NY) coated with poly-D-lysine (66.7 μg/mL) and laminin (6.7 μg/mL; 354232, Corning). For astrocyte cultures, we continued to shake the mixed glial cells at 260 rpm and 37 °C overnight (i.e., for an additional ~16-18 hours). On the next day, the supernatant, which contains non-astroglial cells, was discarded and flasks were refilled with freshly warmed DMEM. After an additional 2-3 days, astroglia were trypsinized and plated at a density of 20,000 cells per well in a 96-well plate coated with poly-D-lysine (66.7 μg/mL) and laminin (6.7 μg/mL). Primary astrocytes were passaged a maximum of four times or for one month after being harvested from the brain, whichever was earlier.

2. **Primary neuron cultures**

Neurons were harvested from the hippocampus of postnatal day 0-2 Sprague Dawley rat pups, based on the method described in prior work (Volpicelli-Daley et al., 2014), with slight modifications. Briefly, tissue from the hippocampus was harvested in ice-cold Hibernate A (HA; BrainBits, LLC., Springfield, IL). Following two washes in Hank’s Balanced Salt Solution (HBSS; 14170-161, Invitrogen, Life Technologies), tissue
was incubated for ~30-60 mins at 37 °C and 5% carbon dioxide in papain (20 U/mL; LS 3126, Worthington Biochemical Corporation; Lakewood, NJ), freshly prepared on the day of harvest. Next, tissue was briefly incubated in DNase solution (DN25, Sigma-Aldrich, St. Louis, MO) and triturated in HBSS. After filtering suspension through a 40 µm cell strainer (22-363-547, Fisher Scientific), neurons were plated in Neurobasal-A medium (10888022, Fisher Scientific), supplemented with glutamax (35-050-061, Fisher Scientific), penicillin/streptomycin (15-140-122, Fisher Scientific), B27 (17-504-044, Fisher Scientific), and 10% fetal bovine serum (FBS; S11550H, Atlanta Biologicals). For neuron monolayer cultures or neuron/glia bilayer cultures, neurons were plated at 60,000 cells per well in 96-well plates coated with poly-D-lysine (66.7 µg/mL) and laminin (6.7 µg/mL). For the co-cultures, neurons were always plated on top of the glia. After 4 h of plating neurons, plating media was replaced with freshly warmed neuronal media (same constituents as plating media, but without the serum) (Volpicelli-Daley et al., 2014). Neurons were always plated alone (as a control) or on top of a microglial monolayer, 48 h after microglia were plated (i.e., 24 h after the “first hit”; described below). For sex-stratified microglia/neuron co-cultures, male or female neurons were plated on top of male or female microglia, respectively.

3. In vitro treatments of primary cultures

For the in vitro studies in Aims 1-2, primary neuron cultures were treated with α-synuclein fibrils at 0, 1, or 4 µg/mL, as indicated in the figures. For all the experiments involving α-synuclein fibrils in this dissertation, preformed wild-type mouse α-synuclein fibrils (PFFs) were prepared in the lab of Dr. Kelvin Luk (University of Pennsylvania), as
in prior work (Luk, Kehm, Carroll, et al., 2012; Luk, Kehm, Zhang, et al., 2012; Luk et al., 2009; Volpicelli-Daley et al., 2014; Volpicelli-Daley et al., 2011), or were obtained from StressMarq Biosciences (SPR-324B, Victoria, Canada). PFFs were sonicated in a waterbath (Bransonic series model M1800, Branson Ultrasonics Corporation, Danbury CT) for 60 mins immediately prior to use, based on work by Mason et al. and Nouraei et al. in similar models (Mason et al., 2016; Mason et al., 2019; Nouraei et al., 2018). Neuron cultures were treated with PFFs on day-in-vitro (DIV) 2 and assays were performed after 6 h, or at DIV5, 6, 12, or 16, as indicated. We did not culture neurons for longer than 16 d, as we observed that nuclei clumped together beyond this timepoint, which made automated analyses of cell counts inaccurate. Treatments of primary neurons with Hsp70 inhibitors or with eHsp70 were performed in parallel with PFFs. The Hsp70 inhibitor, VER155008 (3803/10, R&D Systems Inc, Minneapolis, MN) (Chatterjee et al., 2013; Massey et al., 2010; Saykally et al., 2012; Schlecht et al., 2013) was used at 12.5 µM, based on work at this concentration in primary neuron cultures (Crum et al., 2015; Gleixner et al., 2016; Heinemann, Posimo, Mason, Hutchison, & Leak, 2016; J. M. Posimo et al., 2015). The Hsp70 inhibitor MAL3-101 was prepared in the lab of Dr. Peter Wipf (Fewell et al., 2004), and was used at a concentration that did not elicit significant neuronal toxicities in our hands.

eHsp70 was procured from two independent sources for our studies. Recombinant human Hsp70 protein, certified endotoxin-low (< 50 EU/mg purified protein via limulus amebocyte lysate testing), was purchased from Enzo Life Sciences (ADI-ESP-555, Farmingdale, NY) along with Enzo’s specific PBS control as a vehicle. Alternatively, recombinant human Hsp70 protein was synthesized by Dr. Patrick Needham in the lab of
Dr. Jeffrey Brodsky (University of Pittsburgh), according to methods described in prior work (Ireland et al., 2014). As a control for this latter source of eHsp70, we used a “mock” purification plasmid, which was prepared by the same method as the eHsp70, except it was an empty plasmid with no Hsp70 expression. For the in vivo work, we used either recombinant human Hsp70 (Enzo Life Sciences) or, alternatively, a yeast homolog of Hsp70 (i.e., Ssa1, ~74% homology with human Hsp70 (Kushnirov, Kryndushkin, Boguta, Smirnov, & Ter-Avanesyan, 2000)), which was synthesized by Dr. Patrick Needham in the lab of Dr. Jeffrey Brodsky (University of Pittsburgh) (McClellan & Brodsky, 2000). Apart from PBS, we also used denatured eHsp70 as a control in some experiments. Denaturation was performed by boiling eHsp70 at ~95 °C for 10 mins in a water bath and then the eHsp70 protein was immersed in an ice bath for an additional 10 mins. This cycle was repeated two more times, and denaturation was always completed immediately prior to use in vitro or in vivo. For all in vitro and in vivo work, we relied on concentrations or doses of eHsp70 as in prior work (Bobkova et al., 2015; Robinson et al., 2005; Tytell et al., 2018). Additional details are in the Results sections of each Aim.

For studies in Aim 3, at 24 h after plating microglia, we elicited proinflammatory responses with exposure to 100 ng/mL lipopolysaccharide (LPS; L2880, Sigma-Aldrich) and 20 ng/mL interferon-γ (IFNγ; I3275, Sigma-Aldrich), based on prior work (X. Hu et al., 2012). Anti-inflammatory responses were induced with 20 ng/mL interleukin-4 (IL4; I3650, Sigma-Aldrich), based on prior work (X. Hu et al., 2012). To test if α-synuclein fibril exposure propels microglia to proinflammatory or anti-inflammatory phenotypes, we treated cells with 1 µg/mL of PFFs (Roodveldt et al., 2013). Proinflammatory versus anti-inflammatory markers were measured by In-Cell Western assays at 24 h after treatments.
(48 h after plating microglia), as described below. For studies examining the impact of pre-
treatment of microglia (with LPS/IFNγ, IL-4, or PFFs) on PFF-seeded α-synucleinopathy 
in co-cultured neurons, the pre-treatment (“first hit”) was washed with two media 
exchanges after 24 h. Primary hippocampal neurons were then plated on top of the 
microglia, as described above. After 48 h of plating neurons, microglia/neuron co-cultures 
and neuronal monolayer cultures were exposed to 0 or 1 µg/mL PFF (“second hit”). Ten 
days after the second hit, cells were formalin-fixed for immunofluorescence assays. Similar 
protocols were adopted for PFF exposure of neuron/astroglia co-cultures. For studies in 
sex-stratified microglia/neuron co-cultures, the co-cultures or neuronal monolayers were 
exposed to 0 or 1 µg/mL PFFs at 48 h after plating neurons, and cells were formalin-fixed 
10 d later for immunofluorescence assays. As indicated in Aim 3, in some of the studies in 
sex-stratified co-cultures, microglia were pre-treated with eHsp70 (Enzo) for 24 h, 
following which this first hit was washed with media exchanges and neurons were plated 
alone or on top of microglia. The co-cultures or neuronal monolayer cultures were then 
exposed to 0 or 1 µg/mL PFFs at 48 h after plating neurons, and cells were formalin-fixed 
10 d later for immunofluorescence assays, as mentioned above.

In a separate set of experiments to test microglial phagocytic capacities, microglia 
were exposed to 0.0001% of carboxylate-modified fluorescent microspheres (625/645 nm 
wavelength; 1.0 µm sized microspheres; F8816, Invitrogen) and 0 or 1 µg/mL of PFFs for 
3 h. This concentration of microspheres was based on pilot work to ensure that the 
microspheres were not clumped up, which would have made it difficult to record the 
numbers of microspheres engulfed within microglia. For studies testing if eHsp70 can 
stimulate microglial phagocytosis, eHsp70 was delivered along with the microspheres and
PFFs for 3 h, at a concentration of 0, 10, or 20 µg/mL. To determine the mechanisms underlying microglial phagocytosis in the PFF model, cells were exposed to cytochalasin D at 0, 1, or 10 µM (1233/1, R&D Systems Inc) to test if the microglial uptake of microspheres is abolished in the presence of this established phagocytosis inhibitor that works via actin destabilization (S. M. Kim et al., 2017; Neher et al., 2011; Ribes et al., 2010; Scheiblich et al., 2021; Shi et al., 2013; W. Zhang et al., 2005). Treatments with cytochalasin D were also performed in parallel with PFF exposures. For studies testing microglial uptake of α-synuclein fibrils, primary hippocampal microglia were exposed to 0 or 1 µg/mL waterbath sonicated ATTO 647-conjugated α-synuclein fibrils (StressMarq Biosciences) for 6 h, 16 h, 24 h, 72 h, or 96 h. These fluorescent fibrils were sonicated using the same parameters as the PFFs used for the rest of the studies in this dissertation.

4. **Immunocytochemistry and In-Cell Western assays**

Cells were fixed with 3% formalin (9990244, Thermo Scientific) and 2% sucrose in 0.1 M phosphate buffer for 20 mins at room temperature. In studies assessing extent of detergent-insoluble inclusions (Aim 1, **Fig. 10**), cells were fixed with formalin/sucrose, in the presence of 1% Triton X-100 for 20 mins at room temperature. In either case, formalin was washed with three exchanges of 10 mM freshly filtered phosphate buffered saline (PBS; 10 mins per wash). Cells were then incubated in LI-COR Intercept Blocking Buffer (927-70001, LI-COR Biosciences, Lincoln, NE), supplemented with 0.3% Triton X-100 for 30 mins or in a 1:1 solution of LI-COR Odyssey Blocking Buffer (927-40000, LI-COR Biosciences) in 10 mM PBS, supplemented with 0.3% Triton X-100, for 30 mins. Primary antibodies, as listed in Table 1, were prepared in blocking buffer and PBS, supplemented.
with 0.3% Triton X-100. Cells were incubated overnight at 4 °C with the primary antibody solution. Next, unbound antibodies were washed three times (10 mins each) with 10 mM PBS and cells were incubated for 60 mins with the appropriate infrared wavelength secondary antibodies (for In-Cell Westerns) and visible wavelength secondary antibodies (for higher resolution microscopic analyses), as listed in Table 2. Solutions containing the secondary antibodies were prepared in the same blocking buffer and PBS solutions as the primary antibodies. Unbound secondary antibodies were washed with three exchanges (10 mins each) of PBS. Immunostained cells were immediately scanned on an Odyssey infrared imager (9201-01, Odyssey Classic, LI-COR Biosciences) for In-Cell Western analyses. To assess viability status, we used the infrared nuclear marker DRAQ5 (0.5 μM; 62251, Thermo Scientific) or the nuclear marker Hoechst 33258 (bisBenzimide, 5 μg/mL; B1155, Sigma-Aldrich), both of which were applied at the time of the secondary antibodies. High resolution images were captured with 20× and 40× objectives on an Olympus epifluorescent microscope (Olympus IX73, B&B Microscopes, Pittsburgh, PA), and cell or inclusion counts were performed using the Olympus cellSens software or on ImageJ (NIH Image, Bethesda, MD). As indicated, z-stack images were captured on the Olympus microscope and deconvoluted using cellSens software. Omission of primary antibodies led to the expected loss of signal and these “no primary” antibody wells were used for background subtraction in the In-Cell Western analyses, which were performed using Image Studio Lite Ver 5.2 (LI-COR Biosciences). Experimental and control groups were processed in the same solutions in parallel and were analyzed by blinded observers at identical settings. Additional methods, particularly with respect to image analyses, are described in detail below.
5. Stereotaxic surgeries

CD1 male and female mice (breeders obtained from Charles River, Wilmington, MA) were acclimatized to a 12:12 photoperiod in the Duquesne University Animal Care Facility and were provided with access to food and UV-disinfected water. In this dissertation, there were four experiments in which stereotaxic surgeries were performed:

**Experiment 1** (Aim 2, Fig. 21): Unilateral injections of 5 µg (in 1 µL of PBS) of preformed wild-type mouse α-synuclein fibrils (PFFs) in the AON of aged, 20-month-old male mice (n=20) at AP +4.0 mm, ML +1.0 mm, and DV -2.5 mm from Bregma (from top of skull rather than dura mater). Intranasal infusions of eHsp70 were then begun at 24 h after surgeries and continued every day for 28 d, until sacrifice, as mentioned below.

**Experiment 2** (Aim 2, Fig. 22): Bilateral injections of 5 µg (in 1 µL of PBS) of PFFs (n=24) or 1 µL of PBS (n=7) in the AON of 5-month-old male mice at AP +4.0 mm, ML +/- 1.0 mm, and DV -3.0 mm from Bregma (from top of skull rather than dura mater). In this study, mice were tested in an open field arena at 3 months after PFFs were infused, after which eHsp70 delivery was initiated every 72 h and continued for 18 d, until the pandemic lockdown of ~4-months interrupted this study, as mentioned below.

**Experiment 3** (Aim 3, Fig. 31): Bilateral injections of 5 µg (in 1 µL of PBS) of PFFs (n=10) or 1 µL of PBS (n=10) in the regio inferior (hippocampal CA2/3) of 2.5-month-old male mice at AP -2.5 mm, ML +/- 2.8 mm, and DV -3.6 mm from Bregma (from top of skull rather than dura mater). Note that this experiment was published in (Nouraei et al., 2018). In this dissertation, only the brain tissue from Nouraei *et al.* was used for assessing microglial reactivity.

66
Experiment 4 (Aim 3, Fig. 33-36): Bilateral injections of 5 μg (in 1 μL of PBS) of PFFs (n=20/sex) or 1 μL of PBS (n=9) in the AON of 18-month-old male and female mice at AP + 4.0 mm, ML +/- 1.0 mm, and DV -3.0 mm from Bregma (from top of skull rather than dura mater). In this experiment, PFF injections were performed on day 7 after the initiation of microglial depletion, as mentioned further below.

Regardless of the specific experiment/study, stereotaxic coordinates were always obtained by performing practice injections with blue food dye in spare mice of the same age and we did not solely rely on the stereotaxic atlases. Mice were always anesthetized with ~2-2.5% isoflurane, placed on a microwave-heated pad, stabilized in a stereotaxic frame, and then PBS or PFFs were injected into the specific coordinates, as mentioned above. Scalps were shaved prior to incision, disinfected with betadine and 70% isopropyl alcohol, and the infusions were performed using a Hamilton syringe (80,085, Hamilton Company, Reno, NV), attached to a motorized injection pump (Stoelting, Wood Dale, IL) at a rate of 0.25 μL/min. Following a rest period of ~3-4 min, the needle was gradually withdrawn from the burr hole. All attempts were made to minimize animal suffering. Animals were injected subcutaneously with 0.05 mg/kg buprenorphine immediately after the surgery and placed on an electric heating pad until complete recovery of ambulation. Furthermore, immediately after the surgery (and for 3 days following surgery), topical 2% lidocaine ointment was applied at the incision site.

6. Intranasal infusions

Mice were lightly anesthetized using isoflurane, and eHsp70 or PBS was infused into the nares using an autoclaved pipet tip, as described (Bobkova et al., 2015; Bobkova
et al., 2014; Demyanenko et al., 2020; M. Evgen'ev et al., 2019; M. B. Evgen'ev et al.,
2017; Tytell et al., 2018). After performing the intranasal infusions, mice were left supine
in the anesthesia chamber so that they could breathe in the infusate, and then the mice were
returned to their cages. For studies in Fig. 19a-b, 3-month-old male mice were infused with
PBS (2 µL) or recombinant human eHsp70 (2 µg, Enzo). For the studies in aged, 17-21-
month-old male and female mice (Fig. 19c-f, Fig. 20), we infused human eHsp70 (6 µg,
Enzo) and sacrificed mice at 3, 24, 48, and 72 h post-infusion, to determine the time-
dependent nose-to-brain entry of eHsp70, and its clearance from the brain after uptake. A
separate sham (0 h) group was included, in which mice did not receive intranasal infusions,
but were anesthetized. The specific sample sizes for each timepoint and each sex are as
indicated in the figures. Initially, in the 3 h group in Fig. 19c, we only included an n=3, but
an additional sample from these more expensive aged mice was added by the time the data
in Fig. 19d-f were generated. In the 0 h group in Fig. 19e and in the 72 h group in Fig.
20d, the spinal cords of one male and female mouse were lost during processing. In Fig.
21, intranasal infusions of 2 µg Ssa1 (1 µg/µL of PBS) or 2 µL PBS were initiated at 24 h
after PFF injections in the OB/AON and continued daily for 28 d until sacrifice. In Fig. 22,
intranasal infusions of 6 µg native human eHsp70 (1 µg/µL of PBS), 6 µg denatured human
eHsp70 (1 µg/µL of PBS), or 6 µL PBS were initiated at ~3-months after PFF injections in
the OB/AON (after verifying behavior deficits in an open field) and continued every 72 h,
based on the kinetics data in Fig. 19. We had intended to continue the infusions for at least
30 d, but we were interrupted on day 18 due to the pandemic lockdown of ~4-months.
These mice were tested on a battery of behavior tests after our return to the labs, but no
fresh eHsp70 exposures were performed after our return, and all mice from this study were sacrificed after the behavior assays were completed.

7. Behavior assays and analyses

We performed tests for olfaction, cognition, motor function, anxiety-related behaviors, and repetitive or compulsive behaviors. Animal behavior was tracked using an overhead camera in a dimly lit arena and behavior videos were either analyzed by blinded investigators or were analyzed using the AnyMaze software (Stoelting). Note that all tests were performed after allowing mice to acclimate to the testing room for at least 20-30 mins. For the studies in Fig. 21, three of the aged mice (n=2 from PBS-infused group and n=1 from eHsp70-infused group) had died before the behavior tests were performed due to aging-related attrition. For the studies in Fig. 22a-i, all 31 mice were tested in the open field prior to initiating eHsp70 infusions, but during the infusion regimen, three mice developed dermatitis or an eye infection and were humanely sacrificed prior to the tests in Fig. 22j-o. For the studies in Fig. 34-36, one PLX-fed aged male mouse and one PLX-fed aged female mouse had to be euthanized early due to age-related dermatitis (in the male) and an abdominal abscess (in the female), and so are not included in the behavior assays. Additional behavior test-specific exclusion criteria are described below.

The buried and exposed pellet tests were performed to assess olfactory function, as in prior work (Fleming et al., 2008; Mason et al., 2019). Latencies to contact food pellets exposed on top of the bedding (exposed pellet test) and, separately, when buried ~1 cm underneath the bedding (buried pellet test) were measured. For the tests in Fig. 21, no animals were excluded as all the mice that were tested showed an interest in contacting the
exposed food pellet. However, buried pellet data from two mice in Fig. 21 were excluded due to technical problems with the video for one mouse and the presence of food crumbs on top of the bedding for the other mouse. For the test in Fig. 36j-m, all animals contacted the exposed food pellet, but we noticed that many mice did not eat the food. Thus, data in Fig. 36j-m are shown separately for latencies to contact (Fig. 36j-k) versus latencies to eat (Fig. 36l-m) exposed and buried pellets. Animals that did not eat the pellets received a score of 300 s (the maximum time for which the exposed/buried pellet tests were performed). In Fig. 22, olfactory function was assessed via the odor habituation / dishabituation test, as in prior work (Fleming et al., 2008; Lehmkuhl, Dirr, & Fleming, 2014; Rey et al., 2016). A small, stainless-steel tea-leaf holder was placed in the animal’s cage for 24 h prior to performing the test. On the day of the test, mice were exposed to the same odor for six repetitive trials of 50 s each (familiar odor), following which mice were exposed to a novel odor for the 7th trial. The inter-trial interval was ~5 mins. Time spent contacting the odors was recorded. “Contact” was defined as time for which the mouse’s snout was toward the odor and was less than ~1 cm from the odor.

Anxiety-like and hyperactive behaviors were assessed in an open field arena for 8 mins, based on prior work (Frye, Petralia, & Rhodes, 2000; Rey et al., 2016; Seibenhener & Wooten, 2015). The marble burying test was used to assess repetitive, compulsive-like behaviors (Angoa-Perez, Kane, Briggs, Francescutti, & Kuhn, 2013). Briefly, rat cages were filled with ~4 cm of corncob bedding and 20 glass marbles were arranged in five rows of four marbles each. A separate cage was prepared for each mouse and after placing each mouse within the cage, it was left undisturbed for 30 mins. Next, mice were carefully removed and placed back in their home cages, without shifting the marbles, and two
blinded observers counted the number of marbles that were nearly completely buried (>90%), partially buried (>50-60%), and number of marbles that were fully exposed (0% buried). For this test, in Fig. 36i, are average data from the two blinded observers. Motor dysfunction was assessed by the inverted grid test, as per the method described in prior work (Deacon, 2013; Montana et al., 2011). Briefly, mice were placed on a grid mesh, which was inverted, and the latency of the mice to fall from the grid mesh was measured. Each mouse received three trials with an inter-trial interval of ~30 mins and the average data from all three trials were recorded.

To test differences in spatial reference memory, the forced alternation test was performed in a Y-maze, based on the method described in prior work (Dellu, Contarino, Simon, Koob, & Gold, 2000; Melnikova et al., 2006; Wolf, Bauer, Abner, Ashkenazy-Frolinger, & Hartz, 2016). First, mice were allowed to explore only two out of the three arms of the Y-maze, and, following an inter-trial interval of 30 mins, mice were allowed to explore all three arms to determine time spent exploring the novel versus familiar arms. Mice that made less than three arm entries during the first minute of the second trial were excluded from the analyses, as in prior work (Wolf et al., 2016). Apart from the forced alternation test, spatial working memory was also tested in a Y-maze, via the spontaneous alternation test (Magen et al., 2012; Miedel, Patton, Miedel, Miedel, & Levenson, 2017). Briefly, mice were placed in the Y-maze and allowed to explore all three arms (denoted A, B, C) for 7 mins. Spontaneous alternations were calculated as, [(Number of alternations) / (Number of total arm entries)] * 100, wherein an alternation was defined as the entry of the mouse in all three arms (e.g., ABC, CAB, BCA are all alternations, but not BCC). Mice that made fewer than twelve arm entries in total were excluded from the analyses, based
on prior work (Garcia & Esquivel, 2018). Note that for the correlations of Iba1 with novel place recognition or novel object recognition tests in Fig. 31, we used the behavior data that was already published in (Nouraei et al., 2018).

8. Microglia depletion and repopulation

Colony stimulating factor 1 receptor (CSF1R) inhibitor, PLX5622 (Chemgood, Glen Allen, VA), was prepared at 1200 mg/kg in AIN-76A Rodent Diet (Research Diets, Inc., New Brunswick, NJ). First, microglial depletion was confirmed by flow cytometry on brains from three-to-four-month-old male and female CD1 mice administered the PLX5622-containing diet (n=8/sex) or control AIN-76A diet (n=8/sex) for two weeks. In addition, some mice (n=6/sex) were fed the PLX5622-containing diet for two weeks, following which the diet was reverted to the standard rodent chow for an additional six weeks to assess microglial repopulation by flow cytometry. For these studies, the timeline is shown in Fig. 32f. In a separate series of experiments, ~18-month-old male and female CD1 mice were fed PLX5622-containing diet (n=10/sex) or control AIN-76A diet (n=10/sex) for two weeks. Bilateral stereotaxic surgeries of PFFs (5 µg) were performed in the OB/AON after seven days of initiation of the diet, as mentioned in the section on stereotaxic surgeries. Seven days after surgeries (i.e., fourteen days after initiating the PLX5622-containing diet), the diet was reverted to the standard rodent chow for an additional six weeks to allow for microglial repopulation. As mentioned in the Results section of Aim 3, the open field test was performed on a few mice prior to microglial repopulation to determine the effects of microglial depletion alone on hyperactive and
anxiety-like behaviors. Additional tests were performed after microglia had been repopulated and prior to sacrifice (timeline in Fig. 33a).

9. Flow cytometry

At indicated time-points, brains were harvested from isoflurane-anesthetized mice for flow cytometry, which was performed based on the method described in prior work, with slight modifications (Ganesan, Chandwani, Creisher, Bohn, & O'Donnell, 2018). Spleen samples were harvested from sham mice for the unstained and fluorescence minus one (FMO) controls. Brain tissue or spleen tissue was gently dissociated in PBS (D8537, Sigma-Aldrich) through a 70 µm nylon mesh cell strainer (352359, Corning). Next, the cell suspension was passed over a 30/70% Percoll gradient (45001747, Fisher Scientific), centrifuged (acceleration: 1; brake: 0; 5810R, Eppendorf) at 800 \( x \) g for 20 mins at 4 °C, after which mononuclear cells were collected from the interface. Cells were incubated with 0.84% ammonium chloride (AC123340010, Fisher Scientific) in a 37 °C waterbath for 10 mins to lyse contaminating red blood cells, diluted in PBS, and then centrifuged (acceleration: 1; brake: 0) at 800 \( x \) g for 5 mins at 4 °C. Next, the supernatant was discarded, and the cell pellet was resuspended in freshly prepared 1% FBS/PBS and transferred to a v-bottom 96-well plate (0720096, Fisher Scientific) for immunostaining. Cells were pelleted by centrifuging (acceleration: 9; brake: 9) the 96-well plate at 1500 rpm for 7 mins at 4 °C. The pellet was resuspended in a 1% FBS/PBS solution containing fluorophore-conjugated antibodies (Table 3) in the dark at 4 °C. After 60 mins, cells were re-pelleted by centrifuging the plate at 1500 rpm for 7 mins at 4 °C (acceleration: 9; brake: 9). Cell pellets were resuspended in 1% FBS/PBS and analyzed on the Attune NxT Acoustic
Focusing Cytometer (Thermo Scientific) with Attune Nxt software (Thermo Scientific). For each sample, 100,000 events were recorded and gated such that only single cells were analyzed. Additional gates were applied based on unstained and FMO controls. Before analyzing each new batch of samples on the flow cytometer (i.e., on each new day that flow cytometry was performed), color compensation was performed using the AbC Total Antibody Compensation Bead kit (A10513, Thermo Scientific) to control for fluorescence spillover.

