LOCALIZATION OF HEAT SHOCK PROTEINS
HSPA6 (HSP70B’), HSPA1A (HSP70-1) AND HSPA8 (HSC70)
IN CULTURED HUMAN NEURONAL CELLS

By
Catherine Deane

A thesis submitted in conformity with the requirements for the degree of

Doctor of Philosophy

Graduate Department of Cell & Systems Biology
University of Toronto

© Copyright by Catherine Deane (2019)
ABSTRACT

Localization of Heat Shock Proteins HSPA6 (Hsp70B’), HSPA1A (Hsp70-1) and HSPA8 (Hsc70) in Cultured Human Neuronal Cells

Catherine Deane

Doctor of Philosophy

Graduate Department of Cell and Systems Biology
University of Toronto

2019

Heat shock proteins (Hsps) are protein repair agents that detect and refold misfolded proteins and prevent their aggregation. Accumulation of protein aggregates is a key feature of neurodegenerative diseases. Treatments that showed beneficial effects in current animal models of neurodegenerative diseases have repeatedly failed in human clinical trials. A better understanding of the proteostasis machinery in human cells is required to design more effective therapeutic compounds. HSPA6 (Hsp70B’) is a stress-inducible member of the HSPA (Hsp70) multigene family that is absent in rat and mouse genomes and has been little studied compared to inducible HSPA1A (Hsp70-1), or constitutively expressed HSPA8 (Hsc70). HSPAs are involved in refolding proteins that misfold due to stress or disease. Binding to DNAJ (Hsp40) inhibits aggregation of misfolded proteins until they can be refolded by HSPA, but this machine cannot dissociate aggregated proteins. Recent studies have identified a disaggregate function performed by the mammalian HSPH (Hsp110) family in co-operation with HSPA/DNAJ in vitro. To advance knowledge of the mammalian disaggregation/refolding machine, this thesis investigates localization of HSPA6, HSPA1A, and HSPA8 with other components of the machine following stress in differentiated human neuronal SH-SY5Y cells. Unique targeting of HSPA6 to nuclear
perispeckles and differential localization with disaggregation/refolding machine components at the nucleolus compared to HSPA1A or HSPA8 is observed following thermal stress. siRNA knockdown demonstrates that HSPA6 is protective in the neuronal stress response. MG132 proteotoxicity induces HSPA6 which targets the periphery of cytoplasmic protein aggregates with components of the disaggregation/refolding machine and thus may be involved in the response of differentiated neuronal cells to protein aggregation. Constitutively expressed HSPA8 exhibits similar intracellular targeting as inducible HSPA1A and may act as a rapid stress responder in neuronal cells, circumventing the time lag required to upregulate HSPA1A. In cancer cells, elevated levels of Hsps contribute to their ability to resist cell death. The chemotherapeutic agent cisplatin induces components of the disaggregation/refolding machine. Knockdown of HSPA family members enhances the killing effect of cisplatin on human neuroblastoma cells.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr. Ian R. Brown for providing me with the opportunity to undertake PhD research in his laboratory and for the support and guidance he provided me throughout my time in the program. I am grateful for advice and encouragement in guiding my learning of theoretical and technical concepts and for strengthening my analytical skills. Guidance from Dr. Brown has also helped to significantly improve my writing and communication skills.

I would also like to express my appreciation for guidance and encouragement that I received from my supervisory committee members, Dr. Rongmin Zhao and Dr. Maurice Ringuette. Their continued support has provided me with confidence and has helped to expand my knowledge of scientific concepts. My deepest gratitude goes to former lab members Dr. Ari Chow, Hashwin Ganesh, and Dr. Sam Khalouei for training and guidance in many laboratory protocols and theoretical concepts, and for valuable advice and enjoyable experiences. My appreciation goes to former and current graduate (Sadek Shorbagi, Larrissa Becirovic, Aditi Aggarwal, and Paige O’Leary) and undergraduate lab members for their support, intellectual discussions and for making everyday life in the lab enjoyable. Thank you to the staff at the Centre for the Neurobiology of Stress (CNS), Bruno Chue and Bob Temkin, for their assistance with cell culture facilities and microscopy equipment, being an excellent source for troubleshooting and good conversation. My studies were supported by grants from NSERC Canada to Dr. Ian Brown.

This thesis is dedicated to my family and to my fiancé. A special thank you goes to my mother and father, Patricia and Gordon Deane, my twin brother Gordon, my sister Jessica, my nephews Rowan and Wilson, my fiancé Aaron Drew and his family for their unconditional love and support and for being a constant source of encouragement and relief at stressful times.
# TABLE OF CONTENTS

Abstract ......................................................................................................................... ii
Acknowledgements ........................................................................................................ iv
Table of Contents .......................................................................................................... v
List of Figures ................................................................................................................ xii
List of Tables ................................................................................................................ xv
List of Abbreviations ...................................................................................................... xvi
INTRODUCTION ......................................................................................................... 1

CHAPTER 1 Induction of heat shock proteins in differentiated human neuronal cells by co-application of celastrol and arimoclomol

1.1 Introduction ............................................................................................................. 8
1.2 Methods ................................................................................................................. 11
1.3 Results ................................................................................................................... 15
  1.3.1 Co-application of celastrol and arimoclomol enhances induction of a set of Hsps in differentiated human neuronal cells ........................................... 15
  1.3.2 Hsp induction peaks at 12 h after co-application of celastrol and arimoclomol ..... 18
  1.3.3 Comparison of celastrol vs classical heat shock to induce neuronal Hsps when paired with arimoclomol ................................................................. 18
  1.3.4 Neuronal cell viability is not adversely affected by co-application of celastrol and arimoclomol at concentrations that induce Hsps .............................. 23
1.4 Discussion ............................................................................................................. 27
  1.4.1 Rationale for co-application of celastrol and arimoclomol .............................. 27
1.4.2 Low dose co-application of celastrol and arimoclomol as a multi-target strategy to treat neurodegenerative protein misfolding disorders ................................. 28

1.4.3 Upregulation of Hsps could protect against synaptic loss and dysfunction that occur at early stages of neurodegenerative disease progression ......................... 30

CHAPTER 2 Components of a mammalian disaggregation/refolding machine are targeted to nuclear speckles following thermal stress in differentiated human neuronal cells

2.1 Introduction ........................................................................................................ 32

2.2 Methods ............................................................................................................ 35

2.3 Results .............................................................................................................. 37

2.3.1 HSPA (Hsp70) and DNAJ (Hsp40) components of a protein disaggregation/refolding machine co-localize at nuclear speckles following thermal stress in differentiated human neuronal SH-SY5Y cells ................... 37

2.3.2 The disaggregase HSPH1 (Hsp105α) co-localizes with HSPA1A at nuclear speckles after heat shock ................................................................. 42

2.3.3 The small heat shock protein HSPB1 (Hsp27) is targeted to stress-sensitive neuronal sites ................................................................. 42

2.3.4 Constitutively expressed HSPA8 (Hsc70) is also targeted to nuclear speckles with components of the disaggregation/refolding machine following heat shock ....... 47

2.4 Discussion ......................................................................................................... 50

2.4.1 Components of the mammalian protein disaggregation/refolding machine target nuclear speckles following heat shock in differentiated human neuronal cells ...... 50
2.4.2 The presence of class A and class B members of the DNAJ family enhances the efficiency of the mammalian protein disaggregation/refolding machine ............ 51

2.4.3 Localization of HSPH1, but not BAG-1, to nuclear speckles suggests splicing recovery may involve dissociation and refolding of thermally denatured proteins .. 52

2.4.4 The role of the small heat shock protein HSPB1 in protein disaggregation/refolding ............................................................. 53

2.4.5 Constitutively expressed HSPA8 may be a rapid responder to neuronal stress without the time lag required to upregulate inducible HSPA1A ..................... 53

CHAPTER 3 Differential targeting of HSPA6 (Hsp70B') and HSPA1A (Hsp70-1) with components of a protein disaggregation/refolding machine in differentiated human neuronal cells following thermal stress

3.1 Introduction .................................................................................. 55

3.2 Methods ....................................................................................... 57

3.3 Results .......................................................................................... 58

3.3.1 HSPA6 is targeted to sites at the periphery of nuclear speckles (perispeckles) after heat shock, which is not observed for the widely studied HSPA1A ............. 58

3.3.2 Differential association of HSPA6 and HSPA1A with nucleolar structures following thermal stress ................................................................. 63

3.3.3 Constitutive HSPA8 exhibits similar heat shock-induced targeting to neuronal nuclear sites as HSPA1A ................................................................. 66

3.4 Discussion ..................................................................................... 71

3.4.1 HSPA6 exhibits unique localization at nuclear perispeckles following thermal stress .................................................................................. 71
3.4.2 Association of HSPA6 and HSPA1A with the nucleolus .......................... 72
3.4.3 HSPA8 exhibits similar nuclear targeting as HSPA1A after heat shock .......... 72

CHAPTER 4 Intracellular targeting of Hsps in differentiated human neuronal cells following proteotoxic stress

4.1 Introduction .......................................................................................... 74
4.2 Methods ............................................................................................... 76
4.3 Results .................................................................................................... 78
  4.3.1 Induction of HSPA6 in differentiated human neuronal cells by MG132 ........ 78
  4.3.2 Proteotoxic effects of MG132 on differentiated human neuronal cells .......... 83
  4.3.3 Intracellular localization of MG132-induced HSPA6 in human neuronal cells ...... 83
  4.3.4 Targeting of HSPA6 and components of the disaggregation/refolding machine to
      the periphery of protein aggregates in the neuronal cytoplasm ...................... 88
  4.3.5 Constitutively expressed HSPA8 is targeted to the periphery of cytoplasmic
      protein aggregates after MG132 and translocates into the nucleus after subsequent
      heat shock .............................................................................................. 93
  4.3.6 Video representation of the localization of Hsps at the periphery of MG132-
      induced neuronal cytoplasmic protein aggregates ........................................ 98
4.4 Discussion ............................................................................................. 100
  4.4.1 Proteotoxic stress is a potent inducer of HSPA6 in differentiated human neuronal
      cells ........................................................................................................ 100
  4.4.2 HSPA6 targets cytoplasmic protein aggregates with components of the
      disaggregation/refolding machine ............................................................ 101
4.4.3 Constitutively expressed HSPA8 may be important for the neuronal response to multiple stressors ................................................................. 102

CHAPTER 5 Knockdown of heat shock proteins HSPA6 and HSPA1A sensitizes differentiated human neuronal cells to cellular stress

5.1 Introduction ............................................................................................................. 104
5.2 Methods .................................................................................................................. 106
5.3 Results .................................................................................................................... 109

5.3.1 Viability of differentiated human neuronal cells is not affected by knockdown of HSPA6 or HSPA1A ........................................................................... 109

5.3.2 HSPA6 knockdown does not affect the levels of other Hsps, while knockdown of HSPA1A causes upregulation of HSPA6 and downregulation of DNAJB1 .......... 109

5.3.3 HSPA6 and HSPA1A are required for cellular protection against thermal stress in differentiated human neuronal SH-SY5Y cells .............................................. 119

5.3.4 Effect of HSPA1A and HSPA6 knockdown on neuronal cell death after thermal stress ............................................................................................................. 122

5.4 Discussion ............................................................................................................. 127

5.4.1 Upregulation of Hsps by celastrol and arimoclomol enhances neuronal tolerance to thermal stress ............................................................................................................. 127

5.4.2 Effects of HSPA6 and HSPA1A knockdown ........................................................... 127

5.4.3 Different effects of HSPA6 and HSPA1A on neuronal cell death following thermal stress ............................................................................................................. 128
CHAPTER 6 Knockdown of HSPA family members enhances the killing effect of
the chemotherapeutic agent cisplatin on human neuroblastoma cells

6.1 Introduction ........................................................................................................ 130

6.2 Methods .................................................................................................................. 132

6.3 Results ..................................................................................................................... 134

6.3.1 Induction of Hsps by the chemotherapeutic agent cisplatin .......................... 134

6.3.2 siRNA knockdown of HSPA1A and HSPA8 protein levels ......................... 134

6.3.3 Knockdown of inducible HSPA1A or constitutively expressed HSPA8 enhances
the killing effect of cisplatin on human neuroblastoma cells .............................. 141

6.3.4 Cisplatin triggers cell death earlier in neuroblastoma cells compared to
differentiated human neuronal cells .................................................................. 144

6.3.5 Localization of Hsps in cisplatin-treated differentiated human neuronal cells .... 147

6.4 Discussion ............................................................................................................. 152

6.4.1 Hsps and cancer .................................................................................................. 152