10. Perfusions and sacrifice

At indicated time-points, isoflurane-anesthetized mice were perfused through the left ventricle of the heart with ~50 mL of 0.9% saline supplemented with 20 U/mL heparin sodium salt (H3393, Sigma-Aldrich), followed by ~100 mL of 4% formalin (9990244, Thermo Scientific) in 0.1 M phosphate buffer. Brains were post-fixed in formalin for 2-4 h, after which brains were immersed in 30% sucrose in 10 mM PBS for at least 48 h. Brains were cut in the sagittal plane on a freezing microtome (Microm HM450; Thermo-Scientific or on American Optical AO860 Sliding Microtome; Rankin Biomedical Corporation, MI) in a 1-in-5 series of free-floating sections, and the sections were stored in cryoprotectant at -20 °C until immunostaining (Hoffman & Le, 2004). For the immunoblotting studies in Fig. 19-20, brains were rapidly dissected after decapitation of deeply anesthetized mice, stored briefly in dry ice, and then transferred to -80 °C until lysis and immunoblotting procedures were performed. For the DNA repair assays in Fig. 23b-c, dissected OB/AON tissues were weighed, incubated in DMEM (D6046, Sigma-Aldrich), and then transferred to cell culture freezing media. Tissues were shipped on dry ice to LxRepair (Grenoble,
France) for the GlycoSPOT and ExSySPOT assays of DNA repair (Forestier, Sarrazy, Caillat, Vandenbrouck, & Sauvaigo, 2012; Millau et al., 2008; Pons et al., 2010; Prunier, Masson-Genteuil, Ugolin, Sarrazy, & Sauvaigo, 2012).

11. Immunohistochemistry and histological assays

Cryoprotected tissue sections were washed with freshly filtered 10 mM PBS and incubated for 60 mins in a 1:1 solution of LI-COR Odyssey Blocking Buffer (927-40000, LI-COR Biosciences) in 10 mM PBS, supplemented with 0.3% Triton X-100. Sections were incubated overnight at 4 °C with primary antibodies (diluted in the same blocking solution) listed in Table 1. The next day, sections were washed three times with PBS (10 mins/wash) and incubated for 60 mins with visible and infrared wavelength secondary antibodies, as listed in Table 2. After washing off the unbound secondary antibodies with PBS, sections were mounted on glass slides (Superfrost Plus, Fisher Scientific), and the Krystalon mounting medium (23-750008, Thermo-Scientific) was used for coverslipping. After the slides were air-dried, tissue sections were scanned on the LI-COR Odyssey infrared imager (9201-01, Odyssey Classic, LI-COR Biosciences), at a resolution of 21 μm, as in prior work (Nouraei et al., 2018; Nouraei et al., 2016). High-resolution images were captured using a 20× or 40× objective or a 100× oil objective on the Olympus microscope (Olympus IX73, B&B Microscopes). Omission of primary antibodies led to expected loss of signal. Control and experimental groups were processed in parallel in the same solutions and imaged/scanned at identical settings.
12. Western immunoblotting

Human postmortem samples and rodent brain tissue dissections were weighed and sonicated in 1× cell lysis buffer (9803S, Cell Signaling Technology, Danvers, MA), supplemented with 1% protease inhibitor cocktail (P8340, Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (PMSF; P7626, Sigma-Aldrich), and 10 mM sodium fluoride (S7920, Sigma-Aldrich). Based on the tissue wet weight, an equal volume of 1× cell lysis buffer was added for every mg of tissue (i.e., 20 µL/mg). After sonicating lysates for 20 pulses at 1 s each with a probe-tip sonicator (XL2020, Misonix Inc., Farmingdale, NY), samples were diluted in protein loading buffer (928-40004, LI-COR Biosciences), supplemented with β-mercaptoethanol. For immunoblotting of the in vitro samples, cells were scraped in cell lysis buffer (prepared fresh, as above), sonicated, and protein content was measured using the bicinchoninic acid assay (BCA; 23225, Thermo Scientific). Note that we did not centrifuge cell or tissue lysates, except in the studies wherein detergent-insoluble extracts were separated using sequential fractionation, based on methods described in (Volpicelli-Daley et al., 2014). In those studies, we probed for pSer129 and pan-α-synuclein in the Triton-X soluble versus Triton-X insoluble (i.e., after resuspending in SDS) fractions.

Equal amounts of cell or tissue lysates were always loaded on polyacrylamide gels and transferred onto nitrocellulose (926-31092, LI-COR) or Immobilon-FL PVDF (IPFL0010, Millipore, Burlington, MA) membranes. For assessments of pan-α-synuclein and pSer129, membranes were fixed with 4% formalin (9990244, Fisher Scientific) and 0.01% glutaraldehyde (G7651, Sigma-Aldrich) on a shaker at room temperature for 30 mins, prior to immunostaining (B. R. Lee & Kamitani, 2011; Sasaki, Arawaka, Sato, &
Kato, 2015). In cases wherein the Total Protein Stain was used as a loading control, membranes were incubated in the Revert 700 Total Protein Stain (926-11011, LI-COR), based on manufacturer’s instructions. After imaging the Total Protein Stain, membranes were destained (926-11013, LI-COR), washed in tris-buffered saline (TBS), and blocked for 60 mins at room temperature in LI-COR Intercept Blocking Buffer or in a 1:1 solution of TBS and LI-COR Odyssey Blocking Buffer. Membranes were incubated overnight at 4 °C with primary antibodies, as listed in Table 1, prepared in TBS and blocking buffer, and supplemented with 0.1% Tween-20. After washing the unbound primary antibodies, membranes were incubated with secondary antibodies (listed in Table 2), diluted in the same solution as the primary antibodies, for 60 mins at room temperature. Membranes were washed with TBS and 0.1% Tween-20 and scanned on an Odyssey infrared imager (9201-01, Odyssey Classic, LI-COR).

13. Image analyses

First, we begin with the image analyses protocols that we followed for all the in vitro work in this dissertation. In-Cell Western data were acquired from Image Studio Lite Ver 5.2 (LI-COR Biosciences), and the signal of the target protein was normalized by the signal of DRAQ5 in the same well or by microscopic counts of numbers of cells in the same well, as specified. During each run, wells that did not have primary antibodies were always included, and the signal of the target protein was obtained after subtracting the background signal from these “no primary” antibody (secondary antibody only) wells. For microscopic images, the same camera exposures and fixed scaling settings were used for the same protein of interest during each run as well as in the representative images shown
in this dissertation. Images were quantified using the ‘manual threshold’ function on the cellSens software (Olympus) and the same threshold was maintained for the same protein of interest for each 96-well plate (i.e., each litter of pups). To separate thresholded cell clumps, the “auto split” function was used. In this manner, we were able to obtain total numbers (i.e., “count” in cellSens), average sizes (i.e., “area” in cellSens), and area fractions. Note that the area fraction of inclusions is denoted as the total area occupied by the inclusions to separate this readout from average inclusion sizes. For Hoechst+ cells, we excluded all nuclei with fragmented and intensely stained chromatin (i.e., nuclei <20 µm² in area and >600 in mean gray intensities as these may be apoptotic/dying cells), as per prior work (Bhatia et al., 2019; Eidet, Pasovic, Maria, Jackson, & Utheim, 2014; Gleixner et al., 2016). If any cell clumps >500 µm² in area remained, despite “auto-split”, these were also excluded. Number of NeuN+ neurons were manually recorded by blinded observers using the cellSens software.

For assessments of uptake of α-synuclein fibrils into neurons, regions of interest (ROIs) were generated using the “manual threshold” function on NeuN images. ROIs were auto split to avoid cell clumps and the image from the corresponding ATTO₆₄₇-α-synuclein fibril channel was then overlaid on top of the ROIs, and thresholded to generate the numbers of fibrils present within, and not outside the ROIs. Numbers of fibrils were normalized by the numbers of NeuN+ neurons. For the assessment of uptake of fibrils into microglia, we obtained the mean grayscale intensities of the ATTO₆₄₇-α-synuclein fibrils and normalized this value by numbers of microglia. As this assay was performed at several timepoints, we were able to obtain the area under the curve. For the microsphere or bead
phagocytosis assay, numbers of beads were manually counted by a blinded observer and normalized over numbers of microglia.

For in vivo assessments, a blinded investigator recorded the numbers of inclusions and numbers of microglia using the “cell counter” function in ImageJ (NIH Image). Numbers of Hoechst+ and NeuN+ cells were assessed using the cellSens software, as described for the in vitro work, above. For these latter assessments, cells <20 µm² in area were excluded, as a better reflection of viability status. Sections scanned on the Odyssey infrared imager were analyzed using Image Studio Lite Ver 5.2 (LI-COR Biosciences) by tracing specific regions of interest and the signal of the target protein was normalized by the area of the traced region, as in prior work (Nouraei et al., 2018; Nouraei et al., 2016). The stereotaxic atlas was always consulted while drawing the anatomical boundaries of each region of interest. For studies in Fig. 21f-h, we had tried the TrueBlack (23007, Biotium, Fremont, CA) autofluorescence quencher, but there was uneven coverage. To avoid inaccurate measurements, we excluded three mice from the data in Fig. 21f-h. In addition, OBs of two more mice were lost while processing tissue for the data in Fig. 21f-h. Another bin from the 1-in-5 sagittal series was used for the data in Fig. 21i and Fig. 23d-e, wherein we did not employ TrueBlack and all mice had their OBs attached to the tissue section for analyses. Similarly, as mentioned previously, for the studies in Fig. 31, sections from the same mice as in (Nouraei et al., 2018) were used for immunostaining.

14. Postmortem human samples

Postmortem OB and amygdalae from male and female patients with Lewy body disorders and age-matched controls were obtained via NIH NeuroBioBank (UCLA and
University of Miami Brain Banks). Demographic information of samples is in Table 4. Only deidentified tissue samples were obtained and were not subject to consideration by the Duquesne University Institutional Review Board (IRB). All IRB numbers for the NeuroBioBank repositories are available online.

15. RNA-sequencing

Postmortem OB and amygdale from the UCLA Brain Bank were sequenced. The UPMC Health Sciences Sequencing Core performed RNA isolation, library preparation, and sequencing. The Qiagen RNeasy Plus Micro Kit (74034, Qiagen, Germantown, MD) was used for RNA isolation. RNA quality was determined using RNA ScreenTape (5067-5579, Agilent, Santa Clara, CA) on an Agilent 2200 TapeStation. Transcriptome libraries were prepared with Takara SMART-Seq kit (634447, Takara Bio USA, Inc, Mountain View, CA), which is used for samples with high postmortem intervals (for samples with a high likelihood of RNA degradation). The illumina NextSeq 500 was used for sequencing and ~23 million reads per sample were generated. Steps in sequence alignment, gene expression quantification, and differential expression analyses were based on prior work (Dobin et al., 2013; Liao, Smyth, & Shi, 2014; Love, Huber, & Anders, 2014).

16. Statistical analyses

All in vitro data are presented as mean + SD in bar graphs. In vivo data and data from postmortem brain samples of human subjects are presented as mean + SD in bar graphs or as violin plots or box plots with medians and interquartile ranges. Data from replicates were averaged to obtain a single value for each litter of pups (i.e., each “n” is a
different litter altogether) For experiments with only two groups, if data were Gaussian (per the Shapiro-Wilk test), the Student’s two-tailed unpaired $t$-test was performed. For heteroscedastic data, the Welch’s $t$ test was performed. For Gaussian data with $>2$ groups, we performed a one, two, or three-way ANOVA followed by Bonferroni post hoc and obtained the statistical significance for pairwise comparisons, statistical interactions, and main effects. Intervariable statistical interactions with biological sex are shown above some graphs, consistent with recent guidelines on reporting sex differences (Garcia-Sifuentes & Maney, 2021). For non-Gaussian data, the Mann-Whitney $U$ (2 groups) or Kruskal-Wallis ($>2$ groups) test was used. Some non-Gaussian data are also presented as log-transformed values, so that statistical interactions and main effects could be obtained. For correlation testing, either the two-tailed Pearson or Spearman analyses were employed, based on whether data were normally distributed or non-Gaussian. Alpha was always set at 0.05. As far as possible, data were analyzed by blinded observers, except for immunoblotting, wherein samples are always loaded in a preset order. All statistical tests were performed using GraphPad Prism (Version 9) or IBM SPSS Statistics (Version 23). Statistical outliers were never excluded, except in some key datasets, and in these cases the data are always shown with and without the outlier.
Chapter 3

Specific Aim 1: To determine if males versus females differ in their dependence on endogenous Hsp70 defenses in a-synucleinopathic disease

Rationale

Men are at ~1.5-2-fold higher risk of Lewy body disorders, compared to women (Baldereschi et al., 2000; Cerri et al., 2019; de Lau et al., 2004; Elbaz et al., 2002; Gillies et al., 2014; Gowers, 1886; Taylor et al., 2007; Van Den Eeden et al., 2003; Wooten et al., 2004). Mechanisms underlying this sex bias are unclear but may be driven by sex differences in expression and function of cytoprotective molecules, either at baseline or after stress exposure. Lewy body disorders are characterized by pathological inclusions that contain misfolded α-synuclein, which is phosphorylated at Serine 129 (pSer129), under disease conditions (J. P. Anderson et al., 2006; Fujiwara et al., 2002; Spillantini et al., 1997). In response to misfolded proteins, chaperones such as those that belong to the Hsp70 disaggregation machinery are induced, but it is unknown if this stress response pathway is impacted by biological sex, which we examined in the studies of Aim 1.

Prior work suggests that, at baseline, women show twice as much Hsp70 in their peripheral circulation as men (Pockley, Shepherd, & Corton, 1998). Similarly, female rodents show higher Hsp70 levels in their hypothalamus and heart (Bodega, Hernandez, Suarez, Martin, & Fernandez, 2002; Voss et al., 2003). Ovariectomized mice have been shown to display lesser tolerance to heat stress, perhaps because of low levels of Hsp70, both of which can be rescued by estrogen supplementation (Hou, Wei, Luo, & Liu, 2010; Takahashi et al., 2010). Estradiol may increase the levels of Hsp70, by perhaps increasing
the levels of its transcription factor, HSF1 (C. D. Chen et al., 2016; Vydra et al., 2019). Apart from HSF1, estradiol activates the estrogen receptor, which is normally maintained in an Hsp90-bound inactive state (Chambraud, Berry, Redeuilh, Chambon, & Baulieu, 1990; Fliss, Benzeno, Rao, & Caplan, 2000). In the presence of estradiol, the estrogen receptor dissociates from this Hsp90-bound inactive state and induces the transcription of its target genes (Beato & Klug, 2000). Thus, estradiol exposure can activate the estrogen receptor as well as HSF1, which is the transcription factor for many Hsps, including Hsp90 and Hsp70. Studies have shown that Hsp90 is critical for the estrogen receptor to perform its functions and there may be a positive feedback loop between estradiol/estrogen receptor and HSF1 (Fliss et al., 2000; Inano, Curtis, Korach, Omata, & Horigome, 1994; Powell, Wang, Shapiro, & Xu, 2010). Although Hsp90 is the most widely studied Hsp to bind the estrogen receptor, Dhamad et al. discovered that Hsp70 is the most abundant interactor for the estrogen receptor (Dhamad, Zhou, Zhou, & Du, 2016). The role of Hsp70 in this context has not been clarified, but based on its chromatin-bound state, it is hypothesized to interact with the receptor to repress it “on-site”, i.e., when the estrogen-receptor is also chromatin-bound (Dhamad et al., 2016). These findings suggest that Hsps, including Hsp70, and the female sex hormone estradiol regulate each other’s functions, but the role of this loop in neurological disease is unclear.

There is also very little known about levels of Hsp70 in key limbic brain regions, such as the OB and amygdala—areas that show dense α-synuclein pathology in Lewy body disorders. Some studies point toward a decrease in the levels of the constitutive version of Hsp70 (i.e., Hsc70) in the postmortem amygdala of PD subjects, compared to controls (Alvarez-Erviti et al., 2010). However, in that study, the sex and age of the subjects were
not stated. Many studies also do not include age- and sex-matched controls and recent guidelines on reporting sex differences recommend the testing of statistical interactions with biological sex as one variable (Garcia-Sifuentes & Maney, 2021). In our studies, we first examined levels of Hsp70, Hsc70, and a key co-chaperone of Hsp70 (i.e., Hsp40) in the postmortem OB and amygdala of male and female control subjects and age-matched patients that had been clinically diagnosed with Lewy body disorders.

Chaperones, including Hsp70, Hsc70, and Hsp40 are known to colocalize with markers of Lewy pathology in human disease (Auluck et al., 2002; Leverenz et al., 2007; McLean et al., 2002). This neuropathological finding may reflect an entrapment of Hsps within inclusions, which might lead to a loss in the normal function of Hsps due to a decrease in the levels of the soluble forms of these chaperones. Thus, the second goal in this Aim was to test if the Hsp70 defenses are active in α-synucleinopathies. In addition, we sought to assess if neuronal cells from males versus females differ in their reliance on Hsp70 defenses, which might explain the higher risk of α-synucleinopathies in males. We began by characterizing a sex-stratified disease model by harvesting neurons from male versus female rat pups and exposing them to PFFs. Next, PFF-treated male versus female primary neuronal cultures were exposed to two independent pharmacological inhibitors of Hsp70/Hsc70 activities, i.e., VER155008 and MAL3-101. The former is an adenosine-derived, active site inhibitor that binds at and blocks the ATP binding domain of Hsp70/Hsc70 (Schlecht et al., 2013). As ATP binding is critical for Hsp70/Hsc70 to fulfill its functions, even those independent of its chaperone function, VER15008 may be a less selective antagonist of Hsp70 chaperone activities (as discussed in (Crum et al., 2015)). In contrast, the pyrimidinone MAL3-101 is an allosteric inhibitor that blocks the Hsp40-
stimulated ATP hydrolysis step and thus more selectively inhibits the \textit{chaperone} function of Hsp70 (Adam et al., 2014; Braunstein et al., 2011; Fewell et al., 2004; Huryn et al., 2011; Kilpatrick et al., 2013b). We relied on these tools to determine the specific function of Hsp70 that may be involved in \(\alpha\)-synucleinopathies.

\textbf{Results}

\textit{Women express higher levels of Hsp70 in the amygdala, irrespective of disease, but age at death is positively correlated with amygdalar Hsp70 levels only in diseased men.}

Postmortem OB and amygdalae from male and female patients with Lewy body disorders and age-matched unaffected control subjects were acquired from the NIH NeuroBioBank repositories. Age at death (Fig. 4a) and postmortem intervals (Fig. 4b) did not show any differences across groups. Immunoblotting the OB revealed that there were no significant differences in Hsp70 levels across the groups (Fig. 4c-f). In the amygdala, there were significant main effects of biological sex (two-tailed \(p=0.026\)) and disease (two-tailed \(p=0.046\)) on Hsp70 (Fig. 4g-j). To better delineate these findings, we pooled the control and diseased samples to test the effect of biological sex and found that women, regardless of disease, show higher Hsp70 in their amygdalae as compared to age-matched men (Fig. 4g). On combining the male and female samples to test the effect of disease, we found that there was a trend (two-tailed \(p=0.057\)) toward higher levels of Hsp70 with disease (Fig. 4h). This rise in Hsp70 with disease could mainly be attributed to diseased men, based on the two-way ANOVAs (Fig. 4i; two-tailed \(p=0.069\)).

We correlated Hsp70 levels with the age at death or postmortem intervals of the human subjects. No significant correlations were found in the OB (Fig. 5a). In contrast, a
significantly positive correlation was found between amygdalar Hsp70 levels and age at death—but only among diseased subjects. This association between amygdalar Hsp70 and survival among diseased subjects was mainly attributed to the diseased men, and not diseased women (Fig. 5b-c). No significant correlations were noted between postmortem intervals and Hsp70 in the OB or amygdala (data not shown).
We verified that the human samples diagnosed with Lewy body disorders did express high levels of pSer129, a marker of Lewy pathology. Levels of monomeric (~15 kDa) pSer129 were higher with disease in the detergent (Triton X-100) insoluble fraction (Fig. 6a-d) of the male and female amygdala. Triton X-100 can extract soluble proteins, including α-synuclein under physiological conditions, but not the high molecular weight, often insoluble α-synuclein, which is present in proteinopathic inclusions (Stojkovska &
We also report that detergent-insoluble pSer129 aggregates, which we defined as pSer129 bands above the monomeric 15 kDa form, were higher with disease in both the male and female amygdala (Fig. 6e-f). Immunoblots in Fig. 6g show that, as expected, no pSer129 is seen in the detergent (Triton X-100) soluble fraction, which serves as a control to ensure that the sequential fractionation process was effective. Our findings that the diseased subjects express high levels of detergent-insoluble pSer129 are consistent with the clinical and neuropathological diagnoses of Lewy body disorders.

**Figure 6. Diseased subjects express high levels of detergent-insoluble phosphorylated α-synuclein in their amygdalae.** Amygdalae from control and diseased subjects were sequentially extracted using Triton X-100 into detergent soluble (Tx-Soluble) and detergent insoluble (Tx-Insoluble) fractions. These fractions were immunoblotted and probed for phosphorylated α-synuclein (pSer129). Monomeric pSer129 was analyzed separately from oligomeric pSer129 and the corresponding data are shown in a-d and e-f, respectively. Values from males and females were combined in a-b and e, to test the effect of disease on pSer129 levels. Data in panels b and d are log-transformed values of the raw data shown in a and e, respectively. Statistically significant main effects are shown above the graphs in panels d and f. **p ≤ 0.01; + p ≤ 0.05; Data in panel a were analyzed by the two-tailed Mann–Whitney U. Data in panels b, e were analyzed by the Welch’s t-test. Data in panel c were analyzed by the Kruskal–Wallis test. Data in panels d, f were analyzed by the two-way ANOVA followed by Bonferroni post hoc. Representative immunoblots are shown in g.
Biological sex and disease did not significantly impact the levels of constitutive Hsp70 (i.e., Hsc70) or co-chaperone Hsp40 in the OB or amygdala of human subjects

We determined if the impact of sex and disease on the inducible form of the chaperone (Hsp70) could also be extrapolated to the constitutive version of the chaperone (Hsc70) and to co-chaperone, Hsp40. However, no significant differences in levels of Hsc70 or Hsp40 were seen across any of the groups (Fig. 7-8). Postmortem intervals were also not correlated with Hsc70 or Hsp40 in the OB or amygdala (data not shown). However, Hsp40 levels in the amygdala were positively correlated with age at death in males only, irrespective of disease status (Fig. 8h).
Figure 7. Biological sex or disease status does not alter the levels of Hsc70 in the OB or amygdala. The OB and amygdala were obtained from male and female patients with Lewy body disorders or age-matched controls via NIH NeuroBioBank. Values from control and diseased samples were combined to test the effect of sex on Hsc70 levels in the OB (a) and amygdala (e). Values from male and female subjects were combined to test the effect of disease on Hsc70 levels in the OB (b) and amygdala (f). The full extent of the data, analyzed by two-way ANOVAs, are shown for the OB (c) and amygdala (g). Representative full-length immunoblots are shown for the OB (d) and amygdala (h) and these were first probed for the Hsp70 cofactor, Bag1 (not shown), and then reprobed for Hsc70. The Total Protein Stain was used as a loading control. Data in a-b and e-f were analyzed by the two-tailed unpaired Student’s t-test. Data in c and g were analyzed by the two-way ANOVA followed by Bonferroni post hoc. Reprinted by permission from [the Springer Nature Customer Service Centre GmbH]: [Springer] [Neurotherapeutics: The journal of the American Society for Experimental NeuroTherapeutics] [Heat Shock Protein 70 as a Sex-Skewed Regulator of α-Synucleinopathy, Bhatia, T. N., Clark, R. N., Needham, P. G., Miner, K. M., Jamenis, A. S., Eckhoff, E. A., Abraham, N., Hu, X., Wipf, P., Luk, K. C., Brodsky, J. L., & Leak, R. K] [Copyright 2021 by Springer] License number: 5218750410757; License date: December 30, 2021
Collectively, these findings from human postmortem brain tissue indicate that 1) women, in general, show higher levels of Hsp70 in their amygdalae, 2) disease may lead to a slight increase in Hsp70 in the amygdala of male subjects only, and 3) amygdalar Hsp70 is positively correlated with age at death (i.e., lifespan or survival) among males with Lewy body disorders.

*Exposure to preformed α-synuclein fibrils for up to 14 days induces robust pSer129+ inclusions, but no cell loss, in primary hippocampal neuronal cultures.*

We intended to determine the functional consequences of loss of Hsp70 defenses on pSer129+ inclusion pathology. To test this, we characterized a model of PFF-induced α-synucleinopathy by exposing primary neuron cultures from the hippocampi of Sprague Dawley rat pups to preformed α-synuclein fibrils for 10 d (Fig. 9a-c) or 14 d (Fig. 9d-f) in vitro. The hippocampus was used as this in vitro platform is the most efficient at allowing PFFs to seed pathology (Polinski et al., 2018b). As mentioned previously, the extent of PFF-induced α-synuclein pathology may be dependent on levels of endogenous α-
synuclein (Courte et al., 2020; Hijaz & Volpicelli-Daley, 2020; Luna et al., 2018; Rietdijk et al., 2017; Taguchi et al., 2016; K. Taguchi et al., 2019). Several groups have shown that the hippocampus expresses higher endogenous α-synuclein compared to some other brain regions including the neocortex, entorhinal allocortex, olfactory bulb, and striatum (Courte et al., 2020; Jessica M. Posimo, 2015). Thus, neuron cultures from the hippocampus are suitable to study the impact of PFF-seeded pathology.

In our hands, exposure to PFFs for 10 d (Fig. 9a) or 14 d (Fig. 9d) did not induce cell loss, which was confirmed using Hoechst+ pan-nuclear counts and In-Cell Westerns for a mature neuronal marker (NeuN) as outputs. These PFFs (labeled “in-house” in Fig. 9) were kindly provided to us by our collaborator, Dr. Kelvin Luk, at the University of Pennsylvania. The absence of cell loss was confirmed using a second source of PFFs, purchased from StressMarq Biosciences (Fig. 9a, d). Notably, neither low (1 µg/mL) nor high (4 µg/mL) concentrations of PFFs used in this study induced cell loss. Numbers of pSer129+ inclusions and total area occupied by pSer129+ inclusions were measured at 10 d (Fig. 9b, c) and 14 d (Fig. 9e, f) after exposure to either the in-house or StressMarq PFFs, at 1 or 4 µg/mL. Although there was robust pathology in the PFF-treated groups at both concentrations of PFFs, no concentration-dependent increase in inclusion pathology was observed. These findings were verified by two independent monoclonal pSer129 antibodies (clone 81A in Fig. 9b, e and clone EP1536Y in Fig. 9c, f).