6.4.2 Cisplatin induces components of a mammalian disaggregation/refolding machine . 152

6.4.3 Knockdown of inducible HSPA1A or constitutively expressed HSPA8 enhances
the killing effect of cisplatin on human neuroblastoma cells .............................. 153

6.4.4 Localization of cisplatin-induced Hsps to the distal ends of differentiated neuronal
processes ................................................................................................................ 155

7 CONCLUSION ...................................................................................................... 157

7.1 Overview ............................................................................................................... 157

7.1.1 HSPA6 (Hsp70B’) and the stress response of human neuronal cells ............. 161

7.1.2 Constitutively expressed HSPA8: a rapid responder to neuronal stress .......... 167
# LIST OF FIGURES

**Figure 1.** Publications stemming from the PhD thesis ............................................. 6

**Figure 2.** Enhanced induction of a set of Hsps by co-application of celastrol and arimoclomol to differentiated human neuronal cells ........................................... 16

**Figure 3.** Time-course of neuronal induction of Hsps after co-application of celastrol and arimoclomol ................................................................. 19

**Figure 4.** Neural induction of Hsps by co-application of celastrol and arimoclomol compared to classical heat shock paired with arimoclomol ................. 21

**Figure 5.** Effect of co-application of celastrol and arimoclomol on cellular viability and neuronal process morphology of differentiated human SH-SY5Y neuronal cells .......... 25

**Figure 6.** HSPA1A (Hsp70-1) and DNAJB1 (Hsp40-1) are targeted to nuclear speckles by heat shock in differentiated human neuronal cells ........................................... 38

**Figure 7.** The class A protein, DNAJA1 (Hsp40-4), also localizes to nuclear speckles after heat shock ................................................................. 40

**Figure 8.** Targeting of the disaggregase HSPH1 (Hsp105α) to nuclear speckles following thermal stress ........................................................................... 43

**Figure 9.** The small heat shock protein HSPB1 (Hsp27) is targeted by thermal stress to nuclear and cytoplasmic sites in differentiated human neuronal cells ......................... 45

**Figure 10.** Constitutively expressed HSPA8 (Hsc70) targets nuclear speckles following thermal stress and co-localizes with members of the mammalian disaggregation/refolding machine ................................................................. 48

**Figure 11.** HSPA6 (Hsp70B’) targets perispeckles, located at the periphera of nuclear speckles, which is not observed for the widely studied HSPA1A ......................... 59

**Figure 12.** Components of the protein disaggregation/refolding machine do not target HSPA6-positive perispeckles after heat shock in differentiated human neuronal cells ...... 61

**Figure 13.** HSPA1A, but not HSPA6, is targeted to the granular component (GC) layer of the nucleolus at 1 h following heat shock ................................................................. 64

**Figure 14.** At 3 h after thermal stress, HSPA6 co-localizes with nucleophosmin, a marker of the GC layer of the nucleolus ........................................................................ 67

**Figure 15.** Constitutively expressed HSPA8 exhibits similar heat shock-induced targeting as HSPA1A ........................................................................ 69
Figure 16. Induction of HSPA6 and components of the protein disaggregation/refolding machine by proteotoxic stress ................................................................. 79

Figure 17. Effect of MG132 concentration on neuronal induction of HSPA6 .......... 81

Figure 18. Elevation of neuronal poly-ubiquitinated proteins and protein aggregation following proteotoxic stress ............................................................. 84

Figure 19. MG132-induced HSPA6 localizes to the neuronal cytoplasm ............ 86

Figure 20. Targeting of MG132-induced HSPA6 to the periphery of cytoplasmic protein aggregates in differentiated human neuronal cells .......................... 89

Figure 21. Localization of components of the protein disaggregation/refolding machine to the periphery of neuronal cytoplasmic protein aggregates ................ 91

Figure 22. Constitutively expressed HSPA8 targets the periphery of cytoplasmic protein aggregates after MG132 ................................................................. 94

Figure 23. HSPA8 translocates to nuclear structures after thermal stress in MG132-treated human neuronal cells ............................................................... 96

Figure 24. Viability of differentiated human neuronal cells is not affected by HSPA6 and HSPA1A siRNAs ............................................................ 110

Figure 25. siRNA knockdown of HSPA6 in differentiated human neuronal cells .... 112

Figure 26. Effect of HSPA1A siRNA on expression of neuronal Hsps .............. 115

Figure 27. Comparison of the knockdown effects of HSPA6 siRNA vs HSPA1A siRNA on differentiated human neuronal cells ............................................. 117

Figure 28. siRNA knockdown of HSPA6 and HSPA1A sensitizes differentiated human neuronal cells to thermal stress ......................................................... 120

Figure 29. Double knockdown of HSPA1A and HSPA6 ............................... 123

Figure 30. Effect of HSPA6 and HSPA1A knockdown on neuronal cell death after thermal stress ................................................................. 125

Figure 31. Induction of Hsps by cisplatin in human SH-SY5Y neuroblastoma cells ...... 135

Figure 32. Time course of Hsp induction following cisplatin ............................ 137

Figure 33. siRNA knockdown of stress-inducible HSPA1A and constitutively expressed HSPA8 .................................................................................. 139

Figure 34. Enhanced killing effect of cisplatin on human neuroblastoma cells following siRNA knockdown of inducible HSPA1A or constitutively expressed HSPA8 .......... 142
Figure 35. Time course of the effect of cisplatin on cell death in neuroblastoma and differentiated neuronal cells ................................................................. 145

Figure 36. Localization of Hsps in cisplatin-treated differentiated human neuronal cells . 148

Figure 37. Hsp localization in the distal ends of differentiated neuronal cellular processes after cisplatin ................................................................. 150

Figure 38. Schematic of sequential Hsp targeting to nuclear structures and transient co-localization of components of the disaggregation/refolding machine during heat shock recovery in differentiated human neuronal cells .................................................. 162

Supplementary Figure 1 Video representation of HSPA6 (Hsp70B‘) surrounding cytoplasmic protein aggregates after MG132 treatment of differentiated human SH-SY5Y neuronal cells ................................................................. 191

Supplementary Figure 2 Rotational view of HSPA6 surrounding neuronal cytoplasmic protein aggregates ........................................................................... 191

Supplementary Figure 3 Rotational view of HSPA1A (Hsp70-1) localization around the periphery of MG132-induced neuronal cytoplasmic protein aggregates ....................... 191

Supplementary Figure 4 Video presentation of constitutively expressed HSPA8 (Hsc70) localized at the periphery of cytoplasmic protein aggregates following MG132 treatment . 192

Supplementary Figure 5 Video representation of DNAJB1 (Hsp40-1) at the periphery of cytoplasmic protein aggregates ........................................................................... 192

Supplementary Figure 6 Rotational video of HSPB1 (Hsp27) surrounding neuronal cytoplasmic protein aggregates after MG132 treatment .................................................. 193

Supplementary Figure 7 Video representation of HSPH1 (Hsp105α) localized to both the periphery and the core of proteostat-positive protein aggregates after MG132 treatment ........................................................................... 193
LIST OF TABLES

Table 1. List of siRNA target sequences ................................................................. 194
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Arim</td>
<td>Arimoclomol</td>
</tr>
<tr>
<td>BAG-1</td>
<td>Bcl2 associated atahanogene 1</td>
</tr>
<tr>
<td>Cel</td>
<td>Celastrol</td>
</tr>
<tr>
<td>CIS</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNAJ</td>
<td>Hsp40 family</td>
</tr>
<tr>
<td>DNAJA1</td>
<td>DNAJ (Hsp40) heat shock protein subfamily A member 1, Hsp40-4</td>
</tr>
<tr>
<td>DNAJB1</td>
<td>DNAJ (Hsp40) heat shock protein subfamily B member 1, Hsp40-1</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GC</td>
<td>Granular component</td>
</tr>
<tr>
<td>HS</td>
<td>Heat shock</td>
</tr>
<tr>
<td>HSE</td>
<td>Heat shock element</td>
</tr>
<tr>
<td>HSF1</td>
<td>Heat shock transcription factor 1</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSPA</td>
<td>Heat shock protein 70 kDa (Hsp70) family</td>
</tr>
<tr>
<td>HSPA1A</td>
<td>Heat shock protein 70 kDa family member 1A, Hsp70-1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>HSPA6</td>
<td>Heat shock protein 70 kDa family member 6, Hsp70B'</td>
</tr>
<tr>
<td>HSPA8</td>
<td>Heat shock protein 70 kDa family member 8, Hsc70</td>
</tr>
<tr>
<td>HSPB</td>
<td>Small heat shock protein family</td>
</tr>
<tr>
<td>HSPB1</td>
<td>Heat shock protein family B member 1, Hsp27</td>
</tr>
<tr>
<td>HSPC</td>
<td>Heat shock protein 90 kDa (Hsp90) family</td>
</tr>
<tr>
<td>HSPH</td>
<td>Heat shock protein 110 kDa (Hsp110) family</td>
</tr>
<tr>
<td>HSPH1</td>
<td>Heat shock protein 110 kDa family member 1, Hsp105α</td>
</tr>
<tr>
<td>HSR</td>
<td>Heat shock response</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase 1, Hsp32</td>
</tr>
<tr>
<td>KD</td>
<td>Knockdown</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>NB</td>
<td>Neuroblastoma</td>
</tr>
<tr>
<td>NEF</td>
<td>Nucleotide exchange factor</td>
</tr>
<tr>
<td>NPM</td>
<td>Nucleophosmin</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SC35</td>
<td>Splicing component 35 kDa</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sHsp</td>
<td>Small heat shock protein</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SOD1&lt;sup&gt;G93A&lt;/sup&gt;</td>
<td>Mutant superoxide dismutase 1 (glycine 93 mutated to alanine)</td>
</tr>
<tr>
<td>SON</td>
<td>Nuclear speckle marker protein</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
INTRODUCTION

Heat shock proteins (Hsps) are protein repair agents that can detect and repair misfolded proteins and prevent their aggregation (Muchowski and Wacker 2005; Asea and Brown 2008; Paul and Mahanta 2014). Correct folding of proteins is essential for proper protein function (Sweeney et al. 2017; Scannevin 2018). Misfolded and damaged proteins accumulate in the cell during stress conditions resulting in the upregulation of Hsps that help to restore proteostasis to the cell (Morimoto 1993; Goloubinoff and De Los Rios 2007; Asea and Brown 2008; Morimoto 2008). The accumulation of protein aggregates is a key feature of neurodegenerative diseases that have been characterized as ‘protein misfolding disorders’ (Mattson and Magnus 2006; Asea and Brown 2008). Early aggregates are linked to neuronal toxicity by impeding a number of important biological processes in neurons including microtubule formation and intracellular trafficking, membrane stability and endocytosis, and mitochondrial function (Scannevin 2018). Hence upregulation of Hsps has been proposed as a therapeutic strategy to combat protein misfolding and aggregation in neurodegenerative diseases (Westerheide and Morimoto 2005; Sweeney et al. 2017; Scannevin 2018).

The HSPA (Hsp70) family is a multi-gene family that has been highly conserved throughout evolution and maintains proteins in a correctly folded, functional state (Noonan et al. 2007a; Noonan et al. 2007b; Morimoto 2008). DNAJ (Hsp40) family members have been recognized for many years as important co-chaperones of HSPA that facilitate refolding by delivering misfolded proteins and stimulating the ATPase activity of HSPA (Kampinga and Craig 2010; Mayer 2013; Mattoo and Goloubinoff 2014). Misfolded proteins are prone to aggregation due to exposed hydrophobic regions on the protein exterior that preferentially associate with hydrophobic regions on other misfolded proteins (Fink 1998; Munch and
Bertolotti 2010). Binding to DNAJs, also referred to as holdases, prevents aggregation of misfolded proteins until they can be refolded by HSPA, the foldase. However, this machine is not capable of dissociating proteins that have already formed into aggregates (Gao et al. 2015; Nillegoda and Bukau 2015).

Bacteria, fungi, plants, and yeast cells express a well-characterized protein disaggregate (Hsp104 in yeast cells and ClpB in *Escherichia coli*) that can solubilize aggregated proteins, homologs of which are lacking in mammalian cells (Glover and Lindquist 1998; Weibezahn et al. 2005; Bosl et al. 2006; Tyedmers et al. 2010; Nillegoda and Bukau 2015). Many therapeutic strategies under investigation are aimed at reducing aggregate accumulation in the brains of patients with neurodegenerative disease, and thereby slow disease progression, but targeting cellular mechanisms for the clearance of neuronal aggregates *in vivo* has remained a challenge (Westerheide and Morimoto 2005; Sweeney et al. 2017; Scannevin 2018). However, recent evidence has revealed that a disaggregate activity is performed by the mammalian HSPH (Hsp110) family in a multi-protein machine that includes HSPA and DNAJ family members (Rampelt et al. 2012; Gao et al. 2015; Nillegoda and Bukau 2015; Nillegoda et al. 2015). The Bukau laboratory in Germany has demonstrated that this mammalian protein disaggregation/refolding machine can restore biological activity to aggregated proteins *in vitro*, including α-synuclein fibrils that are associated with Parkinson’s disease (Gao et al. 2015).

The discovery of the HSPA-DNAJ-HSPH protein disaggregation/refolding machine has been a major advance towards improving our understanding of protein homeostasis in mammalian cells (Rampelt et al. 2012; Gao et al. 2015; Nillegoda and Bukau 2015; Nillegoda et al. 2015; Kirstein et al. 2017). However, the key studies carried out by the Bukau laboratory have been performed in cell-free systems in which purified machine components are mixed and thus
does not reveal where machine components localize in living cells. The identification of intracellular sites where HSPA, DNAJ, and HSPH localize could provide insight into the molecular mechanisms that require repair by the disaggregation/refolding machine following cellular stress. This thesis will advance knowledge of the mammalian protein disaggregation/refolding machine by investigating the targeting of machine components to stress-sensitive sites by immunofluorescence in human neuronal SH-SY5Y cells.

SH-SY5Y is an established human neuroblastoma cell line derived from SK-N-SH cells that originated from a neuroblastoma patient in 1970 (Ross et al. 1983; Ross and Spengler 2007; Kovalevich and Langford 2013). Since neurodegenerative diseases including Alzheimer’s disease (AD), Parkinson’s disease (PD), and amyotrophic lateral sclerosis (ALS), affect differentiated neurons of the adult central nervous system (Muchowski and Wacker 2005; Asea and Brown 2008), SH-SY5Y cells are differentiated by treatment with all-trans-retinoic acid, which results in inhibition of cell division and the growth of neuronal cellular processes (Jacobs et al. 2006; Ross and Spengler 2007; Cheung et al. 2009). Retinoic acid is required in vivo for adult neurogenesis in the rat brain (Jacobs et al. 2006).

The thesis will focus on the association of different members of the HSPA family with components of the disaggregation/refolding machine, specifically the widely studied, stress-inducible member, HSPA1A (Hsp70-1), and also HSPA6 (Hsp70B’), an inducible member that has been little studied in the literature (Chow and Brown 2007; Noonan et al. 2007a; Noonan et al. 2007b; Chow et al. 2010). Unlike HSPA1A that is expressed at low basal levels and strongly upregulated by stress, HSPA6 is not detected in unstressed cells and is strictly inducible (Noonan et al. 2007a). HSPA6 is present in the human genome but is not found in rat or mouse genomes and hence is missing in current animal models of neurodegenerative diseases (Noonan et al.
Potential therapeutic compounds that have been identified in rat and mouse models for the treatment of neurodegenerative diseases have failed to translate into effective therapies in human clinical trials suggesting a deficiency in current animal models (Lang 2010; t Hart et al. 2012; McGonigle and Ruggeri 2014; Sasaki 2015). A better understanding of the Hsp machinery in human cells is required to design more effective therapeutics for neurodegenerative diseases. This thesis also includes investigation of constitutively expressed HSPA8 (Hsc70) that is ubiquitously expressed and performs a variety of housekeeping functions that are essential to maintain proteostasis in the cell under normal conditions (Bukau et al. 2006; Kampinga and Craig 2010; Mayer 2013; Stricher et al. 2013). Interestingly, the Bukau laboratory has recently demonstrated that the disaggregation/refolding machine acts more efficiently with HSPA8 compared to HSPA1A in in vitro assays (Kirstein et al. 2017). Furthermore, our laboratory has reported that HSPA8 is expressed at high levels in neurons relative to other cell types and may pre-protect neurons from cellular stress (Manzerra et al. 1997; Chen and Brown 2007a).

The research goals of this thesis are as follows. Upregulation of Hsps has been proposed as a therapeutic strategy to treat neurodegenerative diseases which have been characterized as protein misfolding disorders. Since Hsps act co-operatively, upregulation of a set of Hsps is a more promising strategy than manipulation of individual Hsps. Chapter 1 develops a protocol to upregulate a set of Hsps, including HSPA6, in differentiated human neuronal cells by treatment with low dose compounds that do not have adverse effects on cell viability or neuronal processes. This protocol is employed in Chapter 2 and 3 to investigate the targeting of HSPA6, HSPA1A, and HSPA8 to intracellular sites of stress in differentiated human neuronal cells by immunofluorescence and co-localization with other components of the mammalian protein disaggregation/refolding machine. Chapter 4 explores HSPA6 expression and localization
compared to HSPA1A and HSPA8 following treatment with the proteasome inhibitor MG132 which induces the accumulation of protein aggregates that are a key feature of neurodegenerative diseases. In Chapter 5, knockdown of inducible HSPA6 and HSPA1A protein expression by small interfering RNA (siRNA) technology is employed to investigate effects on neuronal stress tolerance. In contrast to neurodegenerative diseases in which Hsps are overwhelmed by proteostasis imbalance in differentiated neurons, cancer cells express elevated levels of Hsps that allow them to evade cell death and are required to maintain the cancer phenotype (Calderwood and Ciocca 2008; Ciocca et al. 2013; Chatterjee and Burns 2017). Knockdown of Hsps has been proposed to enhance the sensitivity of cancer cells to chemotherapeutic drugs (Ruggiero et al. 2013; Dasari and Tchounwou 2014). Chapter 6 explores the effect of knockdown of HSPA family members on the killing effect of the chemotherapeutic agent cisplatin that is employed to treat a variety of cancers including neuroblastoma (Ruggiero et al. 2013; Dasari and Tchounwou 2014).

Sections of this PhD thesis have been published as indicated in Figure 1.
Figure 1. Publications stemming from the PhD thesis

References #1-5 have been published and reference #6 was presented at the Society for Neuroscience (SfN) conference in San Diego on November 3-7th 2018. At the end of the Introduction section to each thesis chapter, the relevant publication is cited.
Sections of my PhD studies have been published as follows:


CHAPTER 1

Induction of heat shock proteins in differentiated human neuronal cells by co-application of celastrol and arimoclomol

1.1 Introduction

As average life expectancy increases worldwide, an elevation in the prevalence of neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease has been observed (Martin 1999; Chen and Brown 2007b; Lang 2010; Dunkel et al. 2012). There is increasing urgency for the identification of disease-modifying agents to treat these diseases that are multifactorial in nature (Cavalli et al. 2008; Dunkel et al. 2012; Huang and Mucke 2012; Sheikh et al. 2013). Targeted inhibition of a single disease pathology may be compensated by concurrent deleterious pathways. Few therapies exist for neurodegenerative diseases as compounds that showed beneficial effects in rat and mouse animal models have failed to translate into effective therapies in human clinical trials (Lang 2010; t Hart et al. 2012; McGonigle and Ruggeri 2014; Sasaki 2015). This suggests a deficiency in current animal models that may not encompass all aspects of human neurological diseases. Additionally, there is an urgent need for treatment strategies that impact early stages of disease progression, such as synaptic dysfunction (DeKosky and Marek 2003; Stephan et al. 2012; Chung et al. 2015).

Neurodegenerative diseases are characterized as “protein misfolding disorders” because a common underlying pathology is the accumulation of misfolded, aggregation-prone proteins that disrupt normal cell function leading to cell death (Muchowski and Wacker 2005; Westerheide and Morimoto 2005; Asea and Brown 2008; Richter et al. 2010). The upregulation of Hsps has been proposed as a therapeutic strategy to combat protein misfolding and aggregation in neurodegenerative diseases (Asea and Brown 2008; Pratt et al. 2015). Activation of the heat

Activities of Hsps, such as protein refolding (Fan et al. 2003; Goloubinoff and De Los Rios 2007; Mayer 2013; Mattoo and Goloubinoff 2014; Dekker et al. 2015) and disruption of protein aggregates (Gao et al. 2015; Nillegoda and Bukau 2015), require co-operation between several different classes of Hsps. Therefore, upregulation of a set of Hsps by activation of HSF1 is more effective than genetic manipulation of individual Hsps (Batulan et al. 2003; Liu et al. 2005; Asea and Brown 2008). Celastrol is an HSF1 activator that induces Hsps in neuronal cells (Chow and Brown 2007) and is neuroprotective in a number of animal models of neurodegenerative disease including amyotrophic lateral sclerosis (ALS) (Kiaei et al. 2005), Parkinson’s disease (PD) (Cleren et al. 2005), polyglutamine expansion disease (Zhang and Sarge 2007), and Alzheimer’s disease (AD) (Paris et al. 2010). Celastrol also exhibits potent anti-inflammatory and antioxidant properties (Allison et al. 2001; Jung et al. 2007; Faust et al. 2009; Kim et al. 2009; Venkatesha et al. 2012; Wong et al. 2012; Yang et al. 2014; Sharma et al. 2015) that may provide additional benefits to neurodegenerative disease patients as inflammation and oxidative stress are associated with disease pathology and are thought to exacerbate disease progression (Gao and Hong 2008; Amor et al. 2010; Amor et al. 2014).
Another compound that modulates the heat shock response is arimoclomol, which has been shown to improve motor performance and extend lifespan in SOD1\textsuperscript{G93A} mice, a transgenic animal model of ALS (Kieran et al. 2004; Goloubinoff and De Los Rios 2007; Kalmar and Greensmith 2009; McGoldrick et al. 2013; Poppe et al. 2014). Arimoclomol is a co-inducer of Hsps that prolongs the binding of activated HSF1 to HSEs in the promoter regions of heat shock genes (Hargitai et al. 2003; Kieran et al. 2004; Kalmar and Greensmith 2009). Interestingly, arimoclomol is currently in human clinical trials for ALS (ClinicalTrials.gov identifier: NCT00706147 and NCT03491462) and is well tolerated in ALS patients up to 200 mg/day three times daily (Genc and Ozdinler 2013; Benatar et al. 2018).

The objective of Chapter 1 is to investigate Hsp expression and cell viability in differentiated human neuronal SH-SY5Y cells following co-application of celastrol and arimoclomol. Hsp upregulation by this method is also compared to classical heat shock treatment. Co-application of celastrol and arimoclomol potentiated Hsp induction compared to either compound individually and thereby permits the use of lower effective dosages. Furthermore, the potent anti-inflammatory and antioxidant activities of celastrol could help to combat inflammation and oxidative stress that are associated with neurodegenerative disease pathology, making this a multi-target strategy.

This chapter has been published in *Cell Stress and Chaperones* (2016) as “Induction of heat shock proteins in differentiated human neuronal cells following co-application of celastrol and arimoclomol” by Deane CA and Brown IR. Volume 21, Issue 5, pages 837-848.
1.2 Methods

1.2.1 Maintenance and differentiation of the SH-SY5Y cell line

Human SH-SY5Y neuroblastoma cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Wisent, QC, Canada) with 10% fetal bovine serum (FBS; Wisent) at 37°C in a humidified, 5% CO₂ atmosphere. Cells plated at 4.5 x 10⁴ cells per cm² were allowed to settle onto the growth surface for 24 h prior to experimentation. Cells were grown on 10 cm culture plates for Western blotting experiments or 22 × 22-mm coverslips placed inside six-well plates for immunofluorescence experiments. To generate differentiated SH-SY5Y neuronal cells, media was replaced with serum-free DMEM containing 10 μM all-trans-retinoic acid (R2625; Sigma Aldrich, St. Louis, MO, USA) for 72 h to inhibit cell division and induce extension of neuronal processes (Jacobs et al. 2006; Cheung et al. 2009).

1.2.2 Induction of heat shock proteins

Following 72 h of differentiation, media was replaced with fresh serum-free DMEM with or without celastrol and arimocromol. Celastrol (70950; Cayman Chemical, Ann Arbor, MI, USA) dissolved in DMSO was added directly to the media. DMSO treatment alone was used as a vehicle control for celastrol. Arimocromol (gift from Professor Michael Cheetham, Institute of Ophthalmology, University College London, UK) was prepared fresh for each experiment by dissolving in an appropriate volume of serum-free DMEM and filtering (0.2 μm pore size). The cells were incubated at the indicated concentrations for 24 h unless otherwise specified.
1.2.3 Heat shock treatment

Cells were immersed in a circulating water bath calibrated at 43°C ± 0.2°C for 20 min and returned to a humidified 5% CO₂ atmosphere at 37°C for recovery. The start of heat shock represents the zero time-point (t = 0). To evaluate Hsp induction by heat shock coupled with arimoclomol, arimoclomol was added concurrently with (HS1; t = 0) or subsequent to (HS2; t = 20 min) heat shock.