Collectively, these findings suggest that, in our hands, PFF exposure for up to two weeks does not induce cell loss—at least in primary, mixed-sex neuron cultures harvested from the hippocampus. However, we saw a robust induction of α-synuclein pathology that peaks within 10-14 days after exposure to PFFs.
Figure 9. Exposure to PFFs for up to two weeks induces robust pSer129+ inclusion formation, but not cell loss in mixed-sex, primary hippocampal neuron cultures. Primary mixed-sex neuron cultures were harvested from hippocampi of rat pups and exposed to PFFs for 10 (a-c) or 14 d (d-f). PFFs were obtained from the lab of Dr. Kelvin Luk at University of Pennsylvania (labeled “in-house”) or from StressMarq Biosciences and were used at two concentrations (1 or 4 µg/mL). Cells were treated on DIV2 and fixed and assayed at DIV12 (a-c) or 16 (d-f). Viability was measured via Hoechst nuclear counts or In-Cell Westerns for NeuN (a, d). DRAQ5 was a normalization control for In-Cell Western data. Two independent pSer129 monoclonal antibodies were used for the immunostaining (Clone 81a in b-c and clone EP1536Y in e-f). Numbers of pSer129+ inclusions and total area occupied by pSer129+ inclusions were expressed either alone or as a fraction of Hoechst+ counts (b-c, e-f). Representative photomontages of Hoechst (pseudocolored blue) and pSer129 (pseudocolored red) are shown. Shown are mean + SD of 4 independent experiments, each in triplicate wells. *** p ≤ 0.001 for 0 vs. 1 or 4 µg/mL PFFs; * p ≤ 0.05 for 1 vs. 4 µg/mL PFFs, one-way ANOVA followed by Bonferroni post hoc.

Primary hippocampal neurons harvested from male rat pups develop greater detergent-insoluble pSer129+ inclusions in response to exposure to PFFs.

To extend our findings with sex as a biological variable, we repeated the above studies in sex-stratified primary hippocampal neuron cultures that were harvested from male versus female rat pups. We distinguished male and female rat pups by anogenital distances, which are known to be shorter in females. The accuracy of this technique was confirmed by performing PCR for the Y-chromosome lined Sry gene (Fig 10a). Next, we treated, in parallel, hippocampal neurons from males and females to 1 µg/mL of PFFs for ten days (i.e., from day-in-vitro (DIV) 2 to DIV12). This concentration and timepoint was based on the pilot data shown in Fig. 9. Although some groups have shown PFF-induced loss of cells within 14-21 d, Lewy-related pathology may be reduced by the time massive cell loss becomes apparent (Mahul-Mellier et al., 2020; Volpicelli-Daley et al., 2014; Volpicelli-Daley et al., 2011). Therefore, we exposed neuron cultures to PFFs for 10 d, as Lewy-related pathology peaks in density by this time, and as mentioned in the Introduction, we chose to model early disease stages prior to extensive cell loss to test the therapeutic potential of interventions.

Cell viability in Fig. 10 was measured using two outputs—blinded counts of pan-nuclear marker, Hoechst, and of the mature neuronal marker, NeuN. Exposure to PFFs induced slight cell loss in male neuron cultures only, based on Hoechst+ and NeuN+ cell counts (Fig. 10b-c). Notably, there were no baseline sex differences in numbers, sizes, and total areas occupied by inclusions, unless Triton X-100 was added to extract detergent-soluble proteins. Triton X-100 did not solubilize the mature proteinopathic aggregates—in fact, an increase in the pSer129 immunolabel was noted with Triton X-100, which might
be attributed to greater epitope exposure when the detergent is added during the formalin fixation step (Fig. 10d).
However, we noted sex differences in detergent-insoluble pathology. Numbers of total pSer129+ inclusions, perinuclear inclusions, and total area occupied by inclusions—expressed as a fraction of Hoechst+ or NeuN+ cell counts—all revealed that male cells developed greater detergent-insoluble pathology, compared to females (Fig. 10e-j). In contrast, sex did not impact the average size of detergent-insoluble inclusions (Fig. 10k).

To determine if this sex-skewedness in pathology can simply be explained by sex differences in neuronal uptake of α-synuclein fibrils, we exposed neuron cultures to fluorescent, ATTO647-α-synuclein fibrils, as we were not able to find an antibody that specifically bound the *exogenous* α-synuclein fibrils. Since our postnatal neuron cultures also contain ~10-13% of astroglia (J. M. Posimo et al., 2015), we stained cells for GFAP (marker of astroglia) as well as NeuN, MAP2, and β-III-tubulin (neuronal markers). First, we note that α-synuclein fibrils appeared to colocalize less readily with GFAP+ astroglia than NeuN+ neurons (Fig. 11a; arrows point to instances wherein α-synuclein fibrils may...
be colocalized with GFAP⁺ astroglia). Second, there were many instances wherein α-synuclein fibrils were present at an intranuclear or perinuclear location (Fig. 11b)—consistent with the formation of perinuclear pSer129⁺ inclusions in our model. Third, apart

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**Figure 11. Patterns of uptake of ATTO₆₄⁷-α-synuclein fibrils by primary hippocampal cultures.** Primary hippocampal neuron cultures from male and female rat pups were exposed to 1 µg/mL of ATTO₆₄⁷ α-synuclein fibrils for 4 d. Representative z-stack photomontage in a is of preferential localization of fibrils (pseudocolored gray) within NeuN⁺ neurons (pseudocolored green). Arrows point to rarer instances of colocalization of fibrils with GFAP⁺ astroglia (pseudocolored red). Representative z-stack photomontage in b is of fibrils present in or around Hoechst⁺ nuclei (pseudocolored blue).

from the colocalization of fibrils with NeuN+ neuronal nuclei, α-synuclein fibrils were also present within somatodendritic compartments, based on the MAP2 staining in Fig. 12a. To determine if some of the α-synuclein fibrils were also entering axons, we used a subtractive technique by co-labeling for β-III-tubulin and MAP2 within the same wells. It is known that structures that are β-III-tubulin+ and MAP2- are likely axons (Bigler, Kamande, Dumitru, Niedringhaus, & Taylor, 2017; Hocquemiller et al., 2010). Although there were instances wherein fibrils were localized within β-III-tubulin+/MAP2- processes (likely axons; Fig. 12b), these were relatively rare, suggesting that fibrils are mostly present within or surrounding neuronal nuclei and within somatodendritic compartments. These findings were also confirmed at earlier timepoints (6 h after fibril exposure; data not shown). Note that images in Fig. 11a-b and Fig. 12a-b are z-stacks.

Given that NeuN+ neuronal nuclear boundaries are well-defined in our images and can be more precisely recognized by the “threshold” feature on the cellSens software, we counted the total numbers of fibrils within male and female NeuN+ neurons (Fig. 12c). No significant impact of biological sex was seen, suggesting that the greater detergent-insoluble pathology in male neuronal cultures (Fig. 10) is not due to sex differences in the uptake of α-synuclein fibrils.
Inhibition of endogenous Hsp70 function exacerbates \(\alpha\)-synucleinopathy more robustly in male-derived neuronal cultures.

Based on our above findings, we inferred that biological sex does not impact the uptake of fibrils by neurons, but the detergent-insoluble pathology was slightly higher in male neuronal cultures, compared to their female counterparts. It is possible that this may be due to sex-biasedness in the expression and function of endogenous defenses, such as the Hsp70 disaggregation machinery, but this has never been tested in the context of \(\alpha\)-synucleinopathies. In our sex-stratified in vitro model of \(\alpha\)-synucleinopathy, we inhibited Hsp70 function using two independent pharmacological inhibitors, MAL3-101 (Fig. 13a-h) and VER155008 (Fig. 13i-p) (Adam et al., 2014; Braunstein et al., 2011; Fewell et al., 2004; Huryn et al., 2011; Kilpatrick et al., 2013b; Schlecht et al., 2013). We found that neither MAL3-101 nor VER155008 exacerbated the toxicity of the fibrils in cultures of either sex, although female cells exposed to the inhibitors showed slightly better viability than male cells (Fig. 13b-c, j-k). Some of these effects may be attributed to male-specific baseline toxicity with Hsp70 inhibition, as seen with exposure to VER155008 (Fig. 13j). Exposure to MAL3-101 exacerbated the numbers of pSer129\(^{+}\) inclusions and the total area
occupied by pSer129+ inclusions, but only in male cells (Fig. 13d-g). There was a trend (two-tailed \( p=0.065 \); Fig. 13h) toward slightly bigger inclusion sizes in MAL3-101-treated male cells, compared to females. In principle, female cells did not respond to MAL3-101 with an increase in \( \alpha \)-synucleinopathy.

To extend these findings, we employed a second, independent inhibitor of Hsp70 function, VER155008, which increased the numbers of pSer129+ inclusions, expressed as a fraction of Hoechst+ cells, in both male and female cultures (Fig. 13l). However, the effect was slightly bigger in male cultures. In addition, VER155008 increased total area occupied by the inclusions and the average size of each inclusion in both male and female cultures, though the effect was more prominent in male cells (Fig. 13n-p). It is possible that, unlike MAL3-101, VER155008 may increase pathology in both male and female cultures due to its relative non-selectivity. Nonetheless, together with the MAL3-101 data, these findings suggest that inhibition of Hsp70 function exacerbates pathology more robustly in male neuronal cultures.

Thus, one may infer that male cells are more reliant than female cells on Hsp70 defenses to reduce inclusion formation. An alternative explanation is that blocking Hsp70 function may have a greater impact on the clearance and degradation of inclusions and/or fibrils in males, which may exacerbate pathology more prominently in the males, as compared to the females. These findings are also borne out by the robust statistical interactions noted above the graphs in Fig. 13d-g, n, and p, all of which suggest that biological sex modifies the impact of Hsp70 inhibition on multiple morphological aspects of PFF-induced \( \alpha \)-synucleinopathy.
Figure 13. Pharmacological inhibition of Hsp70 function exacerbates α-synucleinopathy more robustly in male neuronal cultures. Primary hippocampal neuron cultures from male and female rat pups were exposed to 0 or 1 µg/mL of α-synuclein fibrils for 10 d. In parallel, cells were also exposed to two independent inhibitors of Hsp70 function: 1) MAL3-101 at 0 or 5 µM (a-h) and 2) VER155008 at 0 or 12.5 µM (i-p). Representative photomontage in a and i are of Hoechst+ cells (pseudocolored blue), NeuN+ neurons (pseudocolored green), and pSer129+ (pseudocolored red). Blinded counts of Hoechst+ nuclei (b, j) and NeuN+ neurons (c, k). Counts of pSer129+ inclusions, expressed as a fraction of Hoechst+ cells (d, l) or NeuN+ neurons (e, m). Quantification of total area occupied by pSer129+ inclusions, expressed as a fraction of Hoechst+ cells (f, n) or NeuN+ neurons (g, o). Average inclusion size is shown in h and p. Data are expressed as fold-change values of the control groups. Shown are the mean ± SD of 3 independent experiments, each performed in triplicate wells. Significant intervariable statistical interactions are shown above d-g and n, p. ** p ≤ 0.01, *** p ≤ 0.001 for 0 versus 1 µg/mL fibrils; + p ≤ 0.05, ++ p ≤ 0.01, +++ p ≤ 0.001 for vehicle versus inhibitor; ~ p ≤ 0.05, ~ ~ p ≤ 0.01, ~ ~ ~ p ≤ 0.001 for male versus female, two or three-way ANOVA followed by Bonferroni post hoc.

**Female-specific compensatory increase in Hsc70 and Hsp40 with MAL3-101 exposure.**

To determine if the sex-skewed effects with MAL3-101 exposure noted in Fig. 12 are associated with sex-skewed compensatory upregulation of other functionally related chaperones, we immunoblotted cell lysates of fibril-treated male and female neurons exposed to MAL3-101 (Fig. 14). No changes in the levels of Hsp70 were noted across any of the groups (Fig. 14a-b). In contrast, MAL3-101 and fibril-treated neurons showed an increase in the levels of the constitutive form of Hsp70 (i.e., Hsc70)—an effect that was female-specific (Fig. 14c-d). Consistent with these findings, there was also a slight increase in the levels of the co-chaperone, Hsp40, with MAL3-101 and fibril exposure in female, but not male cells (Fig. 14e-f). These changes may thus underlie the failure of MAL3-101 to exacerbate α-synuclein pathology in female neuronal cultures.
Discussion

Through this Aim, we were the first to examine the role of Hsp70 defenses in α-synucleinopathies in studies that systematically included sex as a biological variable. First, we report that women show higher levels of Hsp70 (but not Hsc70 or Hsp40) in the amygdala, compared to age-matched male subjects. However, with disease, males tended to show higher Hsp70 in the amygdala, compared to age-matched control male subjects. Women did not show these disease-specific changes in Hsp70, due perhaps to the higher baseline levels of Hsp70 in women. Although the male-specific increase in Hsp70 with disease was mild, it may still exert protective effects, particularly toward extending the lifespan. Indeed, Tatar et al. have shown that a mere 10-12% increase in Hsp70 levels is sufficient to improve survival of male fruit flies by ~8% (Tatar, Khazaeli, & Curtsinger, 1997). Delivery of exogenous Hsp70 to aged male mice has also been shown to extend their lifespan (Bobkova et al., 2015). Compared to ~35% survival in control, aged males, there was a ~54% survival in Hsp70-treated males (Bobkova et al., 2015). In addition, a polymorphism (T2437C) in an Hsp70 gene (HSPA1L) is known to be negatively linked
with lifespan (O. A. Ross et al., 2003; Singh, Kolvraa, Bross, Christensen, et al., 2006), and this polymorphism also reduces the stability of the interaction between Hsp70 and its client proteins, thereby affecting the chaperone activity of Hsp70 (O. A. Ross et al., 2003; Singh, Kolvraa, Bross, Jensen, et al., 2006; Singh, Kolvraa, & Rattan, 2007). Thus, chaperone functions of Hsp70 may be linked with longevity.

We discovered that Hsp70 levels are positively linked with age at death in males with Lewy body disease, but not in women or control subjects. These sex-specific effects are consistent with early work in Hsp70-knockout mice subjected to sepsis, wherein aged male septic Hsp70\(^{-/-}\) mice showed 100% mortality by day 4 following sepsis, in contrast to 20% mortality in female and 25% mortality in younger male mice (McConnell et al., 2011). Aged male septic Hsp70\(^{-/-}\) mice also showed greater apoptosis, compared to female and younger male mice (McConnell et al., 2011). Thus, aged males may be more dependent than younger males and aged females on Hsp70 defenses to extend their survival under conditions of sepsis. Akin to this work, our data also uncover a probable link between survival and Hsp70 levels in men suffering from Lewy body disorders. Apart from Hsp70, we found that levels of its co-chaperone, Hsp40, are also correlated with age at death in men, but not women. If age at death is indicative of lifespan or survival one may infer that, with disease, survival may be prolonged in those men that harbor higher levels of Hsp70 and that Hsp70 defenses may be engaged more robustly in older men. Alternatively, diseased males that survive longer may have had a stronger induction of Hsp70 for protein quality control. Indeed, in the study on sepsis, the authors inferred that “alterations in HSP70 may be one of the reasons that survival is lower in aged patients who become septic compared to younger patients with the same disease” (McConnell et al., 2011). If
extrapolated to our work, it is thus possible that interference with Hsp70 defenses may have detrimental effects in males with α-synucleinopathies.

Our collective data thus suggest that males may be dependent on Hsp70 defenses under α-synucleinopathic conditions. Note that in our study, the human subjects were diagnosed with different Lewy body disorders and at more than one institution in the US, but the existence of statistically significant differences despite these heterogeneities may suggest that our findings are generalizable across groups. We studied changes in Hsp70 by immunoblotting whole tissue extracts, and so we have not clarified if the changes in Hsp70 can be attributed to specific cell types (neurons versus glia). In future studies, this is important to examine as Hsp70 may show differential functions based on the cell type in which it is primarily expressed or from where it is released (Guzhova et al., 2001; San Gil, Ooi, Yerbury, & Ecroyd, 2017). The postmortem intervals of the human subjects were also long, but this confound is mitigated by an absence of correlations between postmortem intervals and levels of Hsp70, Hsc70, or Hsp40. Nonetheless, longitudinal studies with a larger sample size at different stages of disease or lifespan are warranted. Other groups of chaperones must also be analyzed to establish if the male-specific reliance on Hsp70 defenses can be extended to some other Hsps.

Our other major finding in this Aim is that inhibition of endogenous Hsp70 function intensifies α-synucleinopathy more robustly in male-derived neurons. Notably, our data from this in vitro work supports our findings from human subjects that men (and not women) may be dependent on Hsp70 in α-synucleinopathies. To our knowledge, we are the first to include sex as a biological variable in an in vitro model of PFF-induced α-synucleinopathy. We found that sex differences in this model emerged when detergent-
soluble proteins were removed and is consistent with the in vivo work of Mason et al., wherein PFF-injected female mice had fewer proteinopathic aggregates than PFF-injected male mice. Sex differences also became apparent in our in vitro model when cells were exposed to inhibitors of Hsp70 function. Thus, in culture, males may be more reliant than females on Hsp70 defenses to blunt inclusion formation or to increase the degradation of preexisting inclusions, perhaps because females are able to upregulate Hsc70 and Hsp40 following Hsp70 inhibition. A lack of compensatory increase in these functionally related chaperones may drive an increase in pathology in PFF-treated male cells exposed to Hsp70 inhibitors. Additional work is needed to understand if the protective role of Hsp70 is mediated by the clearance of exogenously added PFFs, prevention of formation of new inclusions, clearance of preexisting inclusions, or a combination of all these mechanisms.

We did not use genetic tools to suppress Hsp70 in our work. Instead, we relied on the established pharmacological inhibitors of Hsp70, i.e., VER155008 and MAL3-101. Of these, MAL3-101 is a more selective inhibitor of Hsp40-stimulated ATP hydrolysis (Adam et al., 2014; Braunstein et al., 2011; Fewell et al., 2004; Huryn et al., 2011; Kilpatrick et al., 2013b), which allowed us to test if it is the chaperone function of Hsp70 that is directly involved in mitigating α-synuclein pathology. As mentioned previously, both the inhibitors have distinct mechanisms of action and, thus, if the inhibitors produce similar outcomes—as they did in our work—one may conclude that the effects are due to the inhibition of the chaperone function of Hsp70 (Crum et al., 2015). It is also important to note that Hsp70 and Hsc70 share structural similarities (Mayer & Bukau, 2005) and thus both inhibitors can have effects on the inducible as well as the constitutive form of the protein. For example, the rise in Hsc70 in female cells with MAL3-101 may be driven by inhibition of
Hsp70, Hsc70, or both. Another caveat is that we cannot measure the inhibitor-induced loss of Hsp70 ATPase activities, as there are many other ATPases in cell lysates (Crum et al., 2015). Furthermore, VER155008 binds at the ATP binding pocket, which is not unique to Hsp70/Hsc70, suggesting that VER155008 may exert non-specific effects. For example, recent work revealed that exposure of PC12 cells to VER155008 elicits a downregulation of the PI3K/AKT and MEK/ERK signaling pathways (F. Xu et al., 2019). It is possible that the non-specificity of VER155008 may underlie the exacerbation of α-synuclein pathology in female cells, given that the more specific inhibitor, MAL3-101 did not worsen pathology in female cultures.

Some of the strengths of our in vitro mechanistic work are that our studies were performed in the absence of other brain cells and the culture media did not include sex hormones. Thus, we were able to study the effects of Hsp70 inhibition on neurons alone, and our data suggest that sex differences may be driven by intrinsic mechanisms or may be a result of the prenatal priming of the brain with sex steroids, as also mentioned in Aim 3. Since there are many baseline sex differences in neural numbers, morphologies, volumes, etc. (Premachandran, Zhao, & Arruda-Carvalho, 2020) in the in vivo brain, it is better to first test mechanistic differences in vitro, wherein postmitotic neurons are harvested in parallel from both sexes and are seeded at the same initial cell densities. Although it is possible that cells from one sex may be more likely to survive culture protocols, this caveat is mitigated by our data wherein we reveal that there are no baseline sex differences in cell numbers after harvesting and plating.

Our mechanistic studies were performed in neuron cultures that were harvested from postnatal (and not embryonic) pups—a developmental stage at which our sexing
technique and our telencephalic dissections would be more accurate (J. M. Posimo, Titler, Choi, Unnithan, & Leak, 2013). Importantly, the prenatal surge in testosterone in male rodents only peaks at ~E18-E19, during which time the male brain becomes primed and sexually differentiated from females (described further in Aim 3 (Weisz & Ward, 1980)). Thus, postnatal cultures are more suitable to study sex differences in vitro, rather than embryonic neuron cultures harvested prior to E18. Furthermore, we were able to culture postnatal neurons for relatively long periods, which may be difficult to achieve in neuron cultures harvested at adult or aged stages, and we optimized our culture protocol based on pilot work to ensure that cells were not clustered or showing signs of extensive chromatin condensation.

As acknowledged previously, we see a ~10-13% astroglial population in our postnatal neuron cultures (J. M. Posimo et al., 2015) and others have suggested that small proportions of astroglia are also seen in E16-E18 neurons (Luna et al., 2018). Although this may be a better model of the in vivo milieu, astroglia are known to engulf α-synuclein fibrils and exert protective effects on nearby neurons (Loria et al., 2017), as we have also found (Aim 3). Some groups have used cytosine arabinofuranoside (Ara-C) to mitigate astrocytic proliferation in vitro, but it is important to note that Ara-C does not kill all astroglia, produces as much as 50% toxicity in NeuN+ neurons when administered alone, and, consistent with this loss in neurons, also produces ~40-50% loss in the area fraction of pSer129 (Luna et al., 2018). To control for the presence of astroglia in our work, nearly all our readouts on inclusion pathology were always expressed as a fraction of Hoechst+ pan-nuclear counts and, separately, of NeuN+ neuronal numbers and the data were largely internally consistent. Such distinct measurements of viability have also been recommended
to reduce the risk of Type I (false positive) errors (Gilbert et al., 2011; J. M. Posimo et al., 2014). In addition, Hsp70 genes (\textit{HSPA1A, HSPA1B, HSPA1L}) and Hsp40 genes (members of the \textit{DNAJ} family) are expressed at higher levels in neurons versus astrocytes of the hippocampus (see DropViz database by (Saunders et al., 2018)), and so effects of Hsp70 inhibition in our work may be largely mediated by loss of Hsp70 function in neurons. We also found that α-synuclein fibrils were more likely to localize with NeuN$^+$ neurons rather than GFAP$^+$ astroglia, perhaps because the LAG3 receptor, which may be involved in uptake of PFFs, is expressed at higher levels in neurons (Mao et al., 2016). In addition, we have not observed pSer129$^+$ inclusions forming in astroglia in culture (unpublished observations). Thus, our model is suitable to examine neuron-seeded α-synucleinopathy.

Collectively, through studies in postmortem human brain tissues and a new sex-stratified \textit{in vitro} model, our data are the first to suggest that males may be more reliant than females on Hsp70 defenses to reduce α-synuclein pathology and that males may be less able to compensate for loss of Hsp70 function.
Chapter 4

Specific Aim 2: To determine if exogenous delivery of Hsp70 (eHsp70) mitigates pathology and behavior deficits in the PFF model of α-synucleinopathic disease

Rationale

In Aim 1, we have shown that endogenous Hsp70 mitigates α-synucleinopathy in an in vitro model of Lewy body disorders, due perhaps to its chaperone function. The effects of Hsp70 are known to be governed by its subcellular localization, as although intracellular Hsp70 may be a chaperone, it may harbor additional immunologic roles when released in the extracellular space (Bianchi, 2007; M. Evgen'ev et al., 2019; Kono & Rock, 2008; Schmitt, Gehrmann, Brunet, Multzoff, & Garrido, 2007). Extracellular Hsp70 is a “danger signal” or a damage-associated molecular pattern (DAMP), released from stressed cells to activate the immune system and enhance the host defense (Bianchi, 2007; Dukay et al., 2019; Giuliano, Lahni, Wong, & Wheeler, 2011; Heil & Land, 2014; Ma, Schenck, Pabon, & Choi, 2018; Wallin et al., 2002). For example, Campisi et al. showed that in a rat model of lipopolysaccharide (LPS)-induced bacterial infection and in rats exposed to the stress of tail shock, levels of extracellular Hsp70 are increased, which may lead to release of cytokines, such as IL6, or inflammatory mediators, such as nitric oxide, thereby promoting host recovery by reducing the numbers of bacterial colonies (Campisi, Leem, & Fleshner, 2003). This was one of the earliest studies to show that Hsp70 in the extracellular space is functional as it helped in resolving inflammation and in encouraging recovery from injury (Campisi et al., 2003). The immunomodulatory roles of Hsp70 are also supported by findings that it can act as an immunologic adjuvant and that it can bridge adaptive and
innate immune responses, and Hsp70-based vaccines have also been tested in multiple
disease models (Gong et al., 2010; W. S. Kim et al., 2018; Pack, Kumaraguru, Suvas, &
Rouse, 2005; Suto & Srivastava, 1995; Tamura et al., 2012). In the extracellular space,
Hsp70 may exist either in the freely soluble form, as a part of exosomes, or it may be bound
to antigenic peptides (Radons, 2016). The uptake of extracellular Hsp70 involves several
receptors or it may enter cells via internalization of exosomes (Radons, 2016). As
mentioned in the Introduction, some receptors for Hsp70 are toll like receptors,
glucocorticoid receptors, scavenger receptors, chemokine receptors, etc., and these are
present on the surfaces of many cells, including neurons, microglia, macrophages, etc.
(Alberti et al., 2021; Calderwood et al., 2007; Dukay et al., 2019; Petta et al., 2016;
Vandevyver et al., 2012). Thus, these reports indicate that in Lewy body disorders, Hsp70
may not only protect against pathology intracellularly via its chaperone function, but it may
also have cytoprotective effects in the extracellular space as a “chaperokine” (Asea, 2005,
2008; Kaur & Asea, 2019). This theory is untested in the PFF model of α-synucleinopathies
and it is unclear if extracellular Hsp70 reduces PFF-seeded pathology and if this can be
used as a therapeutic strategy in Lewy body disorders.

In several informative papers, researchers have examined the role and function of
exogenously delivered Hsp70 (eHsp70) as a potential therapy in models of ageing, stroke,
diabetes mellitus, and AD (Bobkova et al., 2015; Bobkova et al., 2014; Demyanenko et al.,
2020; M. Evgen'ev et al., 2019; M. B. Evgen'ev et al., 2017; Tytell et al., 2018). In these
studies, eHsp70 was delivered through the intranasal route, which is non-invasive and
bypasses the BBB as well as first-pass hepatic metabolism (Erdo, Bors, Farkas, Bajza, &
Gizurarson, 2018; Hanson & Frey, 2008; Kozlovskaya, Abou-Kaoud, & Stepensky, 2014;
Meredith, Salameh, & Banks, 2015; Wen, 2011). Akin to other biotherapeutic agents, Hsp70 may traverse the olfactory and trigeminal nerves to enter the brain after its intranasal delivery (Thorne, Pronk, Padmanabhan, & Frey, 2004). The nose-to-brain uptake of eHsp70 may be relatively rapid, as Bobkova et al. showed that it is present within the OB, neocortex, hippocampus, raphe dorsalis, locus coeruleus, and cerebellum within 3 h of intranasal delivery (Bobkova et al., 2014). However, most of this early work was either performed only in male mice or the sex was not mentioned. Thus, it is unknown if female mice also show nose-to-brain uptake of eHsp70, which we tested in Aim 2. We also tested the temporal kinetics of entry of eHsp70 into the brain and its clearance from the brain after intranasal delivery in aged male and female mice.