1.2.4 Western blotting

Cells were harvested, dissolved in Laemmli buffer and boiled for 10 min. Protein quantification was carried out using the RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. Thirty micrograms of protein were loaded into each lane of a 12% SDS-PAGE gel and separated using the Mini-PROTEAN 3 Electrophoresis Module (Bio-Rad Laboratories) with a 4% stacking gel. Proteins were transferred to nitrocellulose membranes using the Mini Trans-Blot® Module (Bio-Rad Laboratories). Primary antibodies for HSPA1A (SPA-810), HSPA6 (SPA-754), HSPA8 (SPA-815), HSPH1 (SPA-1101), HSPC1 (SPS-771), DNAJB1 (SPA-400), HSPB1 (SPA-803), and HO-1 (Hsp32; OSA-110) were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Primary antibody for β-tubulin (MAB3408) was purchased from EMD Millipore (Billerica, MA, USA). Horseradish peroxidase-conjugated secondary antibodies (Sigma Aldrich, St. Louis, MO, USA) were detected using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA). Densitometry was performed using Quantity One® 1-D Analysis software (Bio-Rad) and
normalized to the β-tubulin control. Data are presented as mean density relative to control ± standard error of the mean (SEM) for a minimum of three independent blots.

1.2.5 Viability determination

For qualitative analysis of cell viability by fluorescence microscopy following co-application of celastrol and arimoclomol, cells were stained with 10 µg/mL propidium iodide (P4864; Sigma Aldrich) to identify compromised cells and then fixed with 4% paraformaldehyde (PFA) for 30 min. PFA was purchased from Canemco Inc. (0173; Conton de Gore, QC, CA). Hoechst 33258 (94403; Sigma Aldrich) at 0.5 µg/mL was used as a counterstain for nuclei.

For analysis of neuronal process morphology, cells were fixed as above and permeabilized in 0.1% Triton X-100 with 100 mM glycine in PBS. The cells were blocked for 1 h in 5% FBS in PBS and incubated with primary antibody against α-tubulin (ab18251; Abcam, Toronto, ON, CA) in 1% FBS in PBS overnight at 4°C. Donkey anti-rabbit AlexaFluor488-conjugated secondary antibody (Molec. Probes, Life Tech., Burlington, ON, CA) was used. DNA was stained with 300 nM DAPI (Invitrogen, Life Technologies). Cells were imaged by structured illumination microscopy using an AxioCam HRm camera with an ApoTome module on an AxioVert 200 M microscope (Carl Zeiss, Toronto, ON, CA).

For quantitative analysis of cell viability, an equal volume of cell suspension was mixed with 0.4% trypan blue (T10282; Life Technologies) and incubated for 2 min. The cell suspension was loaded into a disposable Countess® cell counting chamber slide and the percent of cells stained by trypan blue were quantified using a Countess® automated cell counter (C10281; Invitrogen, Life Technologies). Data are presented as percent viable cells ± SEM relative to the control condition for a minimum of three independent replicates.
1.2.6 Statistical analysis

Two-way analysis of variance (ANOVA) followed by Bonferroni’s test for multiple comparisons was used to test for statistical significance of Western blot densitometry and viability data using Graphpad Prism 5 software (La Jolla, CA, USA). A $p$-value of less than 0.05 (or 0.01 where indicated) was considered statistically significant. Data represent the mean ± SEM for three independent replicates. For Hsp protein levels, optical densities were normalized and plotted as the difference relative to the vehicle control condition.
1.3 Results

1.3.1 Co-application of celastrol and arimoclomol enhances induction of a set of Hsps in differentiated human neuronal cells

Differentiated human SH-SY5Y neuronal cells were treated with celastrol, plus or minus arimoclomol. As shown in Figure 2a, inclusion of arimoclomol with celastrol enhanced the induction of several Hsps, compared to celastrol alone. This included the little studied HSPA6 (Hsp70B′) that is found in the human genome but not in the genomes of rat and mouse, and hence is lacking in current animal models of neurodegenerative diseases (Chow and Brown 2007; Noonan et al. 2007a; Noonan et al. 2007b; Chow et al. 2010). In addition to HSPA6, enhanced induction was also observed following co-application of celastrol and arimoclomol for HSPA1A (Hsp70-1), DNAJB1 (Hsp40-1), HO-1 (Hsp32), and HSPB1 (Hsp27). Induction of HSPA1A and HSPB1 was detected at 0.1 µM celastrol plus arimoclomol, whereas induction of HSPA6 was observed at 0.3 µM celastrol plus arimoclomol. Induction was not observed for constitutively expressed HSPH1 (Hsp105α), HSPC1 (Hsp90α) or HSPA8 (Hsc70). As shown in Figure 2b, statistically significant enhanced induction of HSPA6, HSPA1A, HSPB1, DNAJB1, and HO-1 was observed following co-application of 0.3 µM celastrol and 250 µM arimoclomol, compared to either celastrol or arimoclomol alone.

To determine whether enhanced induction of Hsps could be achieved using a lower concentration of arimoclomol, differentiated human neuronal cells were co-treated with 0.3 µM celastrol plus or minus arimoclomol at concentrations ranging from 50 to 250 µM. As shown in Figure 2c and 2d, levels of induction of HSPA6, HSPA1A, DNAJB1, HO-1, and HSPB1 were
Figure 2. Enhanced induction of a set of Hsps by co-application of celastrol and arimoclomol to differentiated human neuronal cells

a Differentiated SH-SY5Y neuronal cells were treated with celastrol (0.1 - 0.3 µM) with or without arimoclomol (250 µM). Cells were harvested after 24 h and Hsps examined by Western blotting. β-tubulin was used as a loading control b Change in Hsp band intensity relative to the vehicle control for co-application of 0.3 µM celastrol plus 250 µM arimoclomol (Cel + Arim), 0.3 µM celastrol (Cel), and 250 µM arimoclomol (Arim). Induction (# = p<0.01) of HSPA6, HSPA1A, HO-1, HSPB1, and DNAJB1 was seen following application of celastrol alone or in combination with arimoclomol. Significant enhanced induction (* = p<0.05; ** = p<0.01) was observed following co-application of celastrol and arimoclomol, compared to celastrol alone. Levels of constitutively expressed HSPH1, HSPC1, and HSPA8 did not change with celastrol and arimoclomol application alone or in combination c Hsp induction profile following co-application of celastrol (0.3 µM) and arimoclomol (50, 100, 150, 200, or 250 µM). d Comparable levels of Hsp induction were observed when arimoclomol concentration was reduced from 250 to 50 µM for HSPA6, HSPA1A, DNAJB1, HO-1, HSPB1.
maintained when the arimoclomol concentration was lowered five-fold from 250 to 50 µM. Levels of constitutively expressed HSPH1, HSPC1, and HSPA8 were not affected.

1.3.2 Hsp induction peaks at 12 h after co-application of celastrol and arimoclomol

Inducible members of the HSPA family, HSPA6 and HSPA1A, demonstrated maximal levels at 12 h after co-application of 0.3 µM celastrol with 50 µM arimoclomol. As shown quantitatively in Figure 3, HSPA6 levels declined at 18 h whereas HSPA1A levels were maintained. HO-1 attained maximal levels at 12 to 24 h. HSPB1 increased progressively from 6 to 24 h while DNAJB1 reached maximal levels at 10 h. Levels of constitutively expressed HSPH1, HSPC1, and HSPA8 did not show significant change.

1.3.3 Comparison of celastrol vs classical heat shock to induce neuronal Hsps when paired with arimoclomol

Celastrol activates HSF1 monomers to a trimerized form that binds to HSEs in the promoter regions of inducible heat shock genes resulting in their upregulation (Westerheide et al. 2004; Salminen et al. 2010). Heat shock is the classical activator of HSF1, the master regulator of Hsp induction (Morimoto 1993; Sarge et al. 1993). Celastrol and classical heat shock were compared in their ability to induce Hsps in differentiated human neuronal cells when coupled with arimoclomol as co-inducer. As shown quantitatively in Figure 4, co-application of celastrol and arimoclomol at 0.3 µM and 50 µM respectively, resulted in an enhanced induction of HSPA6 compared to celastrol alone. However, classical heat shock applied concurrently with
Figure 3. Time-course of neuronal induction of Hsps after co-application of celastrol and arimoclomol

a Change in Hsp levels over time following co-application of celastrol (0.3 µM) and arimoclomol (50 µM) to differentiated human SH-SY5Y neuronal cells was examined by Western blot. Quantification of the time-course change in Hsp band intensity (* = p<0.05) relative to the vehicle control. Peak induction of HSPA6, HSPA1A, DNAJB1, and HO-1 was observed at 10-12 h. Levels of constitutively expressed HSPH1, HSPC1, and HSPA8 did not change.
Figure 4. Neural induction of Hsps by co-application of celastrol and arimoclomol compared to classical heat shock paired with arimoclomol

a Hsp induction profile following co-application of celastrol (0.3 µM) and arimoclomol (50 µM), or 43°C heat shock for 20 min (HS) plus or minus arimoclomol (50 µM). Arimoclomol was added either concurrently (HS1) or subsequent to (HS2) heat shock. b Quantification of the change in band intensity (* = p<0.01) relative to control. Higher levels (# = p<0.05) of HSPA6, HSPA1A, HO-1, HSPB1, and DNAJB1 were observed following co-application of celastrol and arimoclomol compared to classical heat shock plus arimoclomol.
arimoclomol (HS1), or immediately preceding arimoclomol (HS2), resulted in no detectable induction of HSPA6. Similarly, co-application of celastrol and arimoclomol resulted in a greater induction of HSPA1A, HO-1, HSPB1, and DNAJB1 compared to heat shock paired with arimoclomol. Hence celastrol co-applied with arimoclomol is a more efficient strategy for Hsp upregulation in differentiated human neuronal cells compared to classical heat shock paired with arimoclomol. Upregulation was not observed of constitutively expressed HSPH1, HSPC1 or HSPA8 following celastrol plus arimoclomol or heat shock plus arimoclomol.

1.3.4 Neuronal cell viability is not adversely affected by co-application of celastrol and arimoclomol at concentrations that induce Hsps

Viability assays were conducted to determine the effect of co-application of celastrol and arimoclomol on differentiated human neuronal cells at dosages that resulted in enhanced Hsp induction. Propidium iodide (PI) dye exclusion microscopy showed that neuronal cell viability was observed following co-application of celastrol and arimoclomol at 0.3 µM and 50 µM respectively, but severely impacted if arimoclomol was increased to 250 µM in combination with 0.3 µM celastrol (Figure 5a, cells with PI uptake indicated in pink). Arimoclomol alone at 250 µM or increasing celastrol to 1.5 µM (Figure 5c) resulted in cellular uptake of PI, whereas comparatively little PI uptake was observed for arimoclomol or celastrol alone at 50 µM and 0.3 µM respectively (Figure 5a). As shown in Figure 5d, quantification employing cellular uptake of trypan blue revealed that 84.2% ±3.8 cell viability is maintained following application of celastrol and arimoclomol at the optimized concentrations (0.3 µM and 50 µM, respectively), alone or in combination. Cell viability decreased slightly as celastrol concentration was increased from 0.3 µM to 0.5 µM with a major decrease at 1.5 µM celastrol and at 250 µM arimoclomol.
An important marker of neuronal stress is the integrity of neuronal processes which retract in challenged cells (Kroemer et al. 2009). As shown in Figure 5b, extensive neuronal process morphology (visualized by \(\alpha\)-tubulin staining) was apparent at 0.3 \(\mu\)M celastrol and 50 \(\mu\)M arimoclomol, alone or in combination, but severely impacted when arimoclomol was increased to 250 \(\mu\)M alone or in combination with celastrol. Elevation of celastrol to 1.5 \(\mu\)M resulted in the loss of neuronal process morphology (Figure 5c).
Figure 5. Effect of co-application of celastrol and arimoclomol on cellular viability and neuronal process morphology of differentiated human SH-SY5Y neuronal cells

a Propidium iodide (pink) staining indicated that co-application of celastrol and arimoclomol did not induce neuronal cell death. Hoechst 33258 (blue) was used to identify neuronal nuclei by DNA staining. Differential interference contrast imaging (DIC) was employed to view neuronal morphology. b Neuronal processes were assessed by staining with α-tubulin (green). Nuclei are stained with DAPI (blue). Arrows – long neuronal processes; arrowheads – retracted neuronal processes. c High concentrations of celastrol (1.5 µM) compromised neuronal viability. Upper panel - rounding of neuronal cell bodies (observed by DIC) and uptake of propidium iodide; lower panel - retraction of neuronal processes (arrowhead). d Quantification of cell viability by trypan blue staining. 84.2% ±3.8 neuronal viability (dotted line) was maintained after low dose co-application of celastrol and arimoclomol for 24 h at the optimized concentrations (0.3 µM and 50 µM, respectively). Significant (* = p<0.05; ** = p<0.01) loss of cell viability was observed at 250 µM arimoclomol or 1.5 µM celastrol. Scale bars represent 20 µm.
26
1.4 Discussion

1.4.1 Rationale for co-application of celastrol and arimoclomol

Upregulation of Hsps is proposed as a therapeutic strategy to treat neurodegenerative diseases (Asea and Brown 2008; Pratt et al. 2015). The rationale for employing co-application of celastrol and arimoclomol is as follows.

Celastrol was identified in an NIH sponsored drug screen aimed at identifying potential therapeutic compounds that could suppress a hallmark of neurodegenerative diseases, namely protein misfolding resulting in aggregation (Abbott 2002; Heemskerk et al. 2002). Subsequently, celastrol was shown to be beneficial in a number of animal models of neurodegenerative diseases, including ALS (Kiaei et al. 2005), Parkinson’s Disease (Cleren et al. 2005), polyglutamine expansion disease (Zhang and Sarge 2007), and Alzheimer’s Disease (Paris et al. 2010). Celastrol also exhibits potent anti-inflammatory and antioxidant properties in several animal models of inflammation and apoptosis to combat these other aspects of neurodegenerative disease pathology (Allison et al. 2001; Faust et al. 2009; Kim et al. 2009; Sharma et al. 2015). In human SH-SY5Y neuronal cells, which are employed as a model system in the present study, it has been demonstrated that celastrol prevents the increase in reactive oxygen species that is observed following exposure to the mitochondrial toxin rotenone (Choi et al. 2014). The mechanism of action of celastrol involves activation of HSF1 monomers to a trimerized form that binds to HSEs in the promoter regions of inducible heat shock genes resulting in their upregulation (Westerheide et al. 2004; Salminen et al. 2010; Sharma et al. 2015). Despite abundant promising preclinical data, the occurrence of substantial dose-related side effects in human clinical trials have limited its therapeutic utility (Kalmar and Greensmith 2009; Cascao et al. 2017). The present results demonstrate that combining celastrol with the HSF1 co-activator
arimoclomol could permit the use of lower effective dosages that do not have significant adverse viability effects on neurons and thereby limit potential side effects.

Arimoclomol is a co-inducer that acts by prolonging the binding of heat shock transcription factor HSF1 to HSEs resulting in prolonged transcription of Hsp genes (Hargitai et al. 2003). In a mouse model of ALS, arimoclomol was found to improve motor performance and extend lifespan of SOD1<sup>G93A</sup> transgenic mice (Kieran et al. 2004; Kalmar et al. 2008; McGoldrick et al. 2013; Kalmar et al. 2014; Poppe et al. 2014). Interestingly, arimoclomol is currently in Phase III human clinical trials for ALS (ClinicalTrials.gov identifier: NCT00706147 and NCT03491462) and is well tolerated in ALS patients up to 200 mg/day three times daily (Genc and Ozdinler 2013; Benatar et al. 2018).

1.4.2 Low dose co-application of celastrol and arimoclomol as a multi-target strategy to treat neurodegenerative protein misfolding disorders

Low dose co-application of celastrol and arimoclomol enhanced the induction of a set of Hsps in differentiated human SH-SY5Y neuronal cells relative to either compound individually. This includes up-regulation of HSPA6 (Hsp70B′), a little studied member of the HSPA (Hsp70) family that is present in the human genome, but not in rat and mouse (Chow and Brown 2007; Noonan et al. 2007a; Noonan et al. 2007b; Ramirez et al. 2014). Therapeutic compounds identified in rodent models of neurodegenerative disease have repeatedly failed to translate to effective therapies in human clinical trials (Nestler and Hyman 2010; t Hart et al. 2012; McGonigle and Ruggeri 2014; Sasaki 2015). This could be addressed by the generation of primate animal models employing the common marmoset (Callithris Jacchus) since rodent models fail to encompass the complexity of the human brain (Lang 2010; t Hart et al. 2012;
McGonigle and Ruggeri 2014; Sasaki 2015). Interestingly, the HSPA6 gene is found in the marmoset genome (NCBI gene ID: 100411854). Marmoset models are in early testing stages hence it is important to investigate the effects of potential therapeutic compounds in differentiated human neuronal cells.

Neuronal cell viability and process morphology were maintained following co-application of celastrol and arimoclomol at low dose concentrations which result in enhanced Hsp induction. Higher doses of either arimoclomol or celastrol reduced cell viability, as has been previously reported for celastrol (Kalmar and Greensmith 2009; Jantas et al. 2013). The ability of heat shock or celastrol to induce Hsps in differentiated human neuronal cells, when paired with arimoclomol, was analyzed. Co-application of celastrol and arimoclomol resulted in greater induction of Hsps compared to arimoclomol coupled with heat shock conditions that have previously been used on SH-SY5Y neuronal cells (Chow et al. 2010).

Few effective therapies exist for human neurodegenerative diseases despite a large number of clinical trials (Lang 2010; Dunkel et al. 2012; Pratt et al. 2015). This may be due to the multifactorial nature of neurodegenerative diseases. Targeted inhibition of a single disease pathology may be compensated by concurrent deleterious pathways (Cavalli et al. 2008; Lang 2010; Dunkel et al. 2012; Huang and Mucke 2012; Sheikh et al. 2013). Multi-drug therapies that target several aspects of disease pathology are gaining attention and may provide more effective avenues for treating these multi-pathology diseases (Cavalli et al. 2008; Lang 2010; Dunkel et al. 2012; Huang and Mucke 2012; Sheikh et al. 2013; Veloso et al. 2013a; Veloso et al. 2013b; Veloso et al. 2014). Celastrol exhibits anti-inflammatory and antioxidant properties (Allison et al. 2001; Jung et al. 2007; Faust et al. 2009; Kim et al. 2009; Venkatesha et al. 2012; Wong et al. 2012; Yang et al. 2014; Sharma et al. 2015). In addition to inducing Hsp upregulation to prevent
misfolding and aggregation of mutant, disease-associated proteins, celastrol also has the potential to reduce inflammatory and oxidative stress that accompany neurodegeneration and are thought to exacerbate disease progression (Gao and Hong 2008; Amor et al. 2010; Amor et al. 2014).

1.4.3 Upregulation of Hsps could protect against synaptic loss and dysfunction that occur at early stages of neurodegenerative disease progression

There is an urgent need for treatment strategies that impact early stages of disease progression (DeKosky and Marek 2003; Lang 2010; Dunkel et al. 2012). Synaptic dysfunction and loss have been recognized as an early phenomenon in neurodegenerative diseases (Masliah et al. 2001; Scheff et al. 2006; Shankar and Walsh 2009; Milnerwood and Raymond 2010; Stephan et al. 2012; Wu et al. 2015). Overexpression of HSPA and heat shock preconditioning protects synapses at a functional level (Karunanithi et al. 1999; Karunanithi et al. 2002; Brown 2008; Karunanithi and Brown 2015). Using biochemical isolation of synaptic fractions and electron microscopy immunocytochemistry, it has been demonstrated that HSPA1A (Hsp70), HO-1 (Hsp32), and HSPB1 (Hsp27) localize to synaptic components where they may be involved in synaptic repair and protective mechanisms (Bechtold and Brown 2000; Bechtold et al. 2000). Furthermore, HSPA8 (Hsc70) forms a complex with DNAJB1 (Hsp40) at synapses following hyperthermia in the cerebral cortex (Chen and Brown 2007b). Other studies indicate that overexpression of HSPA1A induces synaptic plasticity in Drosophila neuromuscular junctions that results in higher levels of neurotransmitter release and improved locomotor performance (Xiao et al. 2007). Chen et al. (2014) observed upregulation of pre- and post-synaptic proteins through an HSF1-dependent transcriptional mechanism and reduction of amyloid-β-induced synaptic toxicity and memory impairment.
During normal human brain development, synaptic connections are overproduced and selective elimination (i.e. synaptic pruning) subsequently takes place to shape the developing brain based on activity levels at individual synapses (Stephan et al. 2012; Schafer and Stevens 2015; Wu et al. 2015; Hong et al. 2016). Aberrant reactivation of synaptic pruning mechanisms during neuronal aging has recently been proposed to contribute to early synapse dysfunction and loss that is seen in many neurodegenerative diseases (Stephan et al. 2012; Chung et al. 2015; Wu et al. 2015). Upregulation of Hsps could have beneficial effects on mitigating against synaptic loss and dysfunction during aberrant reactivation of synaptic pruning at early stages in the progression of neurodegenerative diseases.
CHAPTER 2

Components of a mammalian disaggregation/refolding machine are targeted to nuclear speckles following thermal stress in differentiated human neuronal cells

2.1 Introduction

Hsps counter protein misfolding and aggregation by acting co-operatively in multi-protein machines that prevent aggregation and refold proteins denatured by stress or disease mechanisms (Glover and Lindquist 1998; Kampinga and Craig 2010; Rampelt et al. 2012; Bracher and Verghese 2015a). The classical mammalian protein refolding machine is composed of members of the DNAJ (Hsp40) family that act as holdases to detect and bind misfolded proteins, while members of the HSPA (Hsp70) family act as foldases that refold proteins to biologically active states (Hageman et al. 2011; Rampelt et al. 2012; Mattoo and Goloubinoff 2014; Clerico et al. 2015; Nillegoda and Bukau 2015; Nillegoda et al. 2015). The HSPA-DNAJ machine inhibits aggregation of misfolded proteins but cannot dissociate aggregated proteins which accumulate during neurodegenerative diseases and aging (Gao et al. 2015; Nillegoda and Bukau 2015). Bacteria, fungi, and plants express a well-characterized protein disaggregase (Hsp104 in yeast cells and ClpB in Escherichia coli) that can solubilize aggregated proteins, homologs of which are lacking in mammalian cells (Glover and Lindquist 1998; Weibezahn et al. 2005; Bosl et al. 2006; Tyedmers et al. 2010; Nillegoda and Bukau 2015). Recent evidence has revealed that a disaggregase function is performed by the mammalian HSPH (Hsp110) family in co-operation with the HSPA-DNAJ machine (Rampelt et al. 2012; Gao et al. 2015; Nillegoda and Bukau 2015; Nillegoda et al. 2015). The small heat shock protein HSPB1 (Hsp27)
has been shown to enhance protein disaggregation by the machine (Ehrnsperger et al. 1997; Lee et al. 1997; Mogk et al. 2003; Duennwald et al. 2012).

Stress-sensitive sites have previously been identified in differentiated human neuronal cells by tracking the intracellular localization of YFP-tagged HSPA (Hsp70) family members following exposure to heat shock in differentiated human neuronal SH-SY5Y cells (Khalouei et al. 2014a; Khalouei et al. 2014b; Shorbagi and Brown 2016). Using low dose co-application of celastrol and arimoclomol to induce Hsps, this chapter investigates the intracellular targeting of components of the mammalian disaggregation/refolding machine following thermal stress in differentiated human neuronal SH-SY5Y cells. Biochemical reconstitution of the disaggregation/refolding reaction by mixing of the machine components with pre-formed protein aggregates in a cell-free environment in vitro has been employed to study the mammalian disaggregation/refolding machine (Rampelt et al. 2012; Gao et al. 2015; Nillegoda and Bukau 2015; Nillegoda et al. 2015; Kirstein et al. 2017). Knockdown studies in Caenorhabditis elegans (C. elegans) have also been employed to study the machine, but it has not been investigated in a human neuronal cell line. Identification of stress-sensitive sites in human neuronal cells that are targeted by the components of the disaggregation/refolding machine provides insight into neuronal mechanisms that require protection by the machine. The HSPA-DNAJ-HSPH disaggregation/refolding machine has been shown to disassociate amyloid fibrils of α-synuclein in vitro that are associated with Parkinson’s disease (Gao et al. 2015). Given the potential implication for countering neurodegenerative disease-associated protein aggregation, the localization of machine components in stressed human neuronal cells merits investigation.
This chapter has been published in *Cell Stress and Chaperones* (2017) as “Components of a mammalian disaggregation/refolding machine are targeted to nuclear speckles following thermal stress in differentiated human neuronal cells” by Deane CA and Brown IR. Volume 22, Issue 2, pages 191-200. A modified version of Figure 10e is featured on the journal cover.
2.2 Methods

2.2.1 Cell culture, differentiation and Hsp induction

Human SH-SY5Y neuroblastoma cells were plated onto 22 x 22 mm coverslips placed inside 6-well plates and differentiated for 72 h with 10 μM all-trans-retinoic acid as described in the ‘Methods’ section on ‘Maintenance and differentiation of the SH-SY5Y cell line’ in Chapter 1. Following 72 h of differentiation, media containing all-trans-retinoic acid was removed and replaced with fresh serum-free DMEM with or without 0.3 μM celastrol plus 50 μM arimoclomol. Celastrol and arimoclomol were prepared as described in the ‘Methods’ section on ‘Induction of heat shock proteins’ in Chapter 1. After 12 h incubation to allow Hsp induction, the cells were either fixed for immunofluorescence (no HS) or exposed to heat shock (HS). For heat shock, cells were immersed in a water bath equipped with a thermal immersion circulator calibrated at 43 °C (±0.2 °C) for 20 min and subsequently fixed for immunofluorescence.