In neurotoxicant (6-OHDA or lactacystin) models of parkinsonism, intranasal eHsp70 was protective (Pastukhov et al., 2014; Tiefensee Ribeiro et al., 2021), but if eHsp70 mitigates α-synucleinopathy, particularly pSer129+ inclusions, in the PFF model of Lewy body disorders is untested. Thus, our second goal in this Aim was to establish if eHsp70 can diminish α-synuclein pathology in PFF-treated mixed-sex and sex-stratified primary neuron cultures. We performed these studies after pilot work characterizing the recombinant eHsp70 protein from two independent sources at multiple concentrations. Our qualitative findings showed that eHsp70 was taken up by neurons in culture. Since eHsp70 is a multifunctional protein, we sought to determine if the effects of eHsp70 in our in vitro model are dependent on its chaperone function. These studies were based on prior work showing that, apart from chaperone functions, Hsp70 may also, 1) activate the adaptive immune response by upregulating genes involved in antigen presentation and processing, such as genes on major histocompatibility complexes (M. Evgen'ev et al., 2019; M. B.
Evgen'ev et al., 2017), 2) increase uptake of amyloid-β by glia (Kakimura et al., 2002), 3) improve phagocytic capacities of peripheral wound-healing macrophages (Kovalchin et al., 2006), and 4) suppress α-synuclein-stimulated release of inflammatory mediators, such as TNFα (Yu et al., 2018). Furthermore, Hsp70 possesses antioxidant properties, and it may activate genes that participate in glutathione defenses (M. B. Evgen'ev et al., 2017). In addition, Hsp70 may upregulate genes engaged in amine transport, neurotransmission, dopamine biosynthesis, synaptic vesicle release, fatty acid metabolism, detoxification, and immunoproteasome activities (M. Evgen'ev et al., 2019; M. B. Evgen'ev et al., 2017). Finally, Hsp70 is known to strengthen synapses, reduce markers of ageing and senescence, facilitate axonogenesis and neurogenesis, and Hsp70 may also maintain a critical balance between excitatory and inhibitory neurotransmitters in the CNS (Bobkova et al., 2015; Bobkova et al., 2014; Demyanenko et al., 2020; M. Evgen'ev et al., 2019; M. B. Evgen'ev et al., 2017; Tytell et al., 2018). Thus, the salutary effects of Hsp70 in diseases, such as Lewy body disorders, are likely to be widespread at the structural, functional, and biochemical levels.

As a final study in this Aim, we investigated the translational potential of eHsp70, by testing if it mitigates pSer129+ inclusions and behavior deficits in the novel, limbic-predominant model of disease established by Mason et al., wherein PFFs are injected into the OB/AON complex of mice (Mason et al., 2016; Mason et al., 2019). Our findings suggest that nose-to-brain delivery of eHsp70 may be a viable therapeutic approach for limbic-centered diseases, such as Lewy body disorders, where pathology is embedded in the olfactory structures early in the disease (Beach, Adler, et al., 2009; Beach, White, et al., 2009; Braak et al., 2003).
Results

Exogenous Hsp70 (eHsp70) mitigates pSer129+ inclusions in PFF-seeded mixed-sex primary hippocampal neuron cultures.

We began the in vitro studies in this Aim in the mixed-sex, primary hippocampal neuron culture model that we established in Fig. 9. These early, proof-of-concept studies (Fig. 15) were performed using recombinant human eHsp70 protein that was initially not processed for removal of endotoxins and was kindly provided to us by Dr. Patrick Needham and Dr. Jeffrey Brodsky (University of Pittsburgh). Treatments with eHsp70 were always performed in parallel with exposure to 0 or 1 µg/mL of PFFs for 10 d (i.e., from DIV2 to DIV12). Concentrations of eHsp70 were within the range of 12.5-100 µg/mL, based on prior work showing that ~75 µg/mL of eHsp70 is required for survival of motor neurons in culture (Robinson et al., 2005). We also included a mock vehicle control (labeled as “Mock” in Fig. 15) at the same concentrations as the eHsp70. As we noted in the Methods section, this mock control is an empty vector that does not express Hsp70 but goes through the same processing steps as in the purification of recombinant Hsp70. Thus, the mock was included to identify any effects of the residual materials from the synthesis process. Through the photomontage in Fig. 15a, we first report that the anti-human Hsp70 antibody that we used did not cross-react with the endogenous, rodent Hsp70 with any detectable immunoreactivity (note the absence of the green Hsp70 immunolabel in the groups labeled “Untreated” or “Mock Vehicle” in Fig. 15a). In contrast, as per panels labeled “eHsp70”, the anti-human Hsp70 antibody could detect the exogenous human Hsp70 (eHsp70; green) and there also appeared to be a concentration-based increase in the green immunolabel in this group (Fig. 15a). At 100 µg/mL of eHsp70, there was a significant reduction in
numbers of pSer129+ inclusions when expressed as a fraction of Hoechst+ cells (Fig. 15b-c). No changes in the numbers of Hoechst+ nuclei were noted (Fig. 15d).
Consistent with the reduction in the numbers of pSer129+ inclusions, we saw a reduction in the pSer129 protein expression (as per In-Cell Western analyses) at 100 µg/mL of eHsp70 (Fig. 15e). A statistical trend (two-tailed $p=0.053$) toward a slight reduction in the total area occupied by pSer129+ inclusions was noted with 100 µg/mL of eHsp70, compared to the concentration-matched mock control (Fig. 15f-g). In contrast, eHsp70 treatment did not lead to any significant differences in the average size of each pSer129+ inclusion (Fig. 15h). Next, we used the human-specific eHsp70 antibody from Fig. 15a and found that, for the most part, eHsp70+ structures did not harbor pSer129+ inclusions (Fig. 15i-j; a rare exception is noted with the arrowhead wherein eHsp70/pSer129 may be colocalized). These pilot data suggested that eHsp70 is incompatible with inclusion formation in PFF-treated mixed-sex neuron cultures.

We repeated these studies with a recombinant human eHsp70 protein preparation (purchased from Enzo) that was certified endotoxin-low by the manufacturer (Fig. 16). All eHsp70 preparations were confirmed for linearity, purity, and for an absence of...
contaminating degradation products by immunoblotting (Fig. 16a). Primary hippocampal neuron cultures exposed to vehicle or PFFs were treated with eHsp70 (Enzo; 75 µg/mL) or PBS (procured from Enzo as an additional vehicle control) for 10 d (Fig. 16b), after which cells were lysed and sequentially extracted with Triton X-100. Along with the fractionated lysates, PFFs (the positive control) were also loaded on the gel and then immunoblotted for pSer129 and pan-α-synuclein. PFFs were only tagged by the pan-α-synuclein antibody and not pSer129, as PFFs are not phosphorylated (Volpicelli-Daley et al., 2014; Volpicelli-Daley et al., 2011). Unexpectedly, we found that eHsp70 did not reduce pSer129 and α-synuclein levels in the detergent-insoluble fraction (Fig. 16b-c).

This contrasted with the findings in Fig. 16d-e, wherein we treated primary hippocampal mixed-sex neuron cultures with vehicle or PFFs and with eHsp70 (Enzo) at 18.75, 37.5, and 75 µg/mL, after which the cultures were fixed and immunostained for microscopic analyses. No changes in the numbers of Hoechst+ nuclei were noted, but eHsp70 at 75 µg/mL reduced the numbers of pSer129+ inclusions, when expressed alone as well as when expressed as a fraction of Hoechst+ nuclei. There was also a statistical trend toward slightly lower average inclusion sizes with 75 µg/mL of eHsp70 (two-tailed $p=0.061$). Finally, the total area occupied by the inclusions was significantly mitigated with 75 µg/mL of eHsp70, when expressed alone or as a fraction of Hoechst+ nuclei.

In sum, these findings indicate that eHsp70, procured from two independent sources decreases α-synucleinopathy in PFF-treated mixed-sex primary hippocampal neuron cultures. Since the levels of pSer129 and α-synuclein were not reduced in the detergent-insoluble fraction, we speculate that the reduction in inclusion load in situ may be
indicative of changes in the compactness of the proteinopathic aggregates, however, this requires further verification in future studies.
**Figure 16.** Exogenous Hsp70 (eHsp70) decreases pSer129\(^+\) inclusion numbers in fibril-treated, mixed-sex primary hippocampal neuron cultures, but does not reduce detergent-insoluble pSer129/α-synuclein levels. We confirmed the purity and linearity of eHsp70 preparations from the Brodsky lab (with or without endotoxin-removal procedures; labeled “in-house”) or from Enzo Life Sciences via immunoblotting in **a**, the Total Protein Stain was employed as a loading control. Primary hippocampal neuron cultures from rat pups were exposed to 0 or 1 µg/mL of α-synuclein fibrils for 10 d. In parallel, cells were also exposed to eHsp70 (obtained from Enzo) at 75 µg/mL or PBS (also from Enzo) to control against their vehicle-specific effects. Cell lysates were collected, extracted with Triton X-100, and subjected to immunoblotting for pSer129 and pan-α-synuclein (**b-c**). PFFs (0.15 µg) were loaded as a positive control (**c**). Levels of pSer129 and α-synuclein (at 15 kDa or at >15 kDa molecular weight) in the Triton X-100 soluble/insoluble fractions were normalized over the Total Protein Stain. Statistically significant intervariable interactions are shown above the graphs in **b**. Primary hippocampal neuron cultures from rat pups were exposed to 0 or 1 µg/mL of α-synuclein fibrils for 10 d. In parallel, cells were also exposed to eHsp70 (Enzo) at 18.75, 37.5, and 75 µg/mL or PBS (Enzo). UT = untreated. Blinded counts of Hoechst\(^+\) cells, pSer129\(^+\) inclusions, average size of each inclusion, and the total area occupied by pSer129\(^+\) inclusions are shown in **d**. Representative photomontage in **e** is of pSer129\(^+\) inclusions (pseudocolored red) and Hoechst\(^+\) nuclei (pseudocolored blue). Shown are the mean + SD of 3 independent experiments. **p ≤ 0.01, ***p ≤ 0.001 for 0 vs. 1 µg/mL α-synuclein fibrils; + p ≤ 0.05, ++ p ≤ 0.01, +++ p ≤ 0.001 for vehicle vs. eHsp70; − p ≤ 0.05, −− p ≤ 0.01, −−− p ≤ 0.001 for Triton X-100 soluble vs. insoluble fraction; one, two, or three-way ANOVA followed by Bonferroni post hoc. Reprinted by permission from [the Springer Nature Customer Service Centre GmbH]: [Springer] [Neurotherapeutics: The journal of the American Society for Experimental NeuroTherapeutics] [Heat Shock Protein 70 as a Sex-Skewed Regulator of α-Synucleinopathy, Bhatia, T. N., Clark, R. N., Needham, P. G., Miner, K. M., Jamenis, A. S., Eckhoff, E. A., Abraham, N., Hu, X., Wipf, P., Luk, K. C., Brodsky, J. L., & Leak, R. K] [Copyright 2021 by Springer] License number: 5218750410757; License date: December 30, 2021

**eHsp70 is taken up by both, male and female neurons in culture.**

Our findings thus far suggest that eHsp70 reduces α-synucleinopathy in mixed-sex cultures exposed to PFFs. However, based on our data from Aim 1, male cells were more reliant than female cells on endogenous Hsp70 defenses in the PFF-seeded model of α-synucleinopathy. To assess if these sex-specific effects of endogenous Hsp70 can also be extended to exogenous Hsp70, we repeated the studies shown in **Fig. 15-16** in sex-stratified primary hippocampal neuron cultures. First, we identified if both male and female neurons display an uptake of eHsp70 after it is administered in the culture media. We noted that eHsp70 was present extracellularly, within neurites, and bordering NeuN\(^+\) neuronal nuclei in male and female cultures (**Fig. 17a**). In addition, eHsp70 colocalized with shrunken Hoechst\(^+\) nuclei in cells of both sexes (**Fig. 17b**) and, consistent with the qualitative observations in **Fig. 15i-j**, eHsp70 and pSer129 rarely colocalized (**Fig. 17c**). Exceptions
were noted in z-stacks of Fig. 17c, wherein the eHsp70 (pseudocolored green) seemed to ensconce a pSer129\(^+\) inclusion (pseudocolored red). Thus, eHsp70 is taken up neurons of both sexes and although it may occasionally encage pSer129\(^+\) inclusions, the intracellular presence of eHsp70 is largely incompatible with inclusion pathology.
eHsp70 mitigates pSer129+ inclusions in α-synuclein fibril-treated male and female primary hippocampal neuron cultures.

Primary hippocampal neuron cultures from male and female rat pups were exposed, in parallel, to 1 µg/mL α-synuclein fibrils and 75 µg/mL native eHsp70 protein (Enzo) for 10 d. Representative photomontages in a-c are all z-stacks from the same field of view, except that in panel c, the eHsp70 signal (pseudocolored green) was brightened to fully appreciate the near absence of colocalization between pSer129 (pseudocolored red) and eHsp70. In a, the intersecting lines point to examples of neuronal nuclei (NeuN; pseudocolored magenta) surrounded by eHsp70 (pseudocolored green). In b, the intersecting lines point to examples of eHsp70 (pseudocolored green) colocalized with shrunken, dying Hoechst+ nuclei (pseudocolored blue). In b, the arrows point to additional examples of colocalization between eHsp70 and Hoechst. In panel c, the intersecting lines point to examples wherein eHsp70 (pseudocolored green) encage pSer129+ inclusions (pseudocolored red).

findings that denatured eHsp70 might be toxic, we also found that exposure to denatured eHsp70 increased pSer129+ inclusions in PFF-treated cultures (Fig. 18e-i). Denaturation leads to loss in protein conformation and denatured proteins are more likely to aggregate, which may underlie the potential toxicity of denatured eHsp70 in our studies.

In contrast to denatured eHsp70, exposure to the native form of the protein led to a reduction in the numbers of pSer129+ inclusions (Fig. 18e-f). A reduction in the average size of each inclusion was noted with native eHsp70, compared to denatured eHsp70 in both male and female cultures (Fig. 18g). In addition, the total areas occupied by the inclusions were lower in cells of both sexes treated with native eHsp70, compared to denatured eHsp70 (Fig. 18h-i). A closer examination of the data shown in Fig. 18e-g revealed that male cells responded more prominently to native eHsp70 with a reduction in inclusion numbers, when compared to PBS. In contrast, female cells responded more robustly to native eHsp70 with a decrease in the average size of each inclusion, compared to PBS. Given these sex-specific reductions in the numbers (in male cells) and sizes (in female cells) of pSer129+ inclusions, the total area occupied by the inclusions showed no sex-specific effects.

These data indicate that treatment with eHsp70 reduces α-synucleinopathy in both male and female cultures, but that assessing different morphological aspects of pathology is critical in the PFF model as eHsp70 reduced pSer129+ inclusion numbers in males and inclusion sizes in females.
Figure 18. eHsp70 reduces α-synucleinopathy in fibril-treated male and female primary neuron cultures. Primary hippocampal neuron cultures from male and female rat pups were exposed to 0 or 1 µg/mL of fibrils for 10 d. In parallel, cells were also exposed to PBS or eHsp70 (Enzo) in its native or denatured form at 75 µg/mL. Representative photomontage in a is of Hoechst+ cells (pseudocolored blue), pSer129 (pseudocolored red), and NeuN+ neurons (pseudocolored green), all from the same field of view. Blinded measurements of Hoechst+ cells (b) and NeuN+ neurons (c) are shown. The signal of NeuN was measured by In-Cell Western analyses, and expressed as a fraction of NeuN+ neurons (d). Blinded measurements of pSer129+ inclusions, expressed as a fraction of Hoechst+ cells (e) or NeuN+ neurons (f). The average size of each inclusion is shown in (g). The total area occupied by the inclusions is shown as a fraction of Hoechst+ cells (h) or NeuN+ neurons (i). PFF-treated primary hippocampal neuron cultures were exposed to 5 µM MAL3-101 and 75 µg/mL of native eHsp70 (Enzo) or their respective vehicles for 10 d. Representative photomontage in j is of Hoechst+ cells (pseudocolored blue), pSer129 (pseudocolored red), and NeuN+ neurons (pseudocolored green), all from the same field of view. Blinded counts of perinuclear pSer129+ inclusions, expressed as a fraction of Hoechst+ cells (k) or NeuN+ neurons (l). Significant intervariable statistical interactions are shown above the graphs in e-g and k-l. Shown are the mean ± SD of 3–4 independent experiments, each performed in duplicate or triplicate wells. For b–i: * p ≤ 0.05, *** p ≤ 0.001 for 0 versus 1 µg/mL fibrils; + p ≤ 0.05, ++ p ≤ 0.01, +++ p ≤ 0.001 for indicated comparison versus denatured eHsp70; ~ p ≤ 0.05, ~~ p ≤ 0.01, ~~~ p ≤ 0.001 for male versus female, two or three-way ANOVA followed by Bonferroni post hoc. For k-l: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, two-way ANOVA followed by Bonferroni post hoc.

To determine the mechanism underlying the beneficial effects of eHsp70 in this *in vitro* model, we exposed PFF-treated primary hippocampal neuron cultures to eHsp70 and MAL3-101, or their respective vehicles for 10 d. MAL3-101 was chosen as it inhibits Hsp40-stimulated ATP hydrolysis and is thus a more specific inhibitor of the chaperone function of Hsp70 (Adam et al., 2014; Braunstein et al., 2011; Fewell et al., 2004; Huryn et al., 2011; Kilpatrick et al., 2013b). We found that MAL3-101 did not diminish the protective effects of eHsp70 on total numbers of pSer129+ inclusions and the total area occupied by inclusions (data not shown). In contrast, we found that eHsp70 had mitigated the numbers of *perinuclear* inclusions—an effect that was blunted when cells were co-stimulated with MAL3-101 ([Fig. 18j-l](#)). These data and the statistical interactions shown above the graphs in [Fig. 18k-l](#) all indicate that eHsp70 may reduce the numbers of perinuclear pSer129+ inclusions in an Hsp40-dependent (or chaperone function-dependent) manner. Notably, the protective effects of eHsp70 on the numbers of perinuclear inclusions are consistent with the subcellular localization of eHsp70 around the nucleus, as shown in [Fig. 17](#).

*Ehsp70 enters the male, but not the female mouse brain within 3 h of intranasal delivery and is cleared from the male mouse brain by 72 h post-intranasal infusion.*

We have shown that eHsp70 reduces α-synuclein pathology in mixed-sex and sex-stratified primary hippocampal neuronal cultures. As a prelude to testing the translational potential of eHsp70 as a therapy in Lewy body disease, we determined if eHsp70 shows nose-to-brain uptake in young and aged mice. Prior work had shown that intranasally delivered eHsp70 enters the male mouse brain within 3 h (Bobkova et al., 2014). First, we
harvested brain tissue from young (3-month-old) male mice 3 h after the intranasal delivery of endotoxin-low eHsp70 (from Enzo) or PBS. Immunoblotting extracts from the OB and the entorhinal cortex confirmed that eHsp70 enters the OB and travels as far caudally as the entorhinal cortex within 3 h of delivery in young male mice (Fig. 19a-b). We had loaded the recombinant eHsp70 protein as a positive control in these studies, which helped identify the 70 kDa band that was quantified. The higher molecular weight bands are likely aggregated eHsp70 or may be non-specific and the lower (~44 kDa) band is likely the cleaved ATPase domain, which is a major degradation product of Hsp70 (Fan, Lee, & Cyr, 2003; Flaherty, DeLuca-Flaherty, & McKay, 1990).

We repeated these studies in aged, 17-to-21-month-old male and female mice and included additional timepoints to determine the temporal kinetics of eHsp70 entry into the brain. In these latter studies, 6 µg of eHsp70 was delivered intranasally, given the use of high doses of eHsp70 by other groups (Tytell et al., 2018). Brain tissue was harvested from eHsp70-infused male and female mice at 3 h, 24 h, 48 h, and 72 h post-infusion. Sham control mice were also included (labeled “0 h”), which were not subjected to intranasal infusions but were anesthetized. For the data in Fig. 19c-d, we also included the younger PBS-infused controls from Fig. 19a-b. As seen in Fig. 19c-d, even in aged male mice, eHsp70 enters the OB and entorhinal cortex within 3 h of intranasal delivery and gets cleared from the brain by 72 h after intranasal delivery. In these aged mice, we noted that there was a faint ~70 kDa band in the sham (0 h) group but not in the younger, PBS-infused samples, which might be attributed to a mild cross-reaction between the anti-human eHsp70 antibody and the endogenous rodent Hsp70, as endogenous Hsp70 levels are known to rise with age (Crum et al., 2015). In contrast, no cross-reaction was noted via
immunocytochemistry (see the green eHsp70 immunolabel in Fig. 15a) using the same antibody. In addition, the higher molecular weight bands were also brighter in the aged, compared to younger samples. However, only the 70 kDa band (identified with the help of the positive control) was quantified, revealing that eHsp70 enters the OB (Fig. 19c), entorhinal cortex (Fig. 19d), and reaches as far behind as the spinal cord (Fig. 19e) within 3 h of intranasal delivery. We had also probed the brainstem (Fig. 19f), but the levels of eHsp70 in this region were not reproducibly higher in all eHsp70-infused mice (even at 3 h) and so the data were not statistically significant. Entry of eHsp70 into the spinal cord (Fig. 19e) is consistent with prior work showing that the insulin-like growth factor-1 (IGF-1) uses the olfactory and trigeminal nerves for rapid entry into the spinal cord within 30 mins of its intranasal delivery (Thorne et al., 2004). Thus, eHsp70 enters the young and aged male mouse brain within 3 h, lingering for up to ~48 h, and gets cleared within 72 h after intranasal infusions.

Separately, we assessed if eHsp70 also enters the aged female mouse brain after intranasal delivery at the same dose (6 µg) and timepoints (3 h, 24 h, 48 h, and 72 h post-infusion). Surprisingly, we did not find evidence of nose-to-brain uptake of eHsp70 in the OB (Fig. 20a), entorhinal cortices (Fig. 20b), brainstems (Fig. 20c), or spinal cords (Fig. 20d) of aged female mice, although the cross-reaction of the anti-human Hsp70 antibody with the rodent Hsp70 was noted. In sum, our findings suggest that females may require a higher dose of eHsp70, may show a more rapid degradation of eHsp70, or may even be more resistant to brain entry of eHsp70 due to a tighter nose-to-brain barrier. Further studies are warranted to fully tease apart the underlying mechanisms.
Figure 19. eHsp70 enters the male mouse brain within 3 h and gets cleared from the male brain within 72 h of its intranasal delivery. Young, 3-month-old male mice were intranasally infused with PBS or recombinant human eHsp70 protein (2 µg; Enzo; a-b). After 3 h, the OB (a) and entorhinal cortices (b) were harvested and subjected to immunoblotting for Hsp70 using an anti-human Hsp70 antibody. The recombinant eHsp70 protein was loaded in the penultimate lane as a positive control, which helped determine the ~70 kDa band that was quantified and is shown in the violin plots. Higher molecular weight bands may be non-specific or aggregated eHsp70 and the lower band of ~44 kDa is likely the cleaved ATPase domain of Hsp70. GAPDH was used as a loading control. Intranasal infusions were repeated in aged (17-to-20-month-old) male mice and tissue was harvested at 3, 24, 48, and 72 h post-infusion of 6 µg of eHsp70 (Enzo; c-f). Sham controls (0 h) were also included and for the data in c-d, the younger PBS-infused controls from a-b were also included. As a positive control, the recombinant protein was loaded in the last lane and the OB (c), entorhinal cortices (d), cervical spinal cords (e), and brainstems (f) were subjected to immunoblotting for Hsp70 using an anti-human Hsp70 antibody. In c, we first began with n=3 mice in the 3 h group, but an additional sample was added to this group by the time data in d-f were collected. The spinal cord from one male mouse in the sham (0 h) group in e was lost during tissue processing. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Data in a were analyzed by the two-tailed Mann–Whitney U test. Data in panel b were analyzed by the two-tailed unpaired t-test with Welch’s correction. Data in c-f were analyzed by the one-way ANOVA followed by Bonferroni post hoc. Reprinted by permission from [the Springer Nature Customer Service Centre GmbH]: [Springer] [Neurotherapeutics: The journal of the American Society for Experimental NeuroTherapeutics] [Heat Shock Protein 70 as a Sex-Skewed Regulator of α-Synucleinopathy, Bhatia, T. N., Clark, R. N., Needham, P. G., Miner, K. M., Jamenis, A. S., Eckhoff, E. A., Abraham, N., Hu, X., Wipf, P., Luk, K. C., Brodsky, J. L., & Leak, R. K] [Copyright 2021 by Springer] License number: 5218750410757; License date: December 30, 2021
Intranasal delivery of eHsp70 reduces pSer129+ inclusions and lessens behavior deficits in PFF-injected male mice.

Previous work employing intranasal eHsp70 in AD models demonstrated that it is protective only in pathological (disease) conditions but may be toxic in physiological, control conditions (Bobkova et al., 2014; M. Evgen'ev et al., 2019). For example, eHsp70 treatment of non-diseased mice may lead to cell death via pyknosis, karyolysis, cytolysis,
or vacuolization (Bobkova et al., 2014; M. Evgen'ev et al., 2019). These findings were consistent with early work showing that overexpression of Hsp70 in normal fruit flies has several adverse effects on the thermotolerance, development, and growth of flies (Klose, Chu, Xiao, Seroude, & Robertson, 2005; Krebs & Feder, 1997). We tested if intranasal eHsp70 delivery for 28 d mitigates pSer129+ inclusions and behavior deficits in male mice injected with PFFs in the OB/AON. It is known that in this model (Mason et al., 2016; Mason et al., 2019), PFF-injected male mice show only mild cell loss, but robust pathology in the limbic system and smell deficits as per the buried pellet test (Mason et al., 2016; Mason et al., 2019). Other groups have shown that after PFFs are injected in the OB, inclusions form mainly within neurons, peaking at 1-3 months post-injections, after which inclusion numbers may reduce when cell loss becomes extensive (Uemura et al., 2019). Thus, the model by Mason et al. may instead be of early-stage disease but is appropriate for testing the disease-modifying effects of interventions, such as eHsp70.

In this study, we injected PFFs in the OB/AON of aged, 20-month-old male mice. Female mice were not included as eHsp70 did not enter the brains of female mice after intranasal delivery, as per the data shown in Fig. 20. Beginning 24 h after PFF injections, we began daily intranasal delivery of PBS or eHsp70. Intranasal infusions were continued for 28 d (by which time inclusion pathology begins to peak, as per (Uemura et al., 2019)), after which we performed the buried pellet test and then the mice were sacrificed (Fig. 21a). Prior work had shown that daily intranasal eHsp70 delivery in 17-month-old male mice is a feasible approach (Bobkova et al., 2015). The eHsp70 that we used for our proof-of-concept studies was the yeast homologue, SsaI, which shows ~74% homology with HSPA1A (Kushnirov et al., 2000) and exhibits similar properties as human Hsp70 (Huryn
et al., 2011; Ireland et al., 2014). This Ssa1 preparation was provided to us by Dr. Patrick Needham and Dr. Jeffrey Brodsky (University of Pittsburgh).