2.2.2 Immunofluorescence

Cells were fixed with 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.4) for 30 min. The cells were permeabilized with 0.1 % triton X-100 and 100 mM glycine in PBS for 30 min and blocked with 5 % FBS in PBS for 1 h. Primary antibodies were incubated overnight in 1 % FBS in PBS. Primary antibodies for HSPA1A (SPA-810), HSPA8 (SPA-815), DNAJB1 (SPA-400), and HSPB1 (SPA-803) were purchased from Enzo Life Sciences (Farmingdale, NY, USA). DNAJA1 [clone KA2A5.6] (ab3089), HSPH1 (ab109624), BAG-1 (ab7976), SC35 (ab11826), and nucleophosmin (ab37659) primary antibodies were purchased from Abcam (Toronto, ON, CA). Primary antibody for the nuclear speckle marker protein SON
(HPA023535) was purchased from Sigma Aldrich (St. Louis, MO, USA). Cells were then washed and incubated with the appropriate combination of Alexafluor® secondary antibodies (Molecular Probes, Life Technologies, Burlington, ON, CA) and counterstained with 300 nM DAPI (Invitrogen, Life Technologies). Fluorescence images were captured using a Quorum Wave FX-X1 spinning disk confocal microscope (Quorum Technologies, Guelph, ON, CA) equipped with a high resolution Humamatsu Orca R2 camera (Humamatsu Photonics, Japan) using a Plan-APO 63×/1.4 NA oil objective. Excitation lasers = 405, 491, 561, and 644 nm. Emission filters (nm/bandpass): 460/50, 525/50, and 593/40.

2.2.3 Fluorescent image analysis

Image processing and analysis was performed using Volocity 3D image analysis software (PerkinElmer, Waltham, MA, USA). ImageJ software (http://imagej.nih.gov/ij/) was used to perform co-localization analysis using TIFF images exported from Volocity. Background subtracted images were used to generate RGB profile plots representing fluorescence signal intensities in a defined linear region using the RGB Profiler plugin. Images representative of three individual experiments are shown in which 25 cells were analyzed in coverslips harvested from each well of 6-well culture plates.
2.3 Results

2.3.1 HSPA and DNAJ components of a protein disaggregation/refolding machine co-localize at nuclear speckles following thermal stress in differentiated human neuronal SH-SY5Y cells

Differentiated human neuronal SH-SY5Y cells were treated with celastrol and arimoclomol to induce Hsps, including HSPA1A and DNAJB1, as demonstrated in Chapter 1 (Deane and Brown 2016b). Prior to heat shock, HSPA1A was distributed diffusely in the neuronal cytoplasm (Figure 6, no HS panels). Following heat shock (HS), celastrol- and arimoclomol-induced HSPA1A co-localized with nuclear speckles (Figure 6a, merged panel) identified by the marker protein SON (Sharma et al. 2010; Sytnikova et al. 2011). ImageJ line scans confirmed co-localization of the HSPA1A and SON fluorescent signals (arrowheads shown in panels on the right). After heat shock, DNAJB1 co-localized with HSPA1A (Figure 6b) and the nuclear speckle marker SC35 (Figure 6c), confirmed by ImageJ line scans (arrowheads). SC35 and SON have been shown to co-localize with each other in differentiated human neuronal SH-SY5Y cells at nuclear speckles (Khalouei et al. 2014a).

The DNAJ family is a large family of molecular chaperones in mammalian cells and is divided into classes that vary in sequence and structure but include a characteristic J-domain that mediates interactions with HSPA proteins (Kampinga et al. 2009; Kampinga and Craig 2010). The mammalian disaggregation/refolding machine has been reported to more efficiently promote dissociation of protein aggregates in the joint presence of a class A and B member of the DNAJ family (Nillegoda et al. 2015). As shown in Figure 7a, a class A member of the DNAJ family, namely DNAJA1 (Hsp40-4), co-localized after heat shock with DNAJB1 at SON-positive nuclear speckles (Figure 7b), confirmed by ImageJ line scans (arrowheads).
Figure 6. HSPA1A (Hsp70-1) and DNAJB1 (Hsp40-1) are targeted to nuclear speckles by heat shock in differentiated human neuronal cells

Differentiated human neuronal SH-SY5Y cells were treated with celastrol and arimoclomol to induce Hsps. a HSPA1A co-localized with the nuclear speckle marker SON after heat shock, confirmed at arrowheads in ImageJ line scan displaying fluorescence intensity across the region indicated by the dotted line. b DNAJB1 co-localized with HSPA1A after heat shock, confirmed at arrowheads in ImageJ line scan. c The nuclear speckle marker SC35 co-localized with DNAJB1 after heat shock (arrowheads in ImageJ line scan). HS = Heat Shock. DAPI staining is used to identify neuronal nuclei (blue). Scale bars represent 5 μm.
Figure 7. The class A protein, DNAJA1 (Hsp40-4), also localizes to nuclear speckles after heat shock

a DNAJA1 co-localized with DNAJB1 and b the nuclear speckle marker SON (arrowheads) confirmed by ImageJ line scans on the right (scanned region is indicated by the dashed arrow in the merge panel). Scale bars represent 5 μm.
2.3.2 The disaggregate HSPH1 (Hsp105α) co-localizes with HSPA1A at nuclear speckles after heat shock, but not the alternative nucleotide exchange factor, BAG-1

The mammalian disaggregate HSPH1 (Hsp105α) that promotes protein disaggregation in vitro as a member of the disaggregation/refolding machine, acts as a nucleotide exchange factor (NEF) for HSPA proteins (Schuermann et al. 2008; Bracher and Verghese 2015a; Bracher and Verghese 2015b; Nillegoda and Bukau 2015). As shown in the merged panels of Figure 8, and confirmed by ImageJ line scans, HSPH1 co-localized at nuclear speckles with HSPA1A (Figure 8a, arrowheads) and SC35 (Figure 8b, arrowheads) after thermal stress. An alternative HSPA NEF that does not participate in the disaggregation/refolding of aggregated proteins, namely BAG-1 (Rampelt et al. 2012; Bracher and Verghese 2015b; Nillegoda and Bukau 2015), did not co-localize with HSPA1A or SC35 (Figure 8c and d). Targeting of the disaggregate HSPH1 and other components that make up the mammalian disaggregation/refolding machine to nuclear speckles suggests that this machine may contribute to the recovery of RNA splicing following stress-induced inhibition.

2.3.3 The small heat shock protein HSPB1 (Hsp27) is targeted to stress-sensitive neuronal sites

The small heat shock protein HSPB1 (Hsp27) has been shown to facilitate dissociation of protein aggregates by the disaggregation/refolding machine (Mogk et al. 2003; Duennwald et al. 2012; Nillegoda and Bukau 2015). As shown in Figure 9a, HSPB1 co-localized with HSPA1A which was targeted to nuclear speckles after heat shock (Figure 6a). HSPB1 may enhance the activity of the disaggregation/refolding machine at nuclear speckles following heat shock.
Figure 8. Targeting of the disaggregate HSPH1 (Hsp105α) to nuclear speckles following thermal stress

a Co-localization of HSPH1 and HSPA1A after heat shock, confirmed by ImageJ line scans (arrowheads). b HSPH1 and SC35 co-localized after heat shock (arrowheads in ImageJ line scan). c BAG-1 did not co-localize with HSPA1A after heat shock, confirmed by ImageJ line scan. d BAG-1 also did not co-localize with SC35 after heat shock. Scale bars represent 5 μm.
Figure 9. The small heat shock protein HSPB1 (Hsp27) is targeted by thermal stress to nuclear and cytoplasmic sites in differentiated human neuronal cells

a HSPB1 co-localized with HSPA1A in the nucleus after heat shock (arrowheads). b HSPB1 co-localized with the vimentin cytoskeleton in the cytoplasm after heat shock (arrow). Co-localization was confirmed by ImageJ line scans shown on the right. Scale bars represent 5 μm.
cytoplasm, HSPB1 co-localized after heat shock with vimentin, a marker of the cytoskeleton (Figure 9b). The vimentin cytoskeleton assembles into a cage around aggresomes that form in the cell body during times of stress to sequester misfolded, aggregated proteins and reduce their toxicity (Tyedmers et al. 2010). HSPB1 may be involved in stabilizing the vimentin cage that forms during thermal stress recovery in the cytoplasm (Tyedmers et al. 2010).

### 2.3.4 Constitutively expressed HSPA8 (Hsc70) is also targeted to nuclear speckles with components of the disaggregation/refolding machine following heat shock

Interestingly, HSPA8 also exhibited stress-induced co-localization at nuclear speckles with DNAJB1, DNAJA1, and HSPH1 (Figure 10a-c, respectively; and confirmed by ImageJ line scans), as was observed for HSPA1A. This suggests that constitutive HSPA8 has the potential to operate in a disaggregation/refolding machine during the recovery of nuclear speckles from thermal stress. Basal levels of DNAJB1, DNAJA1, HSPH1, and HSPA8 are expressed in differentiated human neuronal SH-SY5Y cells (Deane and Brown 2016b). Co-localization of these machine components in differentiated human neuronal SH-SY5Y cells at nuclear speckles following heat shock was also observed in the absence of celastrol and arimoclomol co-application (Figure 10d-f). This observation suggests that neurons have the potential to rapidly assemble a disaggregation/refolding machine following thermal stress utilizing constitutively expressed HSPA8, without the time lag required for synthesis of stress-inducible HSPA1A.
Figure 10. Constitutively expressed HSPA8 (Hsc70) targets nuclear speckles following thermal stress and co-localizes with members of the mammalian disaggregation/refolding machine

a Following co-application of celastrol and arimoclomol to induce Hsps, HSPA8 co-localized after heat shock with DNAJB1 at SC35-positive nuclear speckles (arrowheads). b HSPA8 also co-localized with DNAJA1 at SON-positive nuclear speckles (arrowheads). c HSPA8 co-localized with HSPH1 (arrowheads). d In the absence of celastrol and arimoclomol co-application, constitutively expressed HSPA8 also targeted nuclear speckles and co-localized with DNAJB1, e DNAJA1 and f HSPH1. Scale bars represent 5 μm.
2.4 Discussion

2.4.1 Components of the mammalian protein disaggregation/refolding machine target nuclear speckles following heat shock in differentiated human neuronal cells

Recent biochemical studies using purified human Hsps have identified a mammalian disaggregation/refolding machine, composed of members of the HSPA (Hsp70), DNAJ (Hsp40), and HSPH (Hsp110) families, that acts to dissociate and refold aggregated proteins \textit{in vitro} (Rampelt et al. 2012; Gao et al. 2015; Nillegoda and Bukau 2015; Nillegoda et al. 2015). Mixing of machine components with pre-formed aggregates in a cell-free environment \textit{in vitro} has been used to demonstrate that the machine can dissociate amyloid fibrils of α-synuclein that are associated with Parkinson’s disease (Gao et al. 2015). However, the machine has not been studied previously in intact human cells.

This chapter presents novel results that demonstrate the targeting of members of the mammalian disaggregation/refolding machine to a specific intracellular structure following heat shock in differentiated human neuronal SH-SY5Y cells, namely nuclear speckles. Using immunofluorescent confocal imaging, co-localization at nuclear speckles following heat shock was observed for the stress-inducible foldase HSPA1A, a class A plus a class B member of the DNAJ holdase family of proteins, namely DNAJA1 and DNAJB1, and the mammalian disaggregase HSPH1. These data enhance the potential relevance of the disaggregation/refolding machine for combating neurodegenerative disease-associated protein aggregation by demonstrating that machine components are expressed and co-localize under stress conditions in a differentiated human neuronal cell line.