First, we report that all the aged mice showed a mild loss in body weight during the course of the 28 d dosing regimen and no significant differences were found between PBS and eHsp70-infused mice (Fig. 21b-c). Second, based on data from two blinded investigators, we noted that eHsp70 infusions significantly reduced the time it takes for PFF-injected aged male mice to contact a food pellet when it is buried under the corncob bedding (Fig. 21d). The exposed pellet test was also performed (Fig. 21e) as a control to determine if the improvement in olfaction noted in Fig. 21d is attributed to differences in motivation/hunger status. A statistical trend (two-tailed \( p=0.088 \); Fig. 21e) toward a slight reduction in the latency to contact the food pellet when it is exposed on top (and not under) the bedding was noted. Given that PFF injections in the male mouse OB/AON led to impairments on the buried pellet test (Mason et al., 2019), we infer that eHsp70 infusions may mitigate this effect and that the beneficial effects of eHsp70 may be mediated by improvements in smell capacities or in the motivation to find the food pellet. Our third major finding in this study is that daily intranasal delivery of eHsp70 for 28 d mitigates the numbers of pSer129\(^+\) inclusions (Fig. 21f) in the AON and, as expected, does not impact neuronal (Fig. 21g) or pan-nuclear (Fig. 21h) viability statuses. Finally, consistent with prior work in the 6-OHDA model (Tiefensee Ribeiro et al., 2021), we found that eHsp70 tended (two-tailed \( p=0.059 \)) to reduce the levels of Iba1—a marker of microglia/macrophage reactivity, implying that eHsp70 may have an anti-inflammatory action (Fig. 21i). Thus, these data show that daily intranasal delivery of eHsp70 reduces \( \alpha \)-synucleinopathy in PFF-injected aged, 20-month-old male mice.
a) 
- α-Synuclein fibril injections in OB/AON
- Intranasal PBS or native eHsp70, every 24 h for 28 days in fibril-injected mice
- 20-mo-old male mice
- 24 h
- 4-weeks
- Buried Pellet Test & Sacrifice

α-synuclein fibrils: PBS eHsp70

b) 
- Body Weight (grams)
- At Surgery At Perfusion
- At Surgery At Perfusion
- Intranasal delivery

Data: PBS eHsp70

* Two-tailed p = 0.088

c) 
- Latency to contact buried peanut (seconds)
  [Average of two observers]

Data: PBS eHsp70 Intranasal delivery

* Two-tailed p = 0.088

d) 
- Latency to contact exposed peanut (seconds)
  [Average of two observers]

Data: PBS eHsp70 Intranasal delivery

* Two-tailed p = 0.088

e) 
- f) 
- g) 
- h) 
- i) 

Data: PBS eHsp70 Intranasal delivery

Two-tailed p = 0.059
To extend these findings, we repeated these studies in younger, 5-month-old male mice that were injected with PBS or PFFs in the OB/AON (Fig. 22a). Three months after surgeries, we confirmed that PFF-injected mice showed impairments in an open field arena (Fig. 22b-i). PFF-injected mice traveled shorter distances (Fig. 22b) at lower speeds (Fig. 22c), indicative of lower exploratory behaviors. In addition, PFF-injected mice showed greater numbers of freezing (Fig. 22d) and immobile (Fig. 22e) episodes, as compared to PBS-injected mice. PFF-injected mice also spent a greater amount of time being inactive (Fig. 22f) and reared less (Fig. 22g). Next, using a lottery-drawing box, all PFF-injected mice were randomly divided into three groups, based on type of intranasal infusate (PBS, native eHsp70, or denatured eHsp70) to be delivered (Fig. 22a). Notably, we confirmed that each group showed equivalent deficits on the open field (Fig. 22h), before beginning the intranasal infusions. For these studies, 6 µg of human eHsp70 (Enzo) was administered intranasally every 72 h, based on the kinetics study in Fig. 19. We had intended for the
work in Fig. 21 to be a long-term in vivo study, wherein we wanted to determine if intranasal eHsp70 rescues PFF-seeded pathology and deficits, by delivering eHsp70 after the emergence of behavior impairments. However, this study was interrupted by the pandemic lockdown of ~4 months and thus we were only able to infuse eHsp70 for 18 d. After our return to the lab, we tested the mice on a range of behavior assays to determine if transient infusions of eHsp70 had exerted long-term improvements in behavior (Fig. 22a).

No improvements on the open field test were seen (data not shown). However, on the Y-maze apparatus, we found that PFF-injected mice that had received native (versus denatured) eHsp70 spent a greater amount of time in the novel arm (Fig. 22j) and, thus, lesser amount of time in familiar arm (Fig. 22k). No significant differences were noted between PBS-injected mice and PFF-injected mice, suggesting that infusions with native eHsp70 were not modifying fibril-induced changes, but exerting baseline improvements in cognition. These data indicate that intranasal infusions of native eHsp70 may have improved baseline spatial reference memory (Kraeuter, Guest, & Sarnyai, 2019).

On the odor habituation/dishabituation test, PBS-injected mice showed both, odor habituation to the familiar odor and dishabituation to the novel odor (Fig. 22l). However, PFF-injected mice that received intranasal infusions of vehicle (PBS control) failed to display odor habituation but showed odor dishabituation—suggestive of impairments in smell with PFF-injections (Fig. 22m). Intranasal infusions of denatured eHsp70 or native eHsp70 did not modify these fibril-induced changes (Fig. 22n-o). Since the behavior data were largely negative, we did not assess brains from this cohort at the histological level.
**Discussion**

Prior work confirms that α-synuclein spreads from cell-to-cell (Luk, Kehm, Carroll, et al., 2012; Schaser et al., 2020; Volpicelli-Daley et al., 2011) and that Hsp70 can prevent the aggregation of α-synuclein (Aprile, Arosio, et al., 2017; Danzer et al., 2011; Dedmon et al., 2005; Kilpatrick et al., 2013a; Klucken et al., 2004; Luk et al., 2008; McLean et al., 2002; Wentink et al., 2020). Much of this work was performed in overexpression models (of Hsp70 and/or α-synuclein), in test tubes using recombinant α-synuclein and Hsp70, or in transformed cell lines. The effects of Hsp70 on PFF-seeded pSer129+ inclusions remain elusive. In Aim 1, we showed that *endogenous* Hsp70 lessens PFF-seeded pSer129+ inclusions in an *in vitro* model of α-synucleinopathy. However, in Aim 2, our major finding...
was that exogenous Hsp70 (eHsp70) mitigates PFF-seeded inclusion pathology in vitro and in vivo and mitigates PFF-induced behavior deficits in vivo. Our in vitro data were confirmed in three independent studies, using eHsp70 at a range of concentrations and in both, mixed-sex and sex-stratified primary hippocampal neuron cultures. We concluded that eHsp70 is an effective therapeutic agent that may be used to modify the underlying pathology in α-synucleinopathies. In many of our studies and experimental readouts, we noted that the salutary effects of eHsp70 were relatively mild. It is known that low levels of the co-chaperone, Hsp40, might be limiting in the fulfilment of Hsp70 functions (Michels et al., 1997). Thus, future studies must examine if the benefits of eHsp70 treatment in this PFF-seeded model can be expanded with the co-administration of additional, exogenous Hsp40.

In our current study, eHsp70 had a greater inhibitory effect on inclusion sizes in female cells and, in male cells, eHsp70 had a greater inhibitory effect on inclusion numbers. These contrasting data suggest that in α-synucleinopathies, one must examine multiple aspects of pathology at the structural level. If we would have solely relied on total inclusion numbers as a readout, we would have wrongly concluded that female cells failed to respond to eHsp70 with a reduction in α-synucleinopathy, despite both males and females showing an uptake of eHsp70 in culture. The mechanisms underlying these opposing data in eHsp70-treated male vs. female neurons are unclear, probably because it is unknown if an increase in inclusion sizes or an increase in inclusion numbers is more toxic. In the future, it may be critical to examine inclusion readouts at multiple time-points after α-synuclein fibrils are applied, as the bigger-sized inclusions would likely form at latter time-points, after the smaller neuritic inclusions have emerged. In our hands, the smaller inclusions did
not colocalize with eHsp70, suggesting that eHsp70 may have either reduced the size of the inclusions (without degrading the inclusions *per se*). As eHsp70 colocalized with the bigger, perinuclear inclusions, it is possible that eHsp70 may be unable to dissolve those bigger inclusions by the time of assay and it may need additional time for the bigger inclusions to be mitigated. Thus, our data may reflect sex differences in the initial seeding of the inclusions *versus* in the maintenance and growth of the inclusions, however these theories would need to be tested in future work at multiple time-points and after separating inclusion readouts based on whether inclusions are perinuclear, neuritic, nuclear, or somal.

In our studies, we compared the effect of native eHsp70 to denatured (boiled) eHsp70, as any residual endotoxins or other materials used in the synthesis of eHsp70 would not be removed by boiling. Prior work suggested that denatured eHsp70 did not differ from other negative control groups (*i.e.*, saline or BSA-treated groups) in their effects on lifespan extension and memory performance in AD and ageing models (Bobkova et al., 2015; Bobkova et al., 2014). In our hands, denatured eHsp70 worsened the α-synuclein pathology, but treatments with native eHsp70 mitigated this effect. We speculate that denatured eHsp70 may be more likely to aggregate and induce toxicity due to a loss in the normal structure and function of the protein. Indeed, the denatured eHsp70 was mildly toxic to neuron cultures in our hands, and, so, as another control, we also included a PBS-treated group to avoid the toxicity induced by denatured eHsp70. We also always use PBS as a control for PFF exposures because unaffected control subjects do not have high levels of monomeric α-synuclein and injecting monomers may produce small amounts of pSer129*+* inclusions, as noted by others (Paumier et al., 2015).
We noted that eHsp70 was localized at a nuclear or perinuclear location in neuron cultures. Although surprising, this finding is consistent with prior work showing that after its intranasal delivery, eHsp70 is confined to a perinuclear zone (Bobkova et al., 2014). Endogenous Hsp40 (and Hsp70) are present at low levels within the nuclear compartment under physiological conditions but can translocate to the nucleus or nucleolus upon heat shock (Hattori, Kaneda, Lokeshwar, Laszlo, & Ohtsuka, 1993; Michels et al., 1997; Welch & Feramisco, 1984). Prior work showed that Hsp70 is colocalized with condensed chromatin in the nucleus (Welch & Feramisco, 1984). Although we did not specifically assess if eHsp70 was bound to condensed chromatin, we did find that in some instances, eHsp70 was colocalized with shrunken nuclei that were brightly stained with Hoechst—potentially apoptotic cells, which may harbor condensed chromatin.

Endogenous Hsp70 is known to be colocalized with markers of Lewy inclusions (Auluck et al., 2002; Leverenz et al., 2007; McLean et al., 2002; Outeiro et al., 2006). To extend this work, we also found that endogenous Hsp70 colocalizes with perinuclear pSer129$^+$ inclusions (Fig. 23a). This intranuclear confinement of Hsp70 is not limited to $\alpha$-synucleinopathies, as it may also be localized within nuclear Ataxin$^+$ aggregates in spinocerebellar ataxia type 1 (SCA1). Despite these collective, but largely qualitative observations, the function of Hsp70 (or eHsp70) at the nucleus is unclear. Some studies suggest that Hsp70 can function as a chaperone protein even within the nucleus (Michels et al., 1997). Although we did not specifically count the numbers of nuclear inclusions, we found that exposures to native eHsp70 protein reduced the numbers of perinuclear inclusions—an effect that was at least partially modulated by the Hsp40-dependent chaperone function of Hsp70. This reduction in numbers of perinuclear inclusions is
consistent with the subcellular localization of eHsp70, suggesting that, at least near the nucleus, eHsp70 is likely to function as a chaperone. It is possible that we may have seen a more robust involvement of the Hsp40-dependent chaperone function of eHsp70 if we had administered Hsp40 in conjunction with eHsp70, as also mentioned above.

Apart from chaperone and immunologic roles, some studies show that Hsp70 may stimulate DNA repair (Bellmann, Jaattela, Wissing, Burkart, & Kolb, 1996; Fujimoto et al., 2017; Gvozdenov, Kolhe, & Freeman, 2019; Knighton & Truman, 2019; Niu et al., 2006; Sottile & Nadin, 2018). Hsp70 is known to interact with the base excision repair (BER) enzyme, apurinic/apyrimidinic endonuclease 1 (APE1), thereby increasing the endonuclease activity of APE1 at abasic sites and promoting DNA repair under oxidative stress (Kenny et al., 2001; Mendez et al., 2000). The N-terminal region of Hsp70 may be critical to stimulate APE1 activities (Mendez et al., 2003). Notably, the N-terminal region also harbors ATPase activities of Hsp70, which are vital for fulfilling its chaperone functions (Lackie et al., 2017). We collected pilot data showing that OB/AON extracts from male mice that received intranasal infusions of eHsp70 display a greater excision of synthetic 8-oxoguanine-cytosine (8oxoG-C) lesions (Fig. 23b), suggestive of better DNA repair activity in eHsp70-infused mice. Extracts from eHsp70-infused mice also tended to show a greater ability to repair glycols (two-tailed $p=0.0580$; Fig. 23c), perhaps by stimulating the activities of BER enzymes, including APE1. We examined the levels of APE1 protein in the aged mice cohort (i.e., in PFF-injected mice that received intranasal PBS or eHsp70) shown in Fig. 21 and found that there was a reduction in APE1 levels in eHsp70-infused mice (Fig. 23d-f). In this study, eHsp70 infusions were initiated immediately after intracerebral injections with PFFs. It is known that α-synuclein fibrils
induce DNA damage (Milanese et al., 2018; Schaser et al., 2019). If eHsp70 directly degrades the PFFs, the DNA damage response will not be initiated, and this may explain the reduction in APE1 protein with intranasal eHsp70. This would perhaps also be consistent with the eHsp70-mediated reduction in Iba1, noted in Fig. 21. As PFFs are known to induce microglia/macrophage reactivity (Aim 3) and an increase in Iba1, we surmise that the reduction in this measure with intranasal eHsp70 may be due to fewer PFFs available to induce microglia/macrophage activation. Further studies are warranted to fully determine the role of Hsp70 in stimulating DNA repair activities in this model. For example, APE1 levels and activities must be assessed in studies wherein eHsp70 infusions are initiated after PFFs elicit DNA strand breaks and after PFFs form dense inclusions, to contrast the data shown in Fig. 23. Nonetheless, our pilot data shown here suggest that eHsp70 may not only directly target misfolded α-synuclein but may also guide DNA repair. These dual functions may be critical in blunting the pathology in Lewy body disorders as these diseases exert both proteotoxic and genotoxic stresses.
Although we had intended to examine the salutary effects of intranasal eHsp70 in PFF-injected mice of both sexes, we found no evidence of nose-to-brain entry of eHsp70 in aged female mice at the same doses and timepoints as in males. This lies in contrast to our *in vitro* data, wherein eHsp70 was taken up by both male and female neurons in culture. Although poorly studied, sex differences in brain entry of exogenous molecules after intranasal delivery have been reported. In 1996, Barna *et al.* showed that intranasal infusions of vesicular stomatitis virus (VSV) led to lower brain penetration in the female,
compared to the male mouse brain (Barna, Komatsu, Bi, & Reiss, 1996). There may be several factors underlying sex differences in the nose-to-brain entry of molecules, such as eHsp70. Females may degrade eHsp70 faster than males, either within the nasal mucosa or after it gains access to the brain parenchyma, perhaps due to higher numbers of glial cells or greater glial reactivity in females (Barna et al., 1996; Oliveira-Pinto et al., 2014), which may clear the infused eHsp70. Alternatively, after intranasal inoculations there may be a greater induction of humoral and cell-mediated immunity, an increase in brain entry of adaptive immune cells, or an increase in the expression of molecules responsible for antigen presentation and processing in females versus males (Barna et al., 1996; Fink, Engle, Ursin, Tang, & Klein, 2018). Recently, sex differences in the nose-to-brain entry of exogenous molecules were reiterated by Nahvi et al. in a preclinical model of post-traumatic stress disorder (PTSD) (Nahvi, Nwokafor, Serova, & Sabban, 2019; Nahvi & Sabban, 2020; Nahvi et al., 2021). In their work, neuropeptide Y (NPY) was provided intranasally as a potential therapy and the authors found that at the same dosages, NPY had a greater effect in males than females at the behavioral and molecular levels (Nahvi et al., 2019; Nahvi & Sabban, 2020). Intriguingly, in a follow-up study the authors found that female rodents required a higher intranasal dosage of NPY to be therapeutically effective, due perhaps to greater cleavage of NPY in the female brain (Nahvi et al., 2021). Thus, there may also be sex differences in the expression and function of proteolytic enzymes responsible for eHsp70 breakdown or in the expression and function of receptors that bind eHsp70. In addition, females may show lower brain entry of eHsp70 due to sex differences in the permeability and integrity of the nose-to-brain barrier. Sex hormones are known to impact BBB permeability and the expression of tight junction proteins (Atallah, Mhaouty-
Kodja, & Grange-Messent, 2017; Bake & Sohrabji, 2004; Burek, Arias-Loza, Roewer, & Forster, 2010; Maggioli et al., 2016; Meng et al., 2011; Mokhtar, Giribabu, & Salleh, 2020; Moon, Lim, Kim, & Moon, 2021; Torres & Bynoe, 2017; Wilson et al., 2008; Zhou, Gilkeson, & Jiang, 2017; Z. Zhou et al., 2017). In a large study (n>20,000) involving subjects aged 1 to 90 years, it was shown that the BBB and blood-cerebrospinal fluid barriers display tighter integrity in females (Parrado-Fernandez et al., 2018). Compared to the BBB, properties of the nose-to-brain barrier are less clear, but if it is also tighter in females, the brain entry of eHsp70 at the same dose as males may be prohibited. Females may thus benefit from linking eHsp70 to some cell permeating peptides, such as trans-activator of transcription (Tat) protein (Nagel et al., 2008; Tunesi et al., 2019) and this must be investigated in future work.

Despite an absence of brain entry of eHsp70 in females, we found that intranasal eHsp70 enters the brains of young and aged male mice and is functional after intranasal delivery, as it reduced PFF-seeded pathology and behavior deficits in males. Although we did not include mice that received intracerebral PBS injections, prior work in this model has confirmed that PFF-injected male mice display olfactory deficits, based on the buried pellet behavior test (Mason et al., 2019). In our work, we found that intranasal infusions of eHsp70 for 28 d significantly reduced the time it takes for PFF-injected mice to contact a buried peanut. Interestingly, a statistical trend (two-tailed $p=0.088$) toward a reduction in the time it takes to contact an exposed peanut after intranasal infusions of eHsp70 was also noted, indicative of slight differences in motivation or hunger statuses. It has been recently suggested that the buried pellet test is not as sensitive as the plethysmography of odor-evoked sniffing to assess deficits in odor detection (Johnson et al., 2020). However, the
buried/exposed pellet tests, as we have performed here, make it possible to determine if the changes are mediated by differences in motivation versus olfaction alone. One must also note that older CD-1 mice show visual deficits and retinal degeneration and may thus rely more on smell even when food is exposed above (and not below) the bedding (Envigo, 2016). Overall, our findings (summarized in Fig. 24) through the studies in Aims 1 and 2 suggest that Hsp70, in its endogenous as well as exogenous form, reduces PFF-seeded α-synucleinopathy in in vitro and in vivo models.

Note that many of the strengths and limitations mentioned in the other Aims, particularly with respect to the model and techniques employed, are also applicable to the studies in Aim 2, but are not repeated here in order to be concise.

Figure 24. Summary schematic for Aims 1 and 2: Impact of endogenous and exogenous Hsp70 on PFF-seeded α-synucleinopathy. Exposure to PFFs elicits greater detergent-insoluble pathology in male versus female neurons, despite equivalent uptake of PFFs by both sexes. Blocking the Hsp40-dependent chaperone function of Hsp70 with MAL3-101 increases pathology more robustly in males. Female cells failed to respond to MAL3-101 with increase in pathology, perhaps due to a compensatory increase in functionally related chaperones, i.e., Hsc70 and Hsp40. Thus, males may be more reliant than females on Hsp70 to mitigate α-synucleinopathy and males may also be less able to compensate for loss of Hsp70 function. In contrast, treatment with exogenous Hsp70 (eHsp70) mitigates pathology in both males and females in a manner that is partly dependent on the chaperone function of Hsp70 in male cells. Intranasal delivery may be a viable route of delivering eHsp70 to the brains of aged male, but not female mice and intranasal eHsp70 reduces pathology and deficits in PFF-injected aged males. Reprinted by permission from [the Springer Nature Customer Service Centre GmbH]: [Springer] [Neurotherapeutics: The journal of the American Society for Experimental NeuroTherapeutics] [Heat Shock Protein 70 as a Sex-Skewed Regulator of α-Synucleinopathy, Bhatia, T. N., Clark, R. N., Needham, P. G., Miner, K. M., Jamenis, A. S., Eckhoff, E. A., Abraham, N., Hu, X., Wipf, P., Luk, K. C., Brodsky, J. L., & Leak, R. K] [Copyright 2021 by Springer] License number: 5218750410757; License date: December 30, 2021
Chapter 5

Specific Aim 3: To investigate if microglia mitigate α-synucleinopathy and behavior deficits in the PFF model and if this effect is modified by biological sex

Rationale

Reactive microglia have long been known to be present in the nigra of PD and in the hippocampus of PDD patients (McGeer et al., 1988). Recent work also reported male-specific microgliosis in the OB of PD patients (Flores-Cuadrado et al., 2021). In contrast to these male-specific changes in the OB, studies have reported a positive link between antibody levels in the sera and disease duration or severity of motor deficits, but only in female and not male PD patients (X. Chen et al., 2021). A negative link between CD4 helper T lymphocytes and severity of motor symptoms or quality of life was also noted in female, but not male PD subjects (X. Chen et al., 2021). Other studies have shown that blood monocytes isolated from drug-naïve PD patients display a stronger enrichment of IFNγ-responsive genes in females with disease, whereas the male response was lesser and more variable (Carlisle et al., 2021). These studies are all suggestive of inflammatory changes in Lewy body disorders, which may be sex-skewed.

Indeed, inflammatory cytokines, including TNFα, IL5, IL2, IL15, IL6, IL1β, IFNγ, etc., are all elevated in PD patients (Mogi, Harada, Kondo, et al., 1994; Mogi et al., 1996; Mogi, Harada, Riederer, et al., 1994; Nagatsu, Mogi, Ichinose, & Togari, 2000a, 2000b; Walker et al., 2015) and there is an infiltration of adaptive immune cells in the brains of PD subjects (Brochard et al., 2009). Some groups suggest that PD may be an autoimmune disease, based on the findings that T lymphocytes in PD patients recognize peptides derived
from α-synuclein as antigenic epitopes (Lindestam Arlehamn et al., 2020; Sulzer et al., 2017). Monocytes are also activated and, along with chemokines, are enriched in the blood of PD subjects (Funk et al., 2013; Grozdanov et al., 2014). These reports all hint toward an immune component in PD and studies have shown that anti-inflammatory drugs or immunosuppressants may mitigate PD risk (Racette et al., 2018; San Luciano et al., 2020). Despite the vast number of studies reporting inflammation or microglial reactivity in PD and other Lewy body disorders, it is unclear if microglial reactivity is a cause or consequence of the disease.

In DLB, microglia are not necessarily reactive but may show dystrophic changes (Bachstetter et al., 2015), reflective of microglial senescence (Streit, Sammons, Kuhns, & Sparks, 2004; Streit & Xue, 2016). Studies that did report microgliosis in DLB also showed a positive link between the numbers of LBs and numbers of reactive microglia (Mackenzie, 2000). In separate studies, a near absence of microglial reactivity (Shepherd, Thiel, McCann, Harding, & Halliday, 2000) or the presence of reactive microglia only in early DLB stages (Surendranathan et al., 2018) were reported. An absence of reactive microglia in latter stages may be due to loss of neurons, microglial exhaustion, and/or an involvement of the adaptive (versus the innate) immune system later in the disease course (Surendranathan et al., 2018). In sum, the studies that investigated microglial reactivity in Lewy body disorders are conflicting and largely descriptive, as they do not prove a causal link between reactive microglia and LB pathology or neurodegeneration.

Consistent with work in postmortem patient brains, it is also known that PFFs induce microglial reactivity in preclinical models (Duffy, Collier, Patterson, Kemp, Luk, et al., 2018; Harms et al., 2017; Thakur et al., 2017; Verma et al., 2021)—a phenomenon
that may precede neuron loss, leading to the hypothesis that stress-reactive microglia may drive neurodegeneration in disease (Duffy, Collier, Patterson, Kemp, Luk, et al., 2018). This theory follows early work in autopsied brain tissue of diseased patients, in which complement activation products processed by microglia were found within LBs and there was also an extension of microglial processes toward degenerating neurons that were laden with LBs (Akiyama & McGeer, 1990; Iseki, Marui, Akiyama, Ueda, & Kosaka, 2000; Togo et al., 2001; Yamada, McGeer, & McGeer, 1992). Some reports show that microglial reactivity also precedes the development of α-synuclein pathology in neurons grafted in the striata of PD patients (Olanow, Savolainen, Chu, Halliday, & Kordower, 2019). Based on these reports, one may speculate that microglia may contribute to the progression of pathology and neuron loss in Lewy body disorders.

To investigate some of these hypotheses, several methods to ablate microglia in vivo have been developed (Eme-Scolan & Dando, 2020; Han, Harris, & Zhang, 2017). Using one such tool—such as the dietary administration of the colony stimulating factor 1 receptor (CSF1R) inhibitor, PLX3397—groups have shown a partial loss in pSer129 following PFF infusions in the striata of microglia depleted mice (Guo et al., 2020). Although this may indicate that microglia drive the formation of pSer129+ inclusions, one must note that PLX3397 is non-selective, shows low brain penetrability, and has off-target effects on non-microglial cells, particularly if administered for long periods (Guo et al. administered PLX3397 for 60 d; (M. R. Elmore et al., 2014; Guo et al., 2020; Y. Liu et al., 2019; Spangenberg et al., 2019)). Furthermore, the sex of the mice was not mentioned in the study by Guo et al. (Guo et al., 2020).
Compared to PLX3397, PLX5622 is more selective and shows ~50-to-100-fold greater selectivity toward CSF1R than other kinases (Dagher et al., 2015; T. S. Kim et al., 2014). In a study that only examined females, delivery of PLX5622 (Y. Liu et al., 2019; Spangenberg et al., 2019), led to an increase in the cell-to-cell transfer of α-synuclein, suggesting that as the resident immune cells of the brain, microglia may normally hinder the spread of α-synuclein (George et al., 2019). An absence of other innate immune cells, such as natural killer (NK) cells, also exacerbates PFF-seeded α-synuclein pathology and motor deficits (Earls et al., 2020). Adaptive immune cells may also impede pSer129 pathology, as PFF infusions in mice that lack T cells, B cells, and NK cells led to an ~8-fold increase in α-synuclein pathology, which was partially rescued by T cell, but not B cell reconstitution (George et al., 2021). Thus, there is ample evidence that innate and adaptive immune responses regulate PD pathology, but there is less consensus on whether the immune response is a cause or consequence of the disease. Despite the characterization of many microglia depleting agents, it is challenging to ascertain if it is the specific microglial response that underlies many of the disease outcomes \textit{in vivo}, wherein multiple cell types or molecules may be at play. There may also be subsets of microglia that may be resistant to CSF1R inhibition, as was recently reported (Zhan et al., 2020), suggesting that the near complete depletion of microglia may not be possible to achieve \textit{in vivo}. To mitigate these caveats, we relied on microglia/neuron co-cultures to test if the mere presence versus absence of microglia affects neuronal α-synucleinopathy.

Sex differences in the neuroimmune system, including in microglia structure and function, have been extensively reviewed (Han, Fan, Zhou, Blomgren, & Harris, 2021; Hanamsagar & Bilbo, 2016; Kodama & Gan, 2019; Lenz & McCarthy, 2015; Osborne,
Sex differences in numbers of microglia may be driven by a prenatal surge in testosterone in male (but not female) rodents, which emerges at ~E18 and can continue until early postnatal stages (Han et al., 2021; Hanamsagar et al., 2017; L. H. Nelson, Saulsbery, & Lenz, 2019). In the preoptic area (POA) of the hypothalamus—a sexually dimorphic area responsible for masculinization and male sex behaviors—this testosterone gets locally aromatized to estradiol, which leads to the synthesis and release of prostaglandins (Lenz et al., 2013; McCarthy, Wright, & Schwarz, 2009). Estradiol-mediated induction in prostaglandins is known to be responsible for male sex behaviors and the masculinization of brain cells (Amateau & McCarthy, 2004; Wright & McCarthy, 2009; M. V. Wu et al., 2009). Due to their short half-lives (Hwa & Martin, 2017), however, it was unknown how prostaglandins remain sustained during the period of brain sexual differentiation.

Microglia are present at greater numbers and at greater levels of reactivity in the male (versus female) brain early during development, and are known to synthesize and release prostaglandins, suggesting that microglia play active roles in brain sexual differentiation (Lenz & McCarthy, 2015; Lenz et al., 2013; Minghetti & Levi, 1998; Minghetti et al., 1997; Welberg, 2013). Prostaglandins also induce microglial reactivity, suggesting that there may be a feed-forward loop whereby prostaglandins are constantly synthesized and released by the dense and reactive microglia of the male brain during the period of sexual differentiation (Lenz et al., 2013; Welberg, 2013). Inhibiting microglial activation, without inducing microglial depletion, during this critical period abolishes 1) the estradiol-induced increase in prostaglandins, 2) sex differences in the numbers of microglia, and 3) the estradiol-induced stimulation of male sexual behaviors (Lenz et al.,
Apart from the male-specific surge in testosterone, high levels of chemokine ligands CCL20 and CCL4 in the male brain during early developmental stages may also drive microglial proliferation in the males (Villa, Vegeto, Poletti, & Maggi, 2016). These reports suggest that sex differences in the brain and in microglia are predetermined and may be mediated by exposure to sex steroids, from prenatal to early postnatal periods (Lenz et al., 2013). Accordingly, microglial sex differences were maintained even after they were harvested and cultured in vitro in the absence of any hormonal cues (Villa et al., 2018). Microglia are also extremely long-lived cells (described further below) and thus it is conceivable that sex dimorphisms in microglia induced by perinatal exposure to sex steroids may be long-lasting and may contribute to the sex-skewed emergence of neurological diseases (such as Lewy body disorders) later in life.

Apart from displaying higher numbers of microglia in some regions early during development, studies show that in males, microglia may have better antigen presentation and processing capacities and may be more responsive to exogenous stimuli, compared to microglia in age-matched females (Guneykaya et al., 2018). Furthermore, recent work revealed that there may be sex differences in the developmental stage at which microglia are impacted under stress (Thion et al., 2018). For example, at early development, male microglia likely mount a more profound stress response, but as the organism matures, this stress response skews toward females, indicating that microglial functions in males may be engaged or needed earlier than females (Thion et al., 2018). Indeed, microglia in males are critical for sexual differentiation in the brain and for the induction of masculinization during the prenatal to early postnatal periods (Lenz et al., 2013). Male (versus female) mice may also have greater numbers of phagocytic microglia in some areas from postnatal day
0 (P0; day of birth) to P4, suggestive of **functional** differences in male vs. female microglia early during development (VanRyzin et al., 2019). In our work, we intended to extrapolate these findings to the PFF model to test if microglia harvested from male rodents at P4 are better able to mitigate inclusions in co-cultured neurons, compared to microglia harvested from age-matched females. The P4 time point was chosen based on work showing that males at P4 harbor greater numbers of total microglia, phagocytic microglia, and activated microglia, compared to females (Schwarz et al., 2012; VanRyzin et al., 2019). Furthermore, this timepoint immediately follows the peak of testosterone surge in male pups (Nissen, 2017; Schwarz et al., 2012).

As the organism matures into adulthood, the induction of an immune response becomes more prominent in females compared to males (S. L. Klein & Flanagan, 2016). Indeed, the male-skewedness in microglial numbers and reactivity noted at P4 switches by P30-P60, timepoints by which females show greater numbers of total, phagocytic, and activated microglia, compared to microglia from age-matched male mice (Schwarz et al., 2012), and this sex dimorphism can continue until the geriatric age (P. R. Mouton et al., 2002). Microglia isolated from ~3-month-old female mice are more protective against ischemic stroke, compared to microglia from males, and these female microglia are able to maintain their sexual phenotype, gene expression, and protective effects even when transplanted into the brains of male mice (Villa et al., 2018). These latter data further suggest that microglia are sexually differentiated prior to adulthood and that this sex dimorphism is either independent of circulating sex steroids and/or may be primed by early exposures to sex steroids. Consistent with the protective effects of female microglia at adulthood, 10-to-14-week-old females (males were not tested) that had their microglia
depleted responded with a rise in spread of α-synuclein (George et al., 2019). In contrast, in a separate study, ~2-month-old males (females were not tested) that had their microglia depleted, responded to rotenone with a decline in neuronal injury, α-synuclein pathology, and cognitive deficits (D. Zhang et al., 2021). Thus, contrary to the benefits afforded by male microglia at early stages, it is the female microglia that are protective at adult stages. However, the role of microglia in disease may also depend on the nature of the toxicant to which these glia are exposed. One must also note that many of these studies did not systematically examine sex as a biological variable, and so it must not be ruled out that male microglia at adulthood (or neonatal female microglia) also have protective effects in α-synucleinopathies. In our studies, males and females were always examined in parallel and statistical interactions with biological sex were always tested.

With ageing, the female mouse brain shows a greater LPS-induced increase in expression of proinflammatory cytokines, compared to aged males and younger females (Murtaj et al., 2019). Microglia in aged females are also much more reactive, and aged female microglia may be unable to fulfill their normal phagocytic roles in the presence of an inflammatory stressor (Murtaj et al., 2019; Yanguas-Casas, Crespo-Castrillo, Arevalo, & Garcia-Segura, 2020). Stress reactivity in aged microglia may be reflective of cellular senescence as microglia may be primed with age, i.e., they may be very easily triggered as they may have a substantially low threshold to reach reactivity (Greenwood & Brown, 2021). For example, the aged female mouse brain shows a greater induction of MHC-I-related genes and complement pathway components, compared to aged males, suggestive of greater levels of inflammation in the brains of aged female mice (Mangold, Masser, et al., 2017; Mangold, Wronowski, et al., 2017). Ageing also leads to higher levels of IL1α,
IL1β, IL6, and TNFα in the female versus male mouse brain (Mangold, Masser, et al., 2017), and this age-related increase in inflammatory genes in females is consistent with prior work in humans (Berchtold et al., 2008). Apart from inflammatory genes, it is also possible that genes involved in disease susceptibility may be differentially present in aged male versus female microglia. For example, microglia from older female (versus male) mice show higher expression of genes, such as *Apoe*, which may contribute to increased vulnerability of females to AD (Chowen & Garcia-Segura, 2021; Kang et al., 2018). The impact of biological sex on the expression of disease-related genes in microglia isolated from subjects with Lewy body disorders is less clear.

Microglia are also long-lived, surviving for about two decades in humans and, in mice, about 50% of the original microglial pool survives for ~26-28 months (Fuger et al., 2017; Reu et al., 2017). Based on microscopic evaluations of microglial cells, Samorajski in 1976, suggested that “Among the neuroglial cells, the microglia undergo the most significant changes with age” (Samorajski, 1976). With age, microglia-specific genes are upregulated across multiple regions and the expression of microglia-specific genes may be the best predictors of biological age (Soreq et al., 2017). Thus, microglia may be more prone to engage age-related cell senescence programs, contributing to the progression of ageing-related neurodegenerative diseases, including Lewy body disorders. As mentioned in the Introduction, Stevenson et al. have reported that almost as many microglia as neurons in the AON of PD subjects harbor pSer129+ inclusions—a proportion that is higher than other non-neuronal cells in the AON (Stevenson et al., 2020). High levels of α-synuclein in microglia may overburden these cells, driving phagocytic exhaustion and an inability to clear the α-synuclein, which may ultimately result in microglia becoming “carriers” and
not “cleaners” of α-synuclein (Bido et al., 2021; Scheiblich et al., 2021). This may also lead to additional toxicities, such as the release of oxidative species from microglia overburdened with α-synuclein, which may lead to loss of even those nearby neurons that do not show extensive pSer129 pathology (Bido et al., 2021).

In addition, with ageing, microglia show lower phagocytosis, lesser protrusion of processes, lesser motility, but greater lipofuscin, greater mitochondrial damage, and greater expression of inflammatory cytokines (Angelova & Brown, 2019; Spittau, 2017). Resetting the ageing (and/or diseased) microglial phenotype by the initiation and latter cessation of CSF1R inhibitors is beginning to receive attention, and has been tested as a therapy in models of ageing, traumatic brain injury (TBI), AD, and MPTP-induced parkinsonism (M. R. P. Elmore et al., 2018; N. R. Johnson et al., 2021; Q. Li et al., 2021; Willis et al., 2020). In these studies, microglial repopulation reduced injury, restored structural morphologies of microglia, and improved neurogenesis, synaptoplasticity, cognition, motor function, etc. (M. R. P. Elmore et al., 2018; N. R. Johnson et al., 2021; Q. Li et al., 2021; Willis et al., 2020). This strategy may be more likely to succeed in the clinic as the near-complete loss of microglia by the sustained use of CSF1R inhibitors may be toxic, may exert off-target effects, and has produced conflicting results, including in models of parkinsonism (George et al., 2019; Guo et al., 2020; Jing et al., 2021; S. J. Oh et al., 2020; X. Yang et al., 2018; D. Zhang et al., 2021). Despite being diseased, microglia do harbor homeostatic roles, as was shown in a recent study wherein diseased microglia were able to adapt to the proteotoxic stress of PFF exposure by forming connections with healthier microglia in the vicinity, to degrade PFFs (Scheiblich et al., 2021). Lewy body disorders are also slowly progressive, suggesting that complete loss of microglia for long periods may not be
therapeutically feasible. Instead, transient, or intermittent depletion of microglia may be more valuable to remove those cells that may have undergone disease- or age-related toxic changes and to replace these cells with a fresh pool of younger, healthier microglia with better phagocytic and degradative machineries.

To our knowledge, the impact of microglial repopulation has not been tested in the new PFF-seeded model of α-synucleinopathy, particularly as a function of biological sex and ageing, which we examined in our studies in this Aim. Our findings suggested that depleting microglia exerted hyperactive and anxiety-like behaviors in PFF-injected male mice, which were not rescued when microglia were repopulated. In contrast, microglial replacement improved cognitive performance in PFF-injected female mice. Thus, targeting microglia may be a promising therapeutic intervention in Lewy body disorders, but may produce sex-specific effects, consistent with prior work in AD (N. R. Johnson et al., 2021).
Results

Characterization of a primary microglia culture model

To determine the role of microglia in a model of PFF-induced α-synucleinopathy, we first characterized primary microglial cultures (Fig. 25). Microglia were harvested from the postnatal rat cortex (Fig. 25a-h) or hippocampus (Fig. 25i-o). First, we verified that these mixed-sex primary cortical and hippocampal microglia respond to classic proinflammatory or anti-inflammatory stimuli with an induction in the expression of established markers of microglial polarization phenotypes. Based on prior work (X. Hu et al., 2012), we used lipopolysaccharide (LPS) and interferon-γ (IFNγ) to induce more proinflammatory phenotypes, and interleukin-4 (IL4) to induce more anti-inflammatory phenotypes. In these studies, we also tested if exposure to PFFs can propel microglia to assume either proinflammatory or anti-inflammatory phenotypes.

Treatments with LPS/IFNγ resulted in mild loss of Hoechst+ cells (Fig. 25a, i) and Iba1+ cells (Fig. 25b, j) in both cortical and hippocampal cultures. Although it is well known that Iba1 marks both microglia and infiltrating macrophages in vivo, early work showed that Iba1 is microglia-specific in culture and does not mark neurons or other glia (Gonzalez Ibanez et al., 2019; Imai, Ibata, Ito, Ohsawa, & Kohsaka, 1996; Ito et al., 1998). Primary microglia cultured by this method were ~86-88% Iba1+, a fraction that did not change with treatment in cortical or hippocampal cultures (Fig. 25c-d, k-l). Iba1 is a marker of reactive microglia, and it likely underestimates rather than overestimates the purity of primary microglia cultures. As expected, IL4 elicited an increase in expression of anti-inflammatory CD206, whereas treatment with LPS/IFNγ elicited an increase in proinflammatory iNOS and CD16 (Fig. 25e-h, m-o). Acute treatment of cortical or
hippocampal microglia with PFFs did not alter either the microglial cell numbers or expression of pro- vs. anti-inflammatory markers.

Figure 25. Characterization of primary cortical and hippocampal microglia. Primary cortical (a-h) and hippocampal (i-o) microglia were treated with 1 µg/mL PFFs, 100 ng/mL lipopolysaccharide (LPS) and 20 ng/mL interferon-γ (IFNγ), or 20 ng/mL interleukin-4 (IL4) and assayed 24 h later. Blinded counts of Hoechst+ cells (a, i) and Iba1+ microglia (b, j) are shown. Numbers of Iba1+ cells expressed as a fraction of Hoechst+ cells (c, k). Representative photomontage of cells stained for Iba1 (pseudocolored green) and Hoechst (pseudocolored blue) is in d, i. Levels of anti-inflammatory CD206 (e, m), proinflammatory iNOS (f, n), and proinflammatory CD16 (g-h, o), expressed as a fraction of DRAQ5 via In-Cell Western analyses. A Rout outlier in g was excluded in the data shown in h. Shown are the mean ± SD of 3–6 independent experiments, each performed in duplicate or triplicate wells. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, one-way ANOVA followed by Bonferroni post hoc.
Primary hippocampal microglia modestly reduce pSer129+ inclusions in co-cultured neurons exposed to preformed α-synuclein fibrils

Next, we tested if the presence of untreated, pro-inflammatory, anti-inflammatory, or PFF-exposed microglia modulates viability and α-synuclein+ inclusions in neighboring neurons. We harvested microglia from the hippocampus and not the cortex, as we and others have characterized hippocampal neuron cultures for the PFF-seeded model of α-synucleinopathy (Aim 1, and (Polinski et al., 2018b)), and we wanted to avoid the confounding effects of region-dependent variations in microglia-neuron interactions. Hippocampal neurons were plated alone or on top of hippocampal microglia that were either untreated (vehicle) or pretreated with LPS/IFNγ, PFFs, or IL4, and then cells were exposed to a “second hit” of PFFs to encourage neuronal inclusion formation (Fig. 26a). The presence of microglia, regardless of pretreatment, significantly reduced the numbers of inclusions that formed in co-cultured neurons, but only when data were expressed as a fraction of Hoechst+ cells and as fold change values of the PFF-treated neuron culture group (Fig. 26b-f). Microglia did not significantly alter Hoechst+ pan-nuclear viability (Fig. 26g). No statistically significant changes in numbers of NeuN+ neurons were noted (Fig. 26h), even when data were normalized (not shown) to mitigate the impact of culture-to-culture variation, which reflects technical rather than biological variance, such as in plating densities and staining intensities. No significant changes in the average sizes of the inclusions or the total area occupied by the inclusions were noted, irrespective of whether the raw (Fig. 26i-k) or normalized data (not shown) were analyzed. In contrast, pretreatment of microglia with anti-inflammatory IL4 reduced perinuclear inclusions in cocultured neurons, when expressed as a fraction of Hoechst+ cells (Fig. 26l). A statistical
trend (two-tailed; \( p = 0.052 \)) toward a reduction in this measure was noted when data were expressed as a fraction of NeuN\(^+\) neurons (Fig. 26m). Together, these findings reveal that acute prestimulation of microglia with PFFs does not condition them to reduce the formation of inclusions in neighboring neurons, but their pretreatment with IL4 might inhibit the formation of the mature, perinuclear \( \alpha \)-synuclein aggregates.
Primary astrocytes mitigate pSer129+ inclusions in co-cultured primary neurons exposed to preformed α-synuclein fibrils.

As per Fig. 27c, microglia mitigated neuronal pSer129+ inclusions to a modest degree, regardless of pre-treatment stimulus. This direct neuroprotective effect was not microglia-specific, as we also found that primary astrocytes reduce pSer129+ inclusion numbers in co-cultured neurons exposed to preformed α-synuclein fibrils, irrespective of whether Hoechst+ cell counts (Fig. 27a-b) or the fluorescence signal of neuron-specific β-III tubulin (Fig. 27c-d) were used to account for plating cell density differences across independent cultures and across wells (i.e., interexperimental and intraexperimental variance in cell attachment). Notably, monolayer cultures of astroglia did not form pSer129+ inclusions in response to PFFs, consistent with negligible expression of α-synuclein by astrocytes (Kam et al., 2020; H. J. Lee et al., 2010; Y. Zhang et al., 2014; Y. Zhang et al., 2016). Together with the microglia/neuron co-culture data in Fig. 26, the
studies in Fig. 27 show that macroglia may also fulfill their neuroprotective roles in α-synucleinopathic disease conditions, consistent with prior work (Loria et al., 2017).

**Figure 27.** Primary astrocytes reduce the numbers of pSer129+ inclusions in co-cultured primary neurons. Primary hippocampal neurons were cultured alone or on top of a monolayer of astrocytes. A separate group of primary astrocytes was also maintained as a control. Neuronal monolayer cultures, neuron/astrocyte bilayer cultures, and astrocytic monolayer cultures were all exposed to vehicle or PFFs. Blinded measurements of numbers of pSer129+ inclusions, expressed as a fraction of Hoechst+ cell counts (a) or of the intensity signal of neuron-specific β-III tubulin (c). Representative photomontages in b and d are of pSer129 (pseudocolored red), β-III tubulin (pseudocolored green), and Hoechst (pseudocolored blue). Shown are mean ± SD of 3 independent experiments, each performed in triplicate wells. ** p ≤ 0.01, *** p ≤ 0.001 for veh versus α-synuclein fibrils; + p ≤ 0.05, ++ p ≤ 0.01, +++ p ≤ 0.001 for indicated comparisons, two-way ANOVA followed by Bonferroni post hoc.

α-synuclein fibrils stimulate the internalization of extracellular microspheres by primary hippocampal microglia.
Exposure of primary hippocampal microglia to α-synuclein fibrils leads to internalization of the fibrils by microglia (Fig. 28a-b). Therefore, we characterized the functional status of primary hippocampal microglia by co-administering α-synuclein fibrils and fluorescent microspheres (beads) to determine if α-synuclein fibril exposure impacts microglial phagocytic/engulfment capacities. Preformed fibril exposure increased the numbers of internalized microspheres (Fig. 28c-e), suggesting that fibril exposure stimulates microglial phagocytic functions. In the presence of the microglial phagocytosis inhibitor, cytochalasin D, which works by stabilizing the actin cytoskeleton (S. M. Kim et al., 2017; Neher et al., 2011; Ribes et al., 2010; Scheiblich et al., 2021; Shi et al., 2013; W. Zhang et al., 2005), the baseline levels of internalization of microspheres were blunted in a concentration-dependent manner (Fig. 28d-e), when raw or fold-change data were analyzed. No changes in the numbers of extracellular microspheres (Fig. 28f-g) or total numbers of microglia were noted (not shown). Initially, we had begun the studies in Aim 3 to determine if the salutary effects of eHsp70, noted in Aim 2, were mediated by the immunomodulatory functions of eHsp70. In this regard, we intended to test if eHsp70 stimulated microglial phagocytic functions, based on prior work showing that Hsp70 stimulates the uptake of amyloid-β by microglia (Kakimura et al., 2002). Treatments with the “Mock” vehicle inhibited the PFF-induced uptake of microspheres by microglia (Fig. 28h-i), but 20 µg/mL eHsp70 mitigated this effect (Fig. 28h-i). On quantifying numbers of extracellular, non-phagocytosed microspheres, we found that cells co-stimulated with the Mock and PFFs reduced this measure, suggesting that PFFs were still pro-phagocytic (Fig. 28j-k). Treatments with 20 µg/mL eHsp70 reduced baseline levels of extracellular microspheres, suggestive of a pro-phagocytic effect of eHsp70 (Fig. 28j-k).
Finally, we tested if pretreatment of sex-stratified microglia with eHsp70 reduces PFF-seeded pathology in co-cultured sex-stratified neurons, but no differences were noted across groups (Fig. 28l-m), perhaps because the pro-phagocytic effects of eHsp70 were mild. Instead, we noted that there may be baseline sex differences in the neuroprotective potential of microglia, which we examined further in Fig. 29 (see below).
Primary hippocampal microglia from male, but not female rat pups, reduce pSer129+ inclusions in co-cultured neurons seeded with preformed α-synuclein fibrils.

Male, but not female PD patients display microglial activation in the OB, relative to control subjects (Flores-Cuadrado et al., 2021). In addition, prior work has shown that male rodents harbor greater numbers of total, activated, and phagocytic microglia early during development—including at postnatal day 4 (P4)—a timepoint that follows the testosterone surge in male rat pups (Nissen, 2017; Schwarz et al., 2012; VanRyzin et al., 2019). To our knowledge, however, the sex-specific functional impact of microglia on α-synucleinopathy is untested. Thus, we exposed hippocampal microglia/neuron co-cultures from male versus female rat pups to α-synuclein fibrils (Fig. 29a). For this work, microglia...
were harvested at P4, and pups were sexed based on the technique that we described in Fig. 10. Male, but not female microglia slightly reduced the numbers of inclusions per Hoechst$^+$ cell in co-cultured neurons from the same sex, but no statistically significant differences in Hoechst$^+$ cell or NeuN$^+$ neuron viability were noted (Fig. 29b-d). Male, but not female microglia also slightly reduced total numbers of inclusions when expressed as a fraction of NeuN$^+$ neurons, but only when the fold-change data were analyzed (Fig. 29e). Note that the data in Fig. 29d-e are the same as Fig. 28l-m, but were re-analyzed without the respective eHsp70 groups. Average sizes of the inclusions were higher when female microglia were co-cultured with female neurons (Fig. 29f). The total area occupied by the inclusions was lower in male (but not female) co-cultures, when expressed as fold-change (Fig. 29g-i). Consistent with these effects, male microglia also reduced the numbers of perinuclear inclusions in male neurons (Fig. 29j-k).

Collectively, these data suggest that male microglia temper pathology in co-cultured male neurons seeded with PFFs. The statistical interactions between biological sex and microglial presence in Fig. 29 suggest that biological sex modifies the effect of microglia on various aspects of neuronal $\alpha$-synucleinopathy. In contrast, female microglia either had no effects or may even have exacerbated some aspects of pathology in co-cultured female neurons seeded with $\alpha$-synuclein fibrils. No sex differences in the levels of $\alpha$-synuclein fibrils in microglia were noted at 6, 16, 24, 72, and 96 h post-treatment (Fig. 30a-c). Thus, sex differences in the abilities of male versus female microglia to reduce inclusions in co-cultured neurons are consistent with the reported beneficial effects of male...
microglia during early development but may not be readily explained by the differential abilities of male versus female microglia to engulf fibrils, at least in vitro.
Figure 29. Male microglia reduce pSer129\(^{+}\) inclusions in co-cultured male neurons seeded with \(\alpha\)-synuclein fibrils. Primary hippocampal microglia were harvested from P4 male or female rat pups and primary hippocampal neurons from the same sex were co-cultured on top of the microglia. A separate group of neuron monolayers was also maintained. Neuron monolayer cultures and neuron/microglia bilayer cultures were exposed to vehicle or \(\alpha\)-synuclein fibrils (schematic in a) for 10 d. Blinded counts of Hoechst\(^{+}\) cells (b) and NeuN\(^{+}\) neurons (c) are shown. Blinded counts of pSer129\(^{+}\) inclusions, expressed as a fraction of Hoechst\(^{+}\) cells (d) and NeuN\(^{+}\) neurons (e) are shown. In panel e, raw data as well as fold-change data of the PFF-treated neuron monolayer cultures are shown. Average sizes of inclusions are shown in f. Representative photomontage of pSer129 (pseudocolored red), Iba1 (pseudocolored green), and Hoechst (pseudocolored blue) from vehicle or fibril-treated male or female neuron monolayer cultures and male or female neuron/microglia co-cultures is shown in g. Total area occupied by pSer129\(^{+}\) inclusions, expressed as a fraction of Hoechst\(^{+}\) cells (h) and NeuN\(^{+}\) neurons (i) are shown as raw data and as fold-change data of the PFF-treated neuron monolayer cultures. Counts of perinuclear pSer129\(^{+}\) inclusions, expressed as a fraction of Hoechst\(^{+}\) cells (j) and NeuN\(^{+}\) neurons (k) are shown as raw data and as fold-change data of the PFF-treated neuron monolayer cultures. Significant intervariable statistical interactions with biological sex are shown above the graphs in f, h, i. Shown are mean ± SD of 3 independent experiments, each performed in duplicate or triplicate wells. ***p ≤ 0.001 for vehicle versus \(\alpha\)-synuclein fibrils; +p ≤ 0.05, + +p ≤ 0.01, + + +p ≤ 0.001 for neuron monolayer cultures versus neuron/microglia bilayer cultures; ~p ≤ 0.05, ~ ~p ≤ 0.01, ~ ~ ~p ≤ 0.001 for male versus female, two- or three-way ANOVA followed by Bonferroni post hoc.