Nuclear speckles are sites rich in RNA splicing factors and were identified by staining with the marker proteins SON and SC35 (Lamond and Spector 2003; Sharma et al. 2010; Spector
and Lamond 2011; Sytnikova et al. 2011) that have been shown to co-localize in the nucleus of differentiated human neuronal cells (Khalouei et al. 2014a). SC35-positive nuclear speckles targeted by HSPA1A after heat shock in differentiated SH-SY5Y cells do not co-localize with Sam68, a marker of nuclear stress bodies (Khalouei et al. 2014a). RNA splicing is disrupted by thermal stress and Hsps are required for splicing recovery (Yost and Lindquist 1986; Corell and Gross 1992; Marin-Vinader et al. 2006; Biamonti and Caceres 2009). Co-localization at nuclear speckles of HSPA, DNAJ, and HSPH components of the mammalian disaggregation/refolding machine after heat shock suggests that dissociation of misfolded and aggregated nuclear speckle proteins could be involved in the recovery of RNA splicing.

2.4.2 The presence of class A and class B members of the DNAJ family enhances the efficiency of the mammalian protein disaggregation/refolding machine

Both DNAJB1, a class B member of the DNAJ family, and DNAJA1, a class A member, co-localized at nuclear speckles following heat shock. The DNAJ family of molecular chaperones delivers substrates to HSPA proteins and stimulates their ATPase activity (Kampinga et al. 2009; Kampinga and Craig 2010; Mattoo and Goloubinoff 2014). It is divided into classes that differ structurally but contain a characteristic J-domain that defines this family and mediates interactions with HSPA proteins (Kampinga et al. 2009; Kampinga and Craig 2010). Class A proteins exhibit the greatest degree of similarity with yeast DNAJ proteins while class B proteins lack the zinc finger domain found in class A proteins (Kampinga et al. 2009; Kampinga and Craig 2010). Nillegoda et al. (2015) have recently shown that efficient dissociation of aggregated proteins by the mammalian disaggregation/refolding machine requires the presence of both a
class A and a class B member of the DNAJ family, and that DNAJA1 and DNAJB1 are effective partners.

2.4.3 Localization of HSPH1, but not BAG-1, to nuclear speckles suggests splicing recovery may involve dissociation and refolding of thermally denatured proteins

HSPA nucleotide exchange factors (NEFs) in humans fall into families that are structurally and functionally distinct, including the HSPH (Hsp110) family, Bcl2-associated athanogene (BAG) family, and Hsp70 binding protein-type (HSPBP-type) NEFs (Schuermann et al. 2008; Kampinga and Craig 2010; Bracher and Verghese 2015a; Bracher and Verghese 2015b). The diversity of HSPA NEFs provides functional specificity by linking HSPA members to distinct cellular pathways (Kampinga and Craig 2010; Hageman et al. 2011; Bracher and Verghese 2015a; Bracher and Verghese 2015b). The results demonstrate that HSPH1 co-localizes at nuclear speckles with components of the disaggregation/refolding machine following thermal stress, whereas the alternative NEF, BAG-1, did not. HSPH1 promotes dissociation of both heat and chemically denatured protein aggregates in vitro (Rampelt et al. 2012; Nillegoda and Bukau 2015) whereas BAG family NEFs promote the degradation of misfolded protein targets by linking HSPA to the protein degradation machinery (Bracher and Verghese 2015a; Bracher and Verghese 2015b). Studies in C. elegans showed that knockdown of Hsp110, but not BAG-1, resulted in the persistence of heat shock-induced luciferase aggregates in YFP-luciferase-expressing animals (Rampelt et al. 2012). Co-localization of HSPH1, but not BAG-1, with HSPA1A, DNAJB1, and DNAJA1 at nuclear speckles following thermal stress suggests that disaggregation and refolding of thermally denatured proteins occurs during splicing recovery, rather than protein degradation.
2.4.4 The role of the small heat shock protein HSPB1 in protein disaggregation/refolding

The small heat shock protein HSPB1 is not an essential member of the disaggregation/refolding machine; however, it has been shown to enhance the in vitro activity of the machine (Mogk et al. 2003; Duennwald et al. 2012; Nillegoda and Bukau 2015). Specifically, small heat shock proteins (sHsps) have been reported to enhance recovery from heat-induced nuclear protein aggregation (Kampinga et al. 1994; Stege et al. 1995) likely by maintaining denatured proteins in a folding competent state (Ehrnsperger et al. 1997; Lee et al. 1997; Duennwald et al. 2012; Rampelt et al. 2012). The present results demonstrate targeting of HSPB1 to nuclear speckles where it may contribute to splicing recovery by enhancing the ability of the machine to dissociate heat-induced aggregates of nuclear speckle proteins (Lamond and Spector 2003; Spector and Lamond 2011). HSPB1 also targeted the vimentin cytoskeleton after heat shock suggesting that it may be involved in stabilization of the “vimentin cage,” which sequesters aggregated proteins following cellular stress (Tyedmers et al. 2010). Interestingly, a recent literature review aimed at identifying modifiers of proteotoxicity identified sHsps as modifiers of neuronal proteotoxicity associated with neurodegenerative diseases (Brehme and Voisine 2016).

2.4.5 Constitutively expressed HSPA8 may be a rapid responder to neuronal stress without the time lag required to upregulate inducible HSPA1A

Constitutively expressed HSPA8 was also targeted to nuclear speckles following heat shock in differentiated human neuronal SH-SY5Y cells and co-localized with DNAJA1, DNAJB1, and HSPH1. Thus, HSPA8 may be involved in recovery of RNA splicing from stress-
induced inhibition. This novel observation suggests that neurons have the potential to rapidly deploy HSPA8 in times of stress to assemble a disaggregation/refolding machine at nuclear speckles, without the time lag required for synthesis of stress-inducible HSPA1A. HSPA8 is abundant in mammalian neurons in vivo compared to non-neural tissues and has been proposed to pre-protect neurons from cellular stress (Manzerra et al. 1997; Chen and Brown 2007a). High levels of HSPA8 in neurons may explain previous observations that neurons are refractory to HSPA induction following heat shock (Manzerra et al. 1997; Drujan and De Maio 1999; Chow et al. 2010). Interestingly, purified HSPA8 has recently been reported to act in vitro as a member of the machine to dissociate α-synuclein amyloid fibrils that form during Parkinson’s disease (Gao et al. 2015) and may be a more efficient HSPA component of the machine compared with HSPA1A (Kirstein et al. 2017). Thus, constitutively expressed HSPA8 could be a key factor as a rapid responder in a disaggregation/refolding machine to combat misfolded, aggregation-prone proteins in human neurodegenerative diseases.
CHAPTER 3

Differential targeting of HSPA6 and HSPA1A with components of a protein disaggregation/refolding machine in differentiated human neuronal cells following thermal stress

3.1 Introduction

The previous chapter demonstrated co-localization of inducible HSPA1A and constitutively expressed HSPA8 at nuclear speckles with components of the mammalian protein disaggregation/refolding machine. This suggests that splicing recovery after thermal stress may involve restoration of nuclear speckle proteins by the disaggregation/refolding machine. The purpose of this chapter is to advance knowledge of HSPA6 that has been little studied relative to HSPA1A or HSPA8. Recent publications on the expression of HSPA6 in human neuronal cells has been reported from our laboratory (Chow and Brown 2007; Chow et al. 2010; Khalouei et al. 2014a; Khalouei et al. 2014b; Becirovic and Brown 2016; Deane and Brown 2016b; Deane and Brown 2016a; Shorbagi and Brown 2016), and from the Hightower laboratory in human cancer cell lines (Noonan et al. 2007a; Noonan et al. 2008). However, it is unknown whether HSPA6 has divergent roles in the stress response from HSPA1A. Chapter 3 will expand on the previous chapter by comparing the intracellular targeting of HSPA6 following heat shock with that observed for HSPA1A and HSPA8. Additionally, by fixing cells at various time points after heat shock, a time-course of Hsp localization during recovery will be established. This could provide insight whether HSPA6 has similar roles in neuronal stress recovery with those of related HSPA family members, or whether it has evolved divergent features that may be lacking in rodent models of neurodegenerative diseases which lack the HSPA6 gene.
This chapter has been published in Frontiers in Neuroscience (2017) as “Differential targeting of Hsp70 heat shock proteins HSPA6 and HSPA1A with components of a protein disaggregation/refolding machine in differentiated human neuronal cells following thermal stress” by Deane CA and Brown IR. Volume 11, Article 227, pages 1-10.
3.2 Methods

3.2.1 Cell culture, heat shock and immunofluorescence

Cells were plated, incubated with celastrol and arimoclomol, and heat shocked as described in the ‘Methods’ section on ‘Sample preparation’ in Chapter 2. Details on fixation, permeabilization, antibody staining, and image acquisition are described in the ‘Methods’ section on ‘Immunofluorescence’ in Chapter 2. RNA polymerase II CTD repeat YSPTSPS (phospho S5) (ab5131) primary antibody was purchased from Abcam (Toronto, ON, CA).
3.3 Results

3.3.1 HSPA6 is targeted to sites at the periphery of nuclear speckles (perispeckles) after heat shock, which is not observed for the widely studied HSPA1A

Following induction of Hsps, including the little studied HSPA6 and the widely studied HSPA1A, by co-application of celastrol and arimoclomol for 12 h, heat shock (43°C) was applied for 20 min to target Hsps to stress-sensitive sites. Prior to heat shock, HSPA6 and HSPA1A were distributed in the neuronal cytoplasm (Figure 11a and 11b, No HS panels). Following heat shock, HSPA6 and HSPA1A translocated into the nucleus and targeted stress-sensitive nuclear structures. At 20 min and 1 h after heat shock, HSPA6 localized to perispeckles (Figure 11a, closed arrowheads) around the periphera of nuclear speckles (open arrowheads) which were identified with the marker protein SON (Sharma et al. 2010; Sytnikova et al. 2011; Khalouei et al. 2014a). In contrast, HSPA1A targeted nuclear speckles at 20 min and 1 h, determined by co-localization with SON (Figure 11b, open arrowheads), confirmed by ImageJ line scans located below the immunocytochemistry panels.

As shown in Figure 12, components of the mammalian disaggregation/refolding machine, namely the holdase DNAJB1 (Figure 12a) and the disaggregase HSPH1 (Figure 12b), did not co-localize after heat shock with HSPA6-positive perispeckles, confirmed by ImageJ line scans. HSPB1 (Figure 12c), that has been shown to facilitate protein disaggregation by the machine, and the constitutive member of the HSPA family, HSPA8 (Figure 12d), also did not co-localize after heat shock with HSPA6-positive perispeckles.
Figure 11. HSPA6 targets perispeckles, located at the periphera of nuclear speckles, which is not observed for the widely studied HSPA1A

a Prior to heat shock, HSPA6 (green) was distributed in the neuronal cytoplasm. After heat shock, HSPA6 localized at 20 min and 1 h to foci at the periphery of nuclear speckles (closed arrowheads) identified by the marker protein SON (red, open arrowheads). ImageJ line scans demonstrated that HSPA6 fluorescent peaks were offset from SON peaks. b Prior to heat shock, HSPA1A (green) was distributed in the neuronal cytoplasm. After heat shock, HSPA1A co-localized with the nuclear speckle marker protein SON (red, open arrowheads) at 20 min and 1 h, confirmed by ImageJ line scans. DAPI (blue) was used to identify neuronal nuclei. Scale bar represents 5 μm. Inset scale bar in a represents 0.5 μm.
Figure 12. Components of the protein disaggregation/refolding machine do not target HSPA6-positive perispeckles after heat shock in differentiated human neuronal cells

Immunocytochemistry and ImageJ line scans demonstrated that a DNAJB1, b HSPH1, c HSPB1 and d HSPA8 did not co-localize with HSPA6-positive perispeckles after heat shock. e HSPA6-positive foci co-localized with the perispeckle marker RNA polymerase II (closed arrowheads), confirmed by ImageJ line scans. DAPI (blue) was used to identify neuronal nuclei. Scale bar represents 5 μm.
HSPA6-positive foci at 20 min and 1 h co-localized with the perispeckle marker RNA polymerase II (Figure 12e; closed arrowheads) that is associated with transcription sites (Ghamari et al. 2013). The antibody recognizes a modified form of RNA polymerase II that is phosphorylated on serine 5 of the C-terminal domain (Pol II CTD-PS5). This modification is associated with the active form of RNA polymerase II that is located at transcription platforms (Ghamari et al. 2013).