Figure 30. Biological sex does not impact fluorescence signal of ATTO647-\(\alpha\)-synuclein fibrils in primary hippocampal microglia at 6, 16, 24, 72, and 96 h post-exposure. Primary hippocampal microglia were harvested from P4 male or female rat pups and exposed to ATTO647-fibrils for 6, 16, 24, 72, and 96 h. Representative photomontage in a is of phase images, Hoechst (pseudocolored blue), \(\alpha\)-tubulin (pseudocolored red), ATTO647-fibrils (grayscale), and Iba1 (pseudocolored green). The signal intensity of ATTO647-fibrils was obtained from microscopic images and the area under the male versus female curve is shown in b. Representative z-stack slice view in c is of ATTO647-fibrils (grayscale) and \(\alpha\)-tubulin (pseudocolored red).
Infusions of preformed a-synuclein fibrils in the CD1 male mouse regio inferior (CA2 + CA3) elicits microglia/macrophage activation, which was negatively correlated with cognitive function

To extend our in vitro findings, we determined if PFF-infused mice show higher levels of Iba1, a marker of microglia/macrophage activation. For this work, we used brain tissue from male CD1 mice (females were not tested) that had been bilaterally injected with PFFs in regio inferior (CA2 + CA3) of the hippocampus (Nouraei et al., 2018). PFF-infusions led to an increase in levels of Iba1 in the whole brain (defined by a trace around the cerebrum and the brainstem regions; Fig. 31a) and dentate gyrus (Fig. 31c). There were statistical trends toward higher Iba1 levels in the CA2/3 (two-tailed \( p=0.06; \) Fig. 31b) and striata (two-tailed \( p=0.056; \) Fig. 31d) of PFF-infused mice, compared to PBS-infused mice. In the nigra (Fig. 31e), entorhinal cortex (Fig. 31f), thalamus (Fig. 31g), sensorimotor cortex (Fig. 31h), AON (Fig. 31i), and cerebellum (Fig. 31j), no significant differences were detected. A statistical trend (two-tailed \( p=0.051 \)) toward slightly higher numbers of Iba1\(^+\) cells was noted in the dentate gyrus of PFF-infused mice, but only if a Rout outlier was excluded from the analyses (Fig. 31m, o). In the caudoputamen, PFF-infused mice showed significantly greater numbers of Iba1\(^+\) cells (Fig. 31n, p).

Notably, PFF-infused mice in this study had not shown cognitive deficits via the novel object or novel place exploration tests (Nouraei et al., 2018). However, there were significantly robust negative correlations between the novel place exploration ratios (at 2-months-post-injection) and Iba1 levels in the CA2/3 region (Fig. 31q) and in the dentate gyrus (Fig. 31r) of PFF-infused, but not PBS-infused mice. At 3-months-post-injection, a negative link between novel place exploration and Iba1 levels in the entorhinal cortex (Fig.
was noted. In contrast, there were no correlations between Iba1 and inclusion counts (not shown) in any of the tested regions.

Thus, PFF injections in the male mouse hippocampal formation led to greater monocyte (Iba1 cannot distinguish between microglia and macrophages) activation in some, but not all brain regions. Notably, a significant rise in Iba1 was seen in the dentate gyrus—a brain area that also showed the densest pathology in the report by Nouraei et al. (note the Y-axes in Fig. 2d of (Nouraei et al., 2018)). Male mice also responded to PFFs with a global increase in Iba1 in the cerebrum and brainstem, underscoring the pervasive nature of the microglial response in α-synucleinopathies. Furthermore, the negative associations between Iba1 levels and memory function indicate that PFF-infused mice with better spatial recognition memory can resist an increase in microglial reactivity or that the PFF-induced microglial reactivity impairs memory function.

**Dietary administration of CSF1R inhibitor, PLX5622, induces comparable microglial depletion in male and female CD1 mice but withdrawal of PLX5622 from the diet leads to greater microglial repopulation in CD1 females.**

As a prelude to determining the role of microglia in an in vivo sex-stratified model of limbic-centered α-synucleinopathy, we added the CSF1R inhibitor, PLX5622 to mouse diets. The CSF1 receptor is critical for microglial viability (M. R. Elmore et al., 2014; B. Hu et al., 2021; Oosterhof et al., 2018), and prior work has established that ad libitum access to 1200 mg/kg of PLX5622-containing chow elicits microglial depletion (Dagher et al., 2015; Spangenberg et al., 2019; Q. Zhang et al., 2019).
We performed flow cytometric analyses on the brains of male and female mice that had been fed PLX5622 or control diet for two weeks (Fig. 32a-f). As withdrawal of PLX5622 from the diet is known to lead to microglial repopulation (M. R. P. Elmore et al., 2018; N. R. Johnson et al., 2021; Q. Li et al., 2021; Willis et al., 2020), we also included sex-balanced groups for an additional six weeks, during which time all mice received the chow without PLX5622 (Fig. 32f). First, we found that more than 94% of all CD45^+CD11b^+F4/80^+ monocytes were positive for TMEM119—a microglia-selective marker (Fig. 32g) (F. C. Bennett et al., 2018; M. L. Bennett et al., 2016). Biological sex or the PLX5622 diet also did not influence the proportion of monocytes that were microglial in nature (Fig. 32g). Second, we found that PLX5622 induced equivalent microglial depletion in males and females, irrespective of whether the TMEM119 marker was included in the analyses (Fig. 32h-j). We noted that male and female CD1 mice responded to PLX5622 with ~60% loss of microglia, consistent with work by other groups in C57BL/6J mice (N. R. Johnson et al., 2021).
Figure 3. PLX5622 leads to equivalent microglial depletion in male and female CD1 mice, but cessation of PLX5622 leads to greater microglial repopulation in females. The efficacy of microglial depletion (for 2 weeks) and subsequent repopulation (for six weeks) using CSF1R inhibitor, PLX5622, was tested by flow cytometry (a-f) on brain tissue harvested from the CD1 male and female mouse brains. Gating strategy is shown in a-e. Single cells were analyzed (a-b) and sequential gates for monocytic markers were applied (CD45, CD11b, F4/80, & TMEM119; c-e). Timeline is shown in f. Data in g reflect the proportion of CD45+CD11b+F4/80+ monocytes that are microglial (TMEM119+) in nature. Numbers of monocytes and microglia are in h-j. Mean fluorescence intensity of TMEM119 is in k. Numbers of TMEM119+ cells in the brain and spleen are shown in l. Significant intervariable statistical interactions with biological sex are shown above panels h-j. Shown are mean ± SD of 6-8 mice per group. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 for indicated comparisons; + p ≤ 0.05, +++ p ≤ 0.001 for male versus female, one- or two-way ANOVA followed by Bonferroni post hoc.
Reversal to the standard chow induced microglial repopulation in both sexes—although, surprisingly, the rebound was higher in females (Fig. 32h-j). Changes in numbers of TMEM119+ cells noted in Fig. 32j were not paralleled by robust changes in fluorescence intensity of TMEM119 (Fig. 32k). As further validation of the TMEM119 antibody, we found that the spleen had much lower numbers of TMEM119+ cells than the brain (Fig. 32l), as expected. Our findings cleared the path to test if microglial depletion and subsequent repopulation reduces PFF-seeded α-synucleinopathy in the model developed by Mason et al., which mimics Stage IIb, limbic-predominant Lewy body disease (Mason et al., 2016; Mason et al., 2019).

**Microglial depletion induces hyperactive behaviors in fibril-injected mice of both sexes, but effects are more robust in males, and are not reversed on microglial repopulation.**

Aged, 18-month-old male and female mice were administered control chow or PLX5622-containing chow for two weeks, after which microglia were repopulated for an additional six weeks (Fig. 33a-b). PFFs were injected into the OB/AON on day 7 after initiation of the PLX5622-containing diet and behavior tests for anxiety, hyperactivity, memory, motor function, olfaction, and repetitive compulsive-like behaviors were performed after microglia had been repopulated (see timeline in Fig. 33a). While the experiments were underway, however, the investigator (T.B.) noted that fibril-injected, microglia-depleted males (but not females) appeared anxious and jumpy in their home cages. Thus, the open field test was performed on a few remaining mice that were still microglia-depleted, immediately prior to microglial repopulation (Fig. 33c-o).
Unlike the males, female mice made a greater number of entries and spent more time in the central squares of the open field arena while their microglia were still depleted (Fig. 33c-e). In contrast, microglia-depleted males made more entries into the corner squares than control diet-fed males, and they also spent more time in the corner squares compared to microglia-depleted females (Fig. 33f-g). If the amount of time spent in corner squares and number of entries made into corner squares were reflective of anxiety, one might speculate that CD1 males may have greater anxiety-like behaviors under conditions of microglia depletion and fibril seeding. Both male and female mice with depleted microglia were hyperactive in terms of distance traveled and speeds (Fig. 33h-i) in the open field. Other measures of activity, such as maximum speed (Fig. 33j), time spent active (Fig. 33k), time spent mobile (Fig. 33l), time spent rearing (Fig. 33m), and number of rears (Fig. 33n), showed an increase with microglial depletion in PFF-injected male but not female mice. A positive correlation (Fig. 33o; Pearson $r=0.6140; p=0.0052$) between the number of corner square entries and number of rears was noted, suggesting that the increase in rearing episodes is indeed associated with an anxiety-like phenotype.

Thus, biological sex may not only modify the effect of PLX5622 on microglia repopulation efficacies but may also modify the link between microglia and hyperactive or anxiety-like behaviors, as noted based on the statistical interactions with biological sex in Fig. 33. Microglia depletion may thus elicit hyperactive behaviors in PFF-injected mice of both sexes, but anxiety-like behaviors only in PFF-injected male mice. Only the open field test was performed while microglia were still depleted. Other tests were completed after microglia had been repopulated to determine if this potential therapeutic strategy modifies behavioral alterations associated with $\alpha$-synucleinopathies.
Figure 3. Depletion of microglia induces hyperactive and anxiety-like behaviors, which were greater in PFF-injected aged male mice, compared to their female counterparts. Aged, 18-month-old male and female mice were fed control chow or chow containing the microglia depleting agent, PLX5622 for two weeks, following which all mice were fed the standard rodent chow for an additional six weeks (a). Bilateral injections of PFFs (5 µg) into the OB/AON were performed 7 d after initiation of the PLX5622 diet. The open field test was performed on a few mice prior to microglia repopulation and this test was repeated after microglia had been repopulated, along with other behavior tests (a). In b are representative brain sections from microglia depleted and microglia repopulated mice stained for the microglia/macrophage activation marker, Iba1 (pseudocolored red). Data from the open field test at the stage when microglia were still depleted are shown in c-o. Numbers of central square entries (c) and time in central squares (d) are shown. Data in d were non-Gaussian and were log-transformed to obtain main effects and statistical interactions. Representative trackplots from AnyMaze are in e. Numbers of corner square entries (f), time in corner squares (g), distance traveled (h), average speeds (i), maximum speeds (j), time active (k), time mobile (l), time spent rearing (m), and number of rears (n) are shown. Numbers of corner square entries were correlated with number of rears in o. Significant intervariable statistical interactions with biological sex are shown above d, f, j, m. Shown are mean ± SD of 4-5 mice per group as bar graphs or interquartile ranges in box plots. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 for control versus microglia depleted; + p ≤ 0.05 for male versus female, Kruskal-Wallis or two-way ANOVA followed by Bonferroni post hoc. Data in o analyzed by Pearson correlation analysis.
To determine if microglial repopulation reverses these deficits, the open field test was performed on all mice after the microglia had been repopulated (Fig. 34a-m). No significant differences in the number of entries into the central squares or time spent in central squares were noted (Fig. 34b-d). Despite microglial repopulation, PFF-injected male mice still made more entries into corner squares, unlike the females (Fig. 34e). However, PFF-injected males with repopulated microglia did not spend more time exploring these corner squares (Fig. 34f), perhaps because they remained hyperactive, as evidenced by an increase in the distance traveled, mean speed, maximum speed, time spent active, time spent mobile, and total numbers of rears (Fig. 34g-m). In sum, we conclude that microglial depletion increases hyperactivity in PFF-injected aged male mice and that microglial repopulation does not modify this effect.
Dietary PLX5622 abolishes sex differences in baseline body weight in aged CD1 mice.

Body weights of PFF-injected aged male and female CD1 mice fed control diet or PLX5622-containing diet were tracked (Fig. 35). PFF-injected aged female mice that were fed control chow showed lower body weight than males. These basal sex differences in body weight were abolished in mice that were exposed to PLX5622 (the “Repop” groups). In addition, within seven days of PLX5622 delivery, a mild but statistically significant loss in body weight was noted in male but not female mice. The loss in body weight in female mice exposed to PLX5622 was delayed and statistically significant only fourteen days after

**Figure 34. Repletion of microglia does not reduce the hyperactive behaviors induced on depleting microglia in PFF-injected aged male mice.** All PFF-injected mice were tested in an open field arena after microglia were repopulated (timeline in a). Representative trackplots from AnyMaze software are in b. Numbers of central square entries (c) and the time spent in central squares (d) are shown. Numbers of corner square entries (e), time spent in corner squares (f), distance traveled (g), average speeds (h), maximum speeds (i), time spent rearing (l), and numbers of rearing (m) are shown. Shown are mean ± SD of 9-10 mice per group. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 for control versus microglia repopulated; † p ≤ 0.05 for male versus female, two-way ANOVA followed by Bonferroni post hoc.

**Figure 35. PLX5622 abolishes sex differences in body weight in PFF-injected aged male and female CD1 mice.** The body weights of aged male and female CD1 mice were measured on the day of PLX5622 initiation, on the day of PFF-injections, on the day that PLX5622 was withdrawn from the diet, and on the day that the open field test was performed after microglia had been repopulated. Shown are mean ± SD of 9-10 mice per group. * p ≤ 0.05 for indicated comparisons; † p ≤ 0.05, ++ p ≤ 0.01 for male versus female, repeated measures two-way ANOVA followed by Bonferroni post hoc.

Dietary PLX5622 abolishes sex differences in baseline body weight in aged CD1 mice.
the PLX5622 diet had been initiated. In contrast, after microglia were repopulated, male and female mice did not lose additional body weight, but the mice also did not recover from the loss in body weight that was noted while PLX5622 was being administered (Fig. 35). We had intended to also quantify food intake, but our CD1 mice showed high food-grinding activities (Kathleen R. Pritchett-Corning, 2013; K. R. Pritchett-Corning, Keefe, Garner, & Gaskill, 2013; Robinson-Junker, O'Hara B, & Gaskill, 2018).

**Microglial replacement improves spatial reference memory in PFF-injected aged female, but not male mice.**

Aside from abovementioned behavior assays, we also performed: 1) the Y-maze test to assess differences in spatial reference memory (forced alternation) and spatial working memory (spontaneous alternation), 2) the buried pellet test to assess olfaction, 3) the inverted grid test to assess differences in motor function, and 4) the marble burying test as a measure of repetitive behaviors. These tests were performed after microglia had been repopulated in PFF-injected aged mice of both sexes (Fig. 36a). The most robust differences were observed on the Y-maze test for spatial reference memory (Fig. 36b-g). Microglial repopulation increased novel arm entries in PFF-injected mice of both sexes (Fig. 36b), compared to mice that were fed the control chow. A concomitant decrease in the number of entries into the familiar arms was only noted in PFF-injected females (Fig. 36c). We speculate that, as in the open field, male mice were also hyperactive in the Y-maze after microglial repopulation, given that they made more entries into both the novel and familiar arms (Fig. 36b-c). When fed the control chow, fibril-injected aged females made greater number of entries into and spent greater time exploring familiar arms than
males (Fig. 36c, e). However, after microglia repopulation, females made more entries into the novel arm (Fig. 36b), fewer entries into the familiar arms (Fig. 36c), spent more time exploring the novel arm (Fig. 36d, f), and entered the novel arm earlier (Fig. 36g). Together with the statistical interactions noted above Fig. 36c and Fig. 36e, these data suggest that PFF-injected aged females may display worse memory than PFF-injected aged males, but this is surmountable with microglia replacement strategies.

A statistical trend (two-tailed \( p=0.060 \)) toward slightly greater spontaneous alternations was noted in microglia repopulated males (Fig. 36h), but only when mice that made <12 arm entries were excluded from the analyses, based on criteria in prior work (Garcia & Esquivel, 2018). Additional exclusion criteria have been described in the Methods section. Thus, microglial replacement produces more robust changes in spatial reference memory compared to spatial working memory, with PFF-injected aged females receiving greater benefits of this potential therapy than males.

No significant differences on the inverted grid test were noted (not shown). PFF-injected aged female mice on the control diet showed a trend (two-tailed \( p=0.051 \)) toward slightly greater burying behaviors than males—a sex difference that was abolished with microglial repopulation (Fig. 36i). No significant differences in the latencies to contact an exposed or buried pellet were noted across any of the groups (Fig. 36j-k). Therefore, we also assessed the latencies to eat the pellet when it was exposed or buried. In both cases, PFF-injected aged females on the control diet (versus males) took slightly longer before they began to eat the pellet, which may either reflect worse olfaction, lesser hunger, or lesser motivation in aged females versus males (Fig. 36l-m). However, no sex differences in the latency to eat the food were noted after microglia repopulation. These latter data in
Fig. 36l-m are consistent with the body weight data shown in Fig. 35, in that the aged females on the control chow display slightly lower body weights, compared to aged males, but not if microglia are repopulated. The data in Fig. 36 indicate that repopulation of microglia may have strong effects at improving memory in PFF-injected female mice, but microglial repopulation did not improve any of the other behavioral measures.
As a final study in this Aim, we examined the levels of Iba1 in the OB (Fig. 37a-d) and amygdala (Fig. 37e-i) of male and female patients with Lewy body disorders or age-matched unaffected controls (same postmortem tissue as in Aim 1). We found that there were no significant differences in Iba1 between control and diseased OB samples (Fig. 37a-b). A Grubbs outlier was noted among controls (see arrow in Fig. 37b), and if this sample was excluded, diseased women showed slightly higher Iba1 in the OB, compared to control women (Fig. 37c). Note that, despite the non-specific bands at very high molecular weights, the polyclonal Iba1 antibody that we used in Fig. 37 was the best available and only the band at ~15 kDa was quantified. To confirm our findings, tissues were probed multiple times for Iba1: a second polyclonal antibody against Iba1 (NB100-1028; Novus Biologicals) showed lot-to-lot variability in specificity and a monoclonal Iba1 antibody (MABN92; EMD Millipore) did not show any bands via immunoblotting.
At the transcriptomic level, bulk RNA-seq showed that the fold-change increase in Iba1 transcripts per million (TPM) with disease in the female OB was greater than in the males (Fig. 37d; AIF1 is the gene for Iba1). In the amygdala, we did not detect any significant differences across groups at the protein and transcriptomic levels (Fig 37e-h). However, amygdalar Iba1 (or microglia/macrophage reactivity) was associated with age at death (Fig. 37i), indicating that ageing is associated with microglial reactivity.
**Discussion**

In this Aim, we made several novel observations that merit discussion. First, we found that hippocampal microglia harvested from P4 male rat pups reduce α-synuclein pathology in co-cultured male neurons seeded with α-synuclein fibrils. Age-matched female microglia either had no effects on α-synuclein pathology or may have increased some aspects of the pathology, such as the average inclusion size in co-cultured female neurons seeded with PFFs. Second, we noted that depletion of microglia with the CSF1R inhibitor, PLX5622, induces hyperactive and anxiety-like behaviors in PFF-injected aged mice of both sexes, but the number of effects were greater in males, and repopulating microglia did not mitigate these deficits in PFF-injected males. Third, we observed that PFF-injected aged female mice fed the control diet had worse spatial reference memories than control chow-fed male counterparts. However, microglia repopulation significantly mitigated these memory deficits in PFF-injected females, based on several readouts, all of which were internally consistent. Finally, an increase in levels of microglia/macrophage activation marker, Iba1, was noted in the postmortem OB of women diagnosed with Lewy body disorders, compared to age-matched controls. Iba1 levels in the amygdala were also positively linked with age at death (*i.e.*, lifespan), irrespective of biological sex or disease.
status. These collective findings thus show that microglial reactivity with natural ageing may have beneficial effects and even a mild (~60%) loss in microglia may worsen disease. We propose that microglial senescence must instead be countered by replacing the aged cells with a pool of younger, newly proliferated cells—a strategy that has already been tested with success in ageing, TBI, AD, and parkinsonism (M. R. P. Elmore et al., 2018; N. R. Johnson et al., 2021; Q. Li et al., 2021; Willis et al., 2020). To our knowledge, none of the studies examining microglial repopulation report worsening of disease outcomes— unlike some of the work on microglia depletion strategies.

It has long been known that microglia may be polarized to more proinflammatory or more anti-inflammatory phenotypes—the simplified M1 vs. M2 paradigm, respectively (Lyu et al., 2021; W. Zhang, Bhatia, & Leak, 2020). Over the years this overtly crude classification, which relies on expression of only a subset of promiscuous markers, has received criticism, as microglia are likely to exist in a continuum of different phenotypic states, and may even express inflammatory and anti-inflammatory markers in parallel (Benusa, George, & Dupree, 2020; Martinez & Gordon, 2014; Ransohoff, 2016). In this dissertation, we refrain from addressing these phenotypes as M1/M2. We found that primary hippocampal microglia reduce the total numbers of inclusions in co-cultured neurons seeded with PFFs, and that pre-treatment with anti-inflammatory IL4 (but not proinflammatory LPS/IFNγ) specifically lowers the numbers of perinuclear inclusions. These latter findings are in line with the work of George et al., in which LPS-injected (but not IL4-injected) mice showed an increase in the cell-to-cell spread of α-synuclein (George et al., 2019).
We also found that microglia respond to PFFs with an increase in engulfment of microspheres, but this PFF-induced increase in phagocytosis was only partially dependent on the polymerization and stabilization of the microglial actin cytoskeleton. This lies in contrast to recent work, in which the internalization of α-synuclein fibrils by microglia was almost completely blocked by cytochalasin D—the inhibitor of actin stability (Haenseler et al., 2017; Scheiblich et al., 2021). However, some differences in the experimental paradigms must be noted. For example, we did not examine if fibril uptake *per se* is dependent on actin polymerization. Instead, we tested if PFF-treatments of microglia show generalized changes in engulfment capacities. Moreover, there were variations in timepoints at which assays were performed (3 h post-treatment in our work *vs.* 5-15 mins post treatment in (Scheiblich et al., 2021)), in experimental readouts (microscopic counts of numbers of phagocytosed beads in our work *vs.* flow cytometric analyses of the proportion of phagocytic microglia in (Scheiblich et al., 2021)), in the type of cells used (primary rodent microglia in our work *vs.* an iPSC-derived macrophage cell line in (Haenseler et al., 2017)), etc. Surface receptors partaking in α-synuclein fibril uptake or bead uptake may also be involved, as some receptors specifically engage the actin cytoskeleton, whereas others may impact the microtubule cytoskeleton (Rosales & Uribe-Querol, 2017). Thus, other mechanisms, such as stabilization of microtubules must be examined, as microtubule stabilizers (*e.g.*, Epothilone D) affect microglia-mediated cell-to-cell transfer of α-synuclein (Valdinocci, Grant, Dickson, & Pountney, 2018) and protect against MPTP-induced parkinsonism (Cartelli et al., 2013).

In our hands, pre-treatment of microglia with PFFs reduced the total numbers of pSer129+ inclusions in neurons. However, the PFF-treatment of microglia alone did not
elicit changes in expression of proinflammatory iNOS and CD16 or anti-inflammatory CD206. In contrast, exposure to monomeric (fibrillar α-synuclein was not examined in that study) α-synuclein is known to lead to an anti-inflammatory phenotype in cultured microglia (N. Li et al., 2020), which might reflect the normal, physiological function of α-synuclein expressed by microglia (S. A. Austin et al., 2006; S. A. Austin et al., 2011). Although additional markers must be examined in our work and PFF exposure timeline or concentrations must be varied, our present data may be in line with findings that fibril-treated macrophages are clustered differently—at least from IFNγ-treated macrophages, based on their gene expression data (E. Xu et al., 2022).

Furthermore, we found that PFF-pretreated microglia were not more efficacious at mitigating neuronal pSer129+ inclusions than untreated microglia, which may be because dual exposure to α-synuclein fibrils may lead to glial fatigue, whereby critical functions may be compromised. Despite being more effective at clearing α-synuclein aggregates than astroglia (H. J. Lee et al., 2008), it is known that excessive α-synuclein can inundate microglia, leading to phagocytic exhaustion and impairments in degradation (Bido et al., 2021; Gardai et al., 2013; Haenseler et al., 2017; Scheiblich et al., 2021). Given that pre-treatments with PFFs, or even with LPS/IFNγ and IL4 did not have any additional beneficial or toxic effects in mixed-sex neuron/microglia co-cultures, we did not pre-treat microglia with any stimulus in studies wherein we used sex-stratified microglia/neuron co-cultures.

Male microglia had more profound effects at reducing α-synucleinopathy in co-cultured male neurons seeded with PFFs, compared to their female counterparts. To our knowledge, we are the first to show that there are sex differences in the abilities of
microglia to mitigate neuronal α-synuclein pathology, even in postnatal cells and under in vitro conditions. As mentioned earlier, these findings are consistent with a body of work showing that in P4 rodents (the age at which microglia were harvested in our work), male microglia are more phagocytic and that this age closely follows the surge in testosterone that is seen in younger male rodents (Nissen, 2017; Schwarz et al., 2012; VanRyzin et al., 2019). In addition, it has been established that in some cases, males may require microglial functions earlier than females—a finding that we can extrapolate to our work (Thion et al., 2018). For example, postnatal male neurons grown in vitro had shown greater PFF-induced detergent-insoluble pathology than female neurons (Aim 1), and male microglia may thus have evolved to offset this insoluble pathology in neurons, although future studies evaluating the extent of detergent-insoluble pathology in PFF-treated microglia/neuron co-cultures are warranted.

The mechanisms that govern these sex differences in vitro must also be studied as they were not explained by sex differences in uptake of fibrils. We cannot rule out more subtle sex differences in other readouts related to phagocytosis, such as in the numbers of phagocytic microglia, numbers of phagocytic cups, numbers of microglia with high vs. low engulfment capacities, etc. In addition, it was recently noted that in males, astroglia also mature much faster than females (Rurak et al., 2022)—similar to the aforementioned findings regarding early maturation of male vs. female microglia. Our neuron cultures do contain ~10-13% astroglia (J. M. Posimo et al., 2015), as also conceded previously, and studies show that microglia interact with astrocytes to exert effects on neurons (Liddelow et al., 2017), including in PD models (Yun et al., 2018). Thus, it is important to note that sex differences in our in vitro co-cultures could be driven by sex differences in astroglia.
We showed that PFFs injected into the hippocampal CA2/3 of male mice elicit an increase in Iba1 in several regions—a finding that follows previous work (Duffy, Collier, Patterson, Kemp, Luk, et al., 2018; Harms et al., 2017; Thakur et al., 2017; Verma et al., 2021). We compared PFF-injected mice to age-matched PBS-injected mice as prior work confirmed that microglial reactivity in response to PFFs is not merely due to exposure to exogenous proteinaceous material, as Duffy et al. have reported that PFF-injections and not injections of rat serum albumin (RSA) induce an increase in MHC immunoreactivity (Duffy, Collier, Patterson, Kemp, Luk, et al., 2018). Furthermore, in their work, there were no differences in the densities of MHC+ cells between PBS and RSA-injected mice (see Fig. 4k in (Duffy, Collier, Patterson, Kemp, Luk, et al., 2018). Together with previously reported caveats associated with using monomeric α-synuclein (see Discussion in Aim 2 and (Paumier et al., 2015)), we continued to rely on PBS exposures as controls.

Apart from higher Iba1, we noted that PFF-injections were negatively linked with cognition, consistent with reports that reactive microglia may impair memory function (Cornell, Salinas, Huang, & Zhou, 2022). Our data, along with reports that microglia may have slow turnover, may be carriers of α-synuclein, and the findings that there may be a generalized impairment in phagocytosis with Lewy body disease (Bido et al., 2021; Fuger et al., 2017; Guo et al., 2020; Haenseler et al., 2017; Reu et al., 2017; Salman, Bergman, Djaldetti, Bessler, & Djaldetti, 1999; Y. Xia et al., 2019), all argue for testing if microglia depletion reduces α-synuclein pathology and improves neurological function.

In this regard, many groups have depleted microglia in vivo in different models of Lewy body disorders (George et al., 2019; Guo et al., 2020; Jing et al., 2021; S. J. Oh et al., 2020; X. Yang et al., 2018; D. Zhang et al., 2021), but, as mentioned previously, these
studies have been largely conflicting, were conducted only in young animals and in one sex (or the sex was left unmentioned). For example, in females, microglial depletion increased cell-to-cell transfer of α-synuclein (George et al., 2019). In males, microglial depletion improved neuron survival and reduced cognitive deficits as well as α-synuclein pathology, in response to rotenone (Jing et al., 2021; D. Zhang et al., 2021). Microglial depletion in males also reduced motor deficits and depression-like behaviors in response to 6-OHDA injections (S. J. Oh et al., 2020). These findings might indicate that adult males exposed to dopamine toxicants or pesticides might benefit from depletion of microglia. However, this theory may be overtly simplistic, as, in response to MPTP, males show an increase in motor deficits, dopaminergic neuron loss, and production of inflammatory mediators if their microglia are depleted (X. Yang et al., 2018). Thus, the microglial response is likely to be dictated by the nature of the neurotoxicant employed. In contrast, the studies that repopulated microglia after short periods of depletion are relatively fewer, but have all shown therapeutically beneficial results in ageing, TBI, AD, and MPTP-induced parkinsonism (M. R. P. Elmore et al., 2018; N. R. Johnson et al., 2021; Q. Li et al., 2021; Willis et al., 2020). However, one must note that these latter studies also did not examine sex as a biological variable—a novelty of our work in Aim 3.

The CSF1 receptor is critical for microglial survival and viability (M. R. Elmore et al., 2014). Mutations in the CSF1R gene underlie a rare form of leukencephalopathy, considered as a “microgliopathy” that may be targeted using microglia replacement therapies (Han, Sarlus, Wszolek, Karrenbauer, & Harris, 2020). Notably, homozygous mutations in CSF1R were recently shown to underlie the near complete congenital loss of microglia, in an infant who survived to less than 12 months after birth (Oosterhof et al.,
2019), highlighting the need to avoid a sustained and near-complete loss of microglia for long periods. Dietary delivery of CSF1R inhibitors is non-invasive and leads to loss of ~99% of microglia within 7 d (M. R. Elmore et al., 2014). Withdrawal of the inhibitor is known to lead to rapid microglial repopulation, with the microglial numbers stabilizing to control levels within 14 d of removal of the inhibitor (M. R. Elmore et al., 2014). We noted that male and female CD1 mice showed equivalent (~60%) loss of microglia. This is relatively mild, compared to the >90% loss of microglia noted in other studies (Y. Liu et al., 2019; Rosin, Vora, & Kurrasch, 2018; Spangenberg et al., 2019; Q. Zhang et al., 2019), but is consistent with recent work in C57BL/6J mice (N. R. Johnson et al., 2021), and suggests that there may be an upregulation of specific subsets of CSF1R-resistant microglia in CD1 mice (or these subsets may be expressed at constitutively higher levels in CD1 mice) (N. R. Johnson et al., 2021; Stratoulias, Venero, Tremblay, & Joseph, 2019).

Furthermore, we note that CD1 mice display food grinding, which may limit the amount of the active drug ingredient that they may intake and future work examining the levels of PLX5622 in the plasma and brain tissue of male vs. female CD1 mice is warranted. Despite equivalent (but incomplete) depletion in males and females, we found that microglia depleted males displayed greater anxiety and hyperactivity than females. Furthermore, females responded to cessation of the PLX5622-containing diet with a more profound re-emergence of microglia, overshooting the numbers noted in mice that had never been exposed to PLX5622. The reasons causing this sex difference are unknown.

Mechanisms by which a fresh pool of microglia repopulate the CNS are currently being debated. Early work with CSF1R inhibitors revealed that there may be Nestin-expressing microglial progenitors in the brain (M. R. Elmore et al., 2014). Nestin marks
neural stem/progenitor cells that belong to the neuroectodermal lineage, and one must note that microglia belong to the myeloid lineage. Although studies have shown that microglia express Nestin in injury (Sahin Kaya, Mahmood, Li, Yavuz, & Chopp, 1999) and that there is a negative link between CSF1R and the pool of Nestin\(^+\) cells (Nandi et al., 2012), the occurrence of cross-lineage differentiation is not likely (Huang, Xu, Xiong, Sun, et al., 2018). Using fate-mapping approaches, recent work revealed that although repopulating microglia show a \textit{transient} expression of Nestin, microglia are not derived from Nestin\(^+\) cells, but re-colonize the brain based on the local proliferation of the few surviving microglia that are resistant to CSF1R inhibition (Huang, Xu, Xiong, Qin, et al., 2018; Huang, Xu, Xiong, Sun, et al., 2018). This study also ruled out the possibilities that myeloid cell progenitors infiltrate the brain after withdrawal of the CSF1R inhibitor or that microglia are derived from neuroectodermal cells, such as astroglia, neurons, \textit{etc.} (Huang, Xu, Xiong, Sun, et al., 2018).

Separately, it was shown that the microglia that resist CSF1R inhibition express high levels of galectin-3 (\textit{a.k.a.,} Mac2) and that these galectin-3\(^+\) microglia mimic the microglial precursors found in the yolk sac (Zhan et al., 2020). Interestingly, it is known that galectin-3 is involved in \(\alpha\)-synuclein-mediated induction of inflammatory cytokines by microglia and \(\alpha\)-synuclein is known to be internalized by activated microglia that are immunopositive for galectin-3 (Boza-Serrano et al., 2014). Future work must thus determine the role of galectin-3 in mediating the behavior deficits that we observed after PFFs were injected in microglia depleted mice. Furthermore, it is unclear if there are sex differences in galectin-3 levels, which must be examined in detail in future work. We used multiple layers of markers, including the recently identified microglia-selective marker
TMEM119 (F. C. Bennett et al., 2018; M. L. Bennett et al., 2016), to show that microglial repletion is higher in females. Despite greater selectivity toward microglia than Iba1, it should be noted that TMEM119 may be expressed by some non-microglial cells, such as by those that are involved in osteoblast differentiation (Hisa et al., 2011; Kaiser & Feng, 2019; K. Tanaka et al., 2012). Our findings that the numbers of TMEM119+ cells are higher in brain vs. spleen and that numbers of TMEM119+ cells in microglia depleted/repopulated mice follow the same pattern as the numbers of CD45+CD11b+F4/80+ cells increase confidence in our results that female CD1 mice show greater microglial repopulation than males, despite comparable microglial depletion.

At microglia depletion, PFF-injected male and female mice showed hyperactivity, as in prior work in other models (Rosin et al., 2018). Notably, we found that the extent of hyperactivity and anxiety-like behaviors was greater in males vs. females, and that these effects were not abolished in males even when microglia were repopulated. Prior work in an AD model and in a separate mouse strain revealed that there may be greater amounts of PLX5622 in the brain and plasma of males vs. females and that microglial depletion may be excitotoxic in males due to an induction of immediate-early genes (N. R. Johnson et al., 2021). Future work using PLX5622 in the PFF model must evaluate the brain-to-plasma concentration ratio of the drug in mice of both sexes and must examine the transcriptome to assess if PFF-injected, microglia-depleted male mice show an induction of excitotoxic genes. This is an important caveat to consider, given the recent work that high amounts of CSF1R inhibitors are likely to exert off-target effects (F. Lei et al., 2020), including on other brain cells and on members of the adaptive immune system (Fengyang Lei et al., 2021; Y. Liu et al., 2019). Furthermore, although the open field test is widely used to assess
locomotion, activity, anxiety, etc., it is important to note that interpretations of anxiety may be confounded by differences in ambulation or locomotion between groups (Seibenhener & Wooten, 2015). In future work, it may be necessary to normalize open field measures by readouts such as the total distance traveled to control for differences in activity across groups.

CSF1R is expressed by multiple cells, including macrophages, Langerhans cells (skin), osteoclasts, oocytes, decidual cells, trophoblastic cells, Paneth cells (intestine), and myoblasts (J. Li, Chen, Zhu, & Pollard, 2006; Mun, Park, & Park-Min, 2020). Consistent with this, CSF1R inhibition at embryogenesis was found to cause craniofacial and dental abnormalities in rodent pups (Rosin et al., 2018). CSF1R knockouts not only show loss of microglia, but also ~50% loss of oligodendroglia (Erblich, Zhu, Etgen, Dobrenis, & Pollard, 2011). These mice also display enlarged ventricles, a disintegrated OB, atrophy of brain tissue, and impaired olfaction and reproduction (Erblich et al., 2011; J. Li et al., 2006). In contrast, some studies continue to show that PLX5622 can be given for as long as ~24 weeks, without underlying toxicities or behavior defects (Spangenberg et al., 2019). Furthermore, it was shown that PLX5622 only exerts off-target effects on oligodendroglia if given for 21 d or longer (Y. Liu et al., 2019). In addition, PLX5622 is in clinical trials as an anti-inflammatory agent for rheumatoid arthritis (NCT01329991). Thus, these reports suggest that PLX5622 may be suitable for microglial depletion for the relatively short timeframe of 14 d, as in our work.

Other methods of microglia depletion have also shown off-target effects (Giuliani, Hader, & Yong, 2005; Lund, Pieber, & Harris, 2017) and Lund et al., have stated that “it has proven difficult to permanently deplete microglia in a specific manner and there are
caveats with all the depletion models employed (Lund et al., 2017). Given all these reasons and the fact that CSF1R inhibitor delivery is simple and non-invasive, we believe that use of CSF1R inhibitors for short-term microglia depletion followed by withdrawal for repopulation may still hold clinical value. However, the off-target effects must be borne in mind when determining the mechanisms underlying the effects. In the future, it may be necessary to have a separate control group, which would be fed the non-brain penetrant analog of PLX5622, i.e., PLX73086 to assess if the effects are mediated by inhibition of peripheral CSF1R-expressing cells and not microglia alone (Bellver-Landete et al., 2019; N. R. Johnson et al., 2021). Our data in the current study, nonetheless, indicate that interference with the normal functions of microglia may be detrimental in PFF-injected aged male mice.

At microglial repopulation, the most robust effects were on memory function in PFF-injected aged female, but not male mice. Microglia are known to sculpt neural circuitry, assist in synaptotrophic and neuroplasticity, and impact memory (Cornell et al., 2022). Furthermore, although we have not determined the reasons underlying a lack of rescue of other behavior deficits, it is possible that some brain areas may be more prone to microglia depletion and repopulation with the CSF1R inhibitors. Indeed, the hippocampus shows higher expression of Csf1r, compared to other areas such as the striatum, globus pallidus, posterior cortex, cerebellum, frontal cortex, nigra, thalamus, (see DropViz database by (Saunders et al., 2018)) etc. Furthermore, recent work reported that after repopulation, microglia first reappear in the subventricular zone and then migrate to other brain regions via white matter tracts (Hohsfield et al., 2021). We only assessed the extent of microglia depletion and repletion in the whole brain and region-dependent changes must
be examined in future work, including in PFF-injected mice. For example, a high proportion of microglia are burdened with inclusion pathology in the AON of PD subjects (Stevenson et al., 2020). Our in vivo model also involves injections of PFFs and formation of dense inclusions in the AON (Mason et al., 2016; Mason et al., 2019). Thus, it may be important to determine the efficacy of microglial depletion and repopulation in the AON. Nonetheless, data in the current study indicate that microglial repopulation may be an effective therapeutic strategy and may have nootropic effects in Lewy body disease—at least in aged females injected with PFFs.

Finally, data from human subjects revealed that women suffering from Lewy body disorders displayed greater microglial reactivity in the OB than control subjects—an effect that was absent in men and was consistent at the protein and transcriptome level. This lies in contrast to prior work showing that it is the men (and not women) with PD that show microgliosis in the OB (Flores-Cuadrado et al., 2021). In this context, it is important to note that the human samples we received from NIH NeuroBioBank suffered from multiple subtypes of Lewy body disorders and are thus more heterogenous than the cohort used by Flores-Cuadrado et al. (Flores-Cuadrado et al., 2021). In fact, in our cohort, only two patients (out of 13) showed a clinical diagnosis of PD, out of which PD was confirmed at autopsy in only one patient. Together with the previously mentioned caveats that microglial reactivity in DLB has shown conflicting results in the literature, our data suggest that sex-specific microglial reactivity may vary based on the specific Lewy body disorder with which the patient is diagnosed.

Moreover, we had to rely on Iba1 to detect microglia/macrophage activation in the human subjects as we were unable to find a TMEM119 antibody that was specific via
immunoblotting. In the amygdala, a positive correlation between Iba1 and age at death of all samples (regardless of disease or biological sex) was noted. These data, together with the findings that, 1) microglial reactivity is negatively linked with memory, 2) preserving the normal levels of Iba1 is vital for synaptoplasticity and memory (Lituma et al., 2021), 3) microglial replacement reduces memory deficits in PFF-injected aged females, 4) greater microglial reactivity is noted in OB of diseased women, and 5) microglial replacement extends lifespan only in female mice with tauopathy (N. R. Johnson et al., 2021), all indicate that microglia repopulation may be therapeutically effective, but may exert sex-specific effects.

Note that many of the strengths and limitations mentioned in the other Aims, particularly with respect to the model and techniques employed, are also applicable to the studies in Aim 3, but are not repeated here in order to be concise.
Chapter 6

Conclusions & Impact

In this work, we examined mechanisms underlying sex differences in Lewy body disorders by systematically including sex as a biological variable in \textit{in vitro} and \textit{in vivo} models of PFF-seeded \textalpha -synucleinopathic disease. Our findings suggest that Hsp70 and microglial defenses against disease may be sex-skewed, and, therefore, our work supports the assessment of biological sex as a variable in studies of neurodegenerative diseases, such as Lewy body disorders. Furthermore, as far as possible, we included control groups for full-factorial ANOVAs, at least for the \textit{in vitro} work in this dissertation. Thus, we were able to test if exposure to eHsp70, Hsp70 inhibitors, or microglia is modifying the disease or only exerting effects on baseline (control) conditions. Analyses of statistical interactions with biological sex have also been provided wherever feasible (\textit{i.e.}, we have statistically tested whether the effect of one variable, such as treatment with eHsp70, is dependent on the biological sex of the cell), based on recent guidelines to mitigate Type I (false positive) errors in reporting of sex differences (Garcia-Sifuentes & Maney, 2021).

First, our findings suggest that male cells display greater dependence on Hsp70 defenses in \textalpha -synucleinopathic disease, as compared to female cells. Using postmortem tissue from age- and sex-matched patients and unaffected controls, we extrapolated our \textit{in vitro} findings, and observed that men with disease tend to engage Hsp70 responses to a greater extent than females, and that men who survive longer with Lewy body disease also show constitutively higher Hsp70 expression. These data are the first to suggest that mechanisms underlying the pathology in Lewy body disorders may be sex-skewed and that
males may be less able to compensate for loss of Hsp70 function. We also observed that women with disease did not show a statistically significant induction in Hsp70, due perhaps to the higher baseline levels of Hsp70 in women under control conditions. Thus, our findings in Aim 1 also highlight the need to include non-diseased controls in in vitro and in vivo models, as baseline sex differences in expression and function of protective molecules may have an impact on the response to disease. It has long been known that women tend to suffer greater adverse drug reactions, due perhaps to greater baseline immune responses in the female sex or due to the disproportionate use of males in drug research (S. L. Klein & Flanagan, 2016; Will et al., 2017; Zucker & Prendergast, 2020). Thus, our findings argue for ensuring that data for females is not deduced from studies that solely include men or solely rely on male-specific model species.

We used the intranasal route of drug delivery and found that males (but not females) show nose-to-brain uptake of eHsp70, and that eHsp70 mitigates α-synuclein pathology and behavior deficits in PFF-injected male mice. The absence of nose-to-brain entry of eHsp70 in females deserves further investigation at other dosages and time-points. Novel drug delivery systems, such as nanoparticle-based formulations, hydrogel-based formulations, or gastric applicators may need to be used to deliver eHsp70 if it continues to fail to penetrate the female nose-to-brain barrier (Brayden & Baird, 2019; Tunesi et al., 2019). Alternatively, eHsp70 may only show brain penetration in females when coupled with cell-permeating peptides, such as the trans-activator of transcription (Tat) protein (Nagel et al., 2008; Tunesi et al., 2019). Apart from being non-invasive and bypassing hepatic metabolism, the intranasal route may be appropriate for delivering therapies that inhibit Lewy pathology, given that in humans, these inclusions initially develop in
olfactory structures before other deeper brain regions. Thus, we delivered eHsp70 intranasally in a model wherein PFFs are also injected in the OB/AON (Mason et al., 2016; Mason et al., 2019). This model shows extensive pathology in the OB/AON but only mild cell loss, indicating that this model is of early-stage disease, and, so, it is suited to test therapeutic potential of interventions, such as eHsp70, as clinical trials suggest that therapies may be more likely to succeed if delivered at early stages, prior to extensive and intractable neurodegeneration. Although our data are proof-of-concept, it is worth noting that the intranasal route is already being used for the daily delivery of drugs, such as desmopressin, via nasal sprays. Future work must determine if eHsp70 can be formulated in a nasal spray or in automated devices that deliver eHsp70 to the brain via the nasal mucosa, for better clinical translation. The pharmacokinetic, pharmacodynamic, and toxicological responses after intranasal eHsp70 must also be examined. Overall, our data from the current work support the continued testing of biologics, such as eHsp70, as therapies that can modify the pathology underlying α-synucleinopathies.

Finally, we found that replacing aged, diseased microglia with a fresh pool of newly colonized microglia improves memory function in female mice injected with PFFs in their OB/AON. Although we have not yet tested the mechanisms that underlie these salutary effects, our work adds to evidence in the literature that microglia repopulation may be a novel therapeutic strategy against neurological diseases. The microglia depleting agent that we used in our work (PLX5622) is already being tested against rheumatoid arthritis in clinical trials (NCT01329991) and although its off-target effects are being increasingly recognized, the dosing regimen that we adopted involved depleting microglia for a short period of 14 d. Future studies testing if >1 cycle of depletion followed by repletion provides
additional benefits at the behavioral level are warranted (Najafi et al., 2018). If positive, this strategy may have significant translational potential, as patients would not need to receive sustained delivery of CSF1R inhibitors. Based on our data, we also speculate that since males were more reliant on microglia to reduce α-synucleinopathy (at least in vitro), the use of PLX5622 may have exerted toxic hyperactive effects in male mice when administered in vivo. Thus, it may be critical to determine the amount of PLX5622 present in the brain and plasma of male mice, which would ultimately help titrate the dose of PLX5622 in mice of both sexes. Alternatively, males may need a different dosing regimen altogether, with shorter periods of depletion than the amount tested here, and/or >1 cycle of depletion/repletion. In addition, it may be more beneficial to deplete and then replete microglia at latter stages of disease (i.e., after PFFs form dense inclusions), at which point microglia may be more fatigued due to an excessive burden of α-synuclein+ aggregates and may thus benefit more from microglia replacement. Lastly, it is also important to consider that interference with the natural functions of microglia may not be feasible in males. Nonetheless, our data that microglia replacement improves memory in PFF-injected aged female mice is promising, given that patients may have little recourse against loss of memory and cognition in Lewy body diseases. Future work must examine if repopulating microglia also elicits a reduction in pathological inclusions in mice injected with PFFs.

In sum, our work provides evidence that Hsp70 may mitigate pathology formed in situ and, if delivered exogenously, it may have the dual effect of inhibiting intracellular pathology as well as inhibiting the cell-to-cell propagation of α-synuclein. Furthermore, our work provides evidence that replacing microglia in aged mice significantly mitigates memory deficits in response to PFF infusions. Replenishing endogenous defenses via the
non-invasive method of oral (PLX5622) or intranasal (eHsp70) delivery may thus modify α-synucleinopathic disease, even in aged rodents.
Chapter 7

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222


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243


Chapter 8
Appendices

Table 1: List of primary antibodies


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<th>Antibody</th>
<th>Host</th>
<th>Company</th>
<th>Catalog No.</th>
<th>Application</th>
<th>Dilution</th>
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Table 2: List of secondary antibodies


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- Donkey anti-Mouse 790: 1:700
- Donkey anti-Mouse 790: 1:30000
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<td>1:700</td>
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<td>Jackson</td>
<td>711-625-152</td>
<td>Immunocytochemistry</td>
<td>1:700</td>
</tr>
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<td>711-625-152</td>
<td>Western Immunoblotting</td>
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<td>Jackson</td>
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<td>Immunocytochemistry</td>
<td>1:700</td>
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<td>Donkey anti-Rat 790</td>
<td>Jackson</td>
<td>712-655-150</td>
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<tr>
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<td>Jackson</td>
<td>706-545-155</td>
<td>Immunocytochemistry</td>
<td>1:700</td>
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<td>Donkey anti-Guinea Pig 680</td>
<td>Jackson</td>
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<td>Immunocytochemistry</td>
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<td>Jackson</td>
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<td>1:700</td>
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<td>Jackson</td>
<td>705-655-147</td>
<td>Immunocytochemistry</td>
<td>1:700</td>
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Table 3: List of antibodies for Flow Cytometry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalog No.</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE Rat anti-Mouse F4/80 (T45-2342)</td>
<td>BD Biosciences</td>
<td>565410</td>
<td>1:50</td>
</tr>
<tr>
<td>PerCP Rat anti-Mouse CD45 (30-F11)</td>
<td>BD Biosciences</td>
<td>557235</td>
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</tr>
<tr>
<td>APC Rat anti-Mouse CD11b (M1/70)</td>
<td>Invitrogen</td>
<td>17-0112-82</td>
<td>1:50</td>
</tr>
<tr>
<td>PE-Cyanine7 Rat anti-Mouse Tnem119 (V3RT1G0sz)</td>
<td>Invitrogen</td>
<td>25-6119-82</td>
<td>500 ng/mL</td>
</tr>
</tbody>
</table>

Table 4: Demographic information of human postmortem samples


<table>
<thead>
<tr>
<th>Number</th>
<th>Age at Death (years)</th>
<th>Gender</th>
<th>Control or Diseased</th>
<th>Clinical Diagnoses</th>
<th>Additional Neuropathological Diagnoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82</td>
<td>M</td>
<td>C</td>
<td>CHF</td>
<td>Unaffected Control, Normal Aging</td>
</tr>
<tr>
<td>2</td>
<td>79</td>
<td>M</td>
<td>C</td>
<td>CA (probable), ARF, Diabetes Type 1</td>
<td>Unaffected Control, Hypoxia (recent, cerebrum)</td>
</tr>
<tr>
<td>3</td>
<td>77</td>
<td>M</td>
<td>C</td>
<td>CA (probable), Heart Attack</td>
<td>Unaffected Control, Hypoxic Changes (intermediate, cerebrum)</td>
</tr>
<tr>
<td>4</td>
<td>82</td>
<td>F</td>
<td>C</td>
<td>CA (probable), Cerebellum</td>
<td>Unaffected Control, Hypoxia (recent, cerebrum)</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>F</td>
<td>C</td>
<td>CA (probable), Cerebellum</td>
<td>Unaffected Control, Atherosclerosis</td>
</tr>
<tr>
<td>6</td>
<td>79</td>
<td>F</td>
<td>C</td>
<td>MS (Clinical only), MBD, Seizure Disorder, COPD, Osteoporosis, Paraplegic, Hydrocephalus, GORD</td>
<td>Unaffected Control</td>
</tr>
<tr>
<td>7</td>
<td>82</td>
<td>M</td>
<td>D</td>
<td>PD, LBD, Depression, Dementia (probable), Atherosclerosis, Sleep Disorder, Hypothyroidism</td>
<td>PD, Atherosclerosis</td>
</tr>
<tr>
<td>8</td>
<td>68</td>
<td>M</td>
<td>D</td>
<td>AD, Dementia (probable), HTN, LBD (probable), Osteoarthritis</td>
<td>AD (probable), Cortical LBD</td>
</tr>
<tr>
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<td>71</td>
<td>M</td>
<td>D</td>
<td>Dementia (probable), Depression, HTN, Diabetes Type 2, Sleep Disorder, Alcohol Abuse</td>
<td>Dementia (probable), Cortical LBD</td>
</tr>
<tr>
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<td>D</td>
<td>AD, OBS, Dementia, Depression, LBD, Ataxia, TIA, ARF, CHF, CA (probable)</td>
<td>AD (probable), Atherosclerosis (probable)</td>
</tr>
<tr>
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<td>79</td>
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<td>D</td>
<td>AD, Cognitive Impairment, Depression, Dementia, COPD</td>
<td>AD, Dementia (probable)</td>
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<tr>
<td>12</td>
<td>75</td>
<td>F</td>
<td>D</td>
<td>Dementia (probable), PD, Vascular Dementia (probable), Hypothyroidism, Chronic LBD, Dementia, Depression, Psychosis, OCD, AD</td>
<td>Dementia (probable)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age at Death (years)</th>
<th>Gender</th>
<th>Control or Diseased</th>
<th>Clinical Diagnoses</th>
<th>Additional Neuropathological Diagnoses</th>
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<tbody>
<tr>
<td>1</td>
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<td>M</td>
<td>C</td>
<td>Unaffected Control</td>
</tr>
<tr>
<td>2</td>
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<tr>
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<td>M</td>
<td>C</td>
<td>Unaffected Control</td>
</tr>
<tr>
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<td>Unaffected Control</td>
</tr>
<tr>
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<td>C</td>
<td>Unaffected Control</td>
</tr>
<tr>
<td>6</td>
<td>82</td>
<td>F</td>
<td>C</td>
<td>Unaffected Control</td>
</tr>
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<td>F</td>
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<td>Unaffected Control</td>
</tr>
<tr>
<td>8</td>
<td>74</td>
<td>M</td>
<td>D</td>
<td>Dementia with Lewy Bodies</td>
</tr>
<tr>
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<td>M</td>
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<td>Dementia with Lewy Bodies</td>
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</tr>
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<td>11</td>
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<td>D</td>
<td>Dementia with Lewy Bodies</td>
</tr>
<tr>
<td>12</td>
<td>73</td>
<td>F</td>
<td>D</td>
<td>Dementia with Lewy Bodies</td>
</tr>
<tr>
<td>13</td>
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</tr>
<tr>
<td>14</td>
<td>83</td>
<td>F</td>
<td>D</td>
<td>Dementia with Lewy Bodies</td>
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</tbody>
</table>

Abbreviations: CHF=Congestive Heart Failure, CA=Cancer, MS-Multiple Sclerosis, MBD=Metachromatic Staphylococcus Aureus, COPD=Chronic Obstructive Pulmonary Disease, GORD=Gastroesophageal Reflux Disease, ARF=Acute Renal Failure, PD=Parkinson’s disease, LBD=Lewy Body Disease, AD=Alzheimer’s disease, HTN=Hypertension, OBI=Organic Brain Syndrome, TI=Transient Ischemic Attack, UTI=Urinary Tract Infection, OCD=Obsessive Compulsive Disorder
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