3.3.2 Differential association of HSPA6 and HSPA1A with nucleolar structures following thermal stress

At 1 h after heat shock, HSPA1A, but not HSPA6, localized to the granular component (GC) layer of the nucleolus (Figure 13a, arrows), identified by the marker protein nucleophosmin (NPM) (Hernandez-Verdun et al. 2010), which is the site of ribosomal RNA processing and ribosomal subunit assembly (Thiry and Lafontaine 2005; Raska et al. 2006; Hernandez-Verdun et al. 2010). The holdase DNAJA1 also co-localized with nucleophosmin at the GC layer of the nucleolus (Figure 13b, arrows). As shown in Figure 13c (arrows), BAG-1 co-localized with HSPA1A and DNAJA1 which were targeted to the nucleolus at 1 h (Figure 13a and 13b, arrows), whereas the disaggregase HSPH1 did not (Figure 13d, arrow). The open arrowheads in Figure 13d represent the targeting of HSPA1A and HSPH1 to nuclear speckles, previously mentioned in Figure 8. It has been reported that BAG-1 does not promote dissociation of aggregated proteins in the presence of other members of the protein disaggregation/refolding machine (Rampelt et al. 2012) but rather targets HSPA substrates for degradation via the proteasome (Bracher and Verghese 2015a; Bracher and Verghese 2015b).
**Figure 13.** HSPA1A, but not HSPA6, is targeted to the granular component (GC) layer of the nucleolus at 1 h following heat shock

a At 1 h, HSPA1A (green, upper panel), but not HSPA6 (green, lower panel) co-localized with nucleophosmin (NPM) (red, arrow), a marker of the GC layer of the nucleolus. This localization was not observed at 20 min, confirmed by ImageJ line scans shown on the right. b DNAJA1 also co-localized with nucleophosmin at 1 h (arrows). c BAG-1 co-localized at 1 h with HSPA1A and DNAJA1 (arrows) that were shown to localize to the nucleolus in a and b, however the disaggregase HSPH1 did not (d, arrow). The open arrowheads represent HSPA1A and HSPH1 targeting to nuclear speckles, previously shown in Figure 7. Scale bar represents 5 μm.
At 3 h after heat shock, HSPA6 (Figure 14a, arrow), but not HSPA1A (Figure 14b), co-localized at the GC layer of the nucleolus with components of a protein disaggregation/refolding machine, namely DNAJB1 and the disaggregase HSPH1 (Figure 14c, arrows), but, interestingly, not BAG-1 (Figure 14d). These results suggest differential targeting of HSPA6 and HSPA1A to nucleolar structures following thermal stress.

3.3.3 Constitutive HSPA8 (Hsc70) exhibits similar heat shock-induced targeting to neuronal nuclear sites as HSPA1A

As shown in Figure 15, HSPA8 localized to SON-positive nuclear speckles at 20 min and 1 h after heat shock (Figure 15a, open arrowheads), and to the nucleophosmin-positive GC layer of the nucleolus at 1 h (Figure 15b, arrows), before returning to the cytoplasm at 3 h. This pattern of heat-induced targeting to neuronal sites was similar to that of HSPA1A (Figure 15a and 15b), but not HSPA6 (Figure 15c and 15d). These results indicate that HSPA8 exhibits similar targeting after thermal stress as inducible HSPA1A. In contrast, HSPA6 exhibits features that are not observed for HSPA1A and HSPA8.
Figure 14. At 3 h after thermal stress, HSPA6 co-localizes with nucleophosmin, a marker of the GC layer of the nucleolus

a HSPA6 co-localized at the 3 h time point with a marker of the GC layer of the nucleolus, nucleophosmin (arrow, upper panel), but not with the nuclear speckle marker SON (open arrowheads, lower panel). b HSPA1A did not co-localize with nucleophosmin (upper panel) or SON (lower panel) at 3 h. c Components of a mammalian disaggregation/refolding machine, including DNAJB1 and HSPH1, were also targeted to the GC layer of the nucleolus at 3 h (arrows), however d BAG-1 was not. DAPI (blue in merged panels and ImageJ line scans) was used to identify neuronal nuclei. Scale bar represents 5 μm.
Figure 15. Constitutively expressed HSPA8 exhibits similar heat shock-induced targeting as HSPA1A

a HSPA8 targeted SON-positive nuclear speckles at 20 min and 1 h after heat shock. b HSPA8 also targeted the GC layer of the nucleolus (identified by the marker protein nucleophosmin) at 1 h and co-localized with HSPA1A. c HSPA8 did not co-localize with HSPA6 at 20 min and 1 h at perispeckles or d at the GC layer of the nucleolus at 3 h. DAPI (blue) was used to identify neuronal nuclei. Scale bar represents 5 μm.
3.4 Discussion

3.4.1 HSPA6 exhibits unique localization at nuclear perispeckles following thermal stress

HSPA6, but not HSPA1A, was rapidly targeted by heat shock to perispeckles located at the periphera of nuclear speckles. Perispeckles are rich in RNA polymerase II and poly(A+)-containing RNA (Bregman et al. 1995; Mortillaro et al. 1996; Hall et al. 2006; Khalouei et al. 2014a) and have been characterized as ‘transcription factories’ (Brown et al. 2008; Rieder et al. 2012; Rieder et al. 2014). Interestingly, components of the disaggregation/refolding machine, namely DNAJB1, and the disaggregase HSPH1, did not co-localize with HSPA6 at perispeckles. This suggests a role for HSPA6 at perispeckles that does not require the elements of the disaggregation/refolding machine. Intriguingly, it has been reported that HSPA6 has high intrinsic ATPase activity and is capable of refolding heat-denatured p53 in the absence of DNAJ proteins (Hageman et al. 2011).

Disruption of transcription can have adverse consequences for neurons which rely on dynamic transcriptional activity to maintain neuronal excitability and synaptic plasticity (Qiu and Ghosh 2008; West and Greenberg 2011). Transcription and splicing of mRNA are inhibited by thermal stress (Yost and Lindquist 1986; Yost and Lindquist 1991; Allen et al. 2004; Espinoza et al. 2004; Biamonti and Caceres 2009; Velichko et al. 2013). The disruption of mRNA transcription has been linked to neurodegenerative diseases (Riley and Orr 2006; Bithell et al. 2009; Caldeira et al. 2013; Xiang et al. 2018). HSPA6 at perispeckles could play a role in the restoration of transcription during neuronal stress recovery.
3.4.2 Association of HSPA6 and HSPA1A with the nucleolus

After thermal stress, HSPA6 and HSPA1A were differentially targeted to the granular component (GC) layer of the nucleolus which is the site of ribosomal RNA processing and ribosomal subunit assembly (Thiry and Lafontaine 2005; Raska et al. 2006; Hernandez-Verdun et al. 2010). At the 1 h recovery time point, HSPA1A, but not HSPA6, co-localized at the GC layer of the nucleolus with DNAJA1 and BAG-1, but not with HSPH1. This suggests a possible role for HSPA1A in BAG-1-directed targeting of heat damaged nucleolar proteins to the proteasome for degradation, which is not observed for HSPA6. Subsequently at the 3 h recovery time point, HSPA6, but not HSPA1A, was targeted to the GC layer of the nucleolus with components of the disaggregation/refolding machine comprised of DNAJB1, and the disaggregase HSPH1. Thus, nucleolar recovery from thermal stress may involve a switch from degradation of damaged nucleolar proteins involving HSPA1A, BAG-1 and DNAJA1 during early stages of recovery to a disaggregation/refolding strategy at later stages involving HSPA6, DNAJB1, and HSPH1.

3.4.3 HSPA8 exhibits similar nuclear targeting as HSPA1A after heat shock

Constitutively expressed HSPA8 is present at high levels in neurons in the mammalian brain relative to other cell types and may pre-protect neurons from stress (Manzerra et al. 1997; Chen and Brown 2007b; Chen and Brown 2007a). Variable levels of HSPA8 in different neuronal populations correlate with the frequency of neurodegenerative diseases in the human population (Chen and Brown 2007a). For example, cortical neurons, associated with Alzheimer’s and high disease frequency, exhibit low levels of cortical HSPA8, and hence reduced buffering
capacity against protein misfolding and aggregation. Whereas, motor neurons, associated with ALS and low disease frequency, demonstrate high levels of constitutively expressed HSPA8, and hence increased buffering capacity against protein misfolding. This further highlights the importance of HSPA8 for neuronal proteostasis. Inducible HSPA members, particularly HSPA1A, have been more widely investigated in studies of protein misfolding and aggregation resulting from cellular stress. However, it has been recognized that constitutive Hsps, including HSPA8, also have stress-related functions (Manzerra et al. 1997; Vos et al. 2008; Stricher et al. 2013).

As demonstrated in Chapters 2 and 3, HSPA8 exhibits targeting features similar to HSPA1A and different from HSPA6, that is, i) co-localization at nuclear speckles with machine components at 20 min and 1 h and ii) targeting to the GC layer of the nucleolus at 1 h. These data suggest that HSPA8 may have a similar role in thermal stress recovery of neuronal cells as inducible HSPA1A, in contrast to HSPA6 that targeted perispeckles at 20 min and 1 h and the nucleolus at 3h suggesting that it may play a different role in recovery. Interestingly, HSPA8 targeted nuclear speckles and the GC layer of the nucleolus after heat shock in cells that did not receive low dose co-application of celastrol and arimoclomol to induce HSPA1A. Thus, HSPA8 acts as a rapid stress responder in neuronal cells without the time lag necessary for upregulation of HSPA1A.
CHAPTER 4

Intracellular targeting of Hsps in differentiated human neuronal cells following proteotoxic stress

4.1 Introduction

As discussed in Chapter 1, despite numerous clinical trials of potential therapeutics for Alzheimer’s disease and other neurodegenerative diseases that appeared promising in animal models, there is currently a paucity of effective therapies (Dunkel et al. 2012; Cummings et al. 2014; Pratt et al. 2015; Bennett 2018; Cummings et al. 2018). This suggests that current animal models may not encompass all aspects of the complex human disease process. There is a critical need to improve our understanding of components of the protein quality control machinery and how they co-operate to maintain proteostasis in human neuronal cells in order to better design therapeutic compounds to treat neurodegenerative diseases. A key feature of these diseases is proteotoxic stress caused by the inability of the protein quality control machinery to manage misfolded proteins and the formation of toxic oligomers (Muchowski and Wacker 2005; Westerheide and Morimoto 2005; Asea and Brown 2008; Labbadia and Morimoto 2015; Yerbury et al. 2016; Sweeney et al. 2017). The presence of insoluble protein aggregates leads to neuronal dysfunction and premature cell death (Muchowski and Wacker 2005; Westerheide and Morimoto 2005; Asea and Brown 2008; Richter et al. 2010; Radwan et al. 2017).

Upregulation of HSPA6 has recently been reported in the brains of patients with Alzheimer’s disease, Parkinson’s disease, and dementia with Lewy bodies (Henderson-Smith et al. 2016; Annese et al. 2018; Santpere et al. 2018). A 9.4-fold increase in HSPA6 expression was observed in the brains of patients with Parkinson’s disease with dementia, compared with a 3.4-
fold increase of HSPA1A (Henderson-Smith et al. 2016). A 6.3-fold increase of HSPA6 expression in Alzheimer’s disease patients and a 30.4-fold increase of HSPA6 expression in Parkinson’s disease patients were reported relative to control patients (Annese et al. 2018). Thus, HSPA6 may play a role in the stress response of neurodegenerative disease-afflicted neurons in vivo. To advance knowledge of HSPA6 under conditions of proteotoxic stress, this chapter investigates the expression and intracellular targeting of HSPA6 in differentiated human neuronal SH-SY5Y cells treated with the proteotoxic stress-inducing agent MG132. MG132 has been extensively employed to induce the formation of intracellular protein aggregates in cell culture studies of protein conformational diseases and Hsp function (Ryhanen et al. 2011; Shen et al. 2011; Jiang et al. 2013; Bang et al. 2014; Crum et al. 2015).

This chapter has been accepted for publication in the Journal of Alzheimer’s Disease (2018) as “Intracellular targeting of heat shock proteins in differentiated human neuronal cells following proteotoxic stress” by Deane CA and Brown IR. Volume 66, Issue 3, pages 1295-1308.

Figure 20a is featured on the homepage of the Journal of Alzheimer’s Disease website.
4.2 Methods

4.2.1 Cell culture, differentiation, and Hsp induction

Human SH-SY5Y neuroblastoma cells were plated onto polystyrene tissue culture plates (for Western blotting and viability assays) or 35 mm glass-bottom plates (for immunofluorescence) and differentiated with 10 µM all-trans-retinoic acid as described in the ‘Methods’ section on ‘Maintenance and differentiation of the SH-SY5Y cell line’ in Chapter 1. Following 72 h of differentiation, media was replaced with fresh serum-free DMEM containing MG132 (at the indicated concentrations), celastrol alone (0.3 µM) or celastrol (0.3 µM) plus arimoclomol (50 µM) for 12 h. MG132 (BML-PI102; Enzo Life Sciences, Farmingdale, NY, USA) dissolved in DMSO was added directly to the media. DMSO was used as a vehicle control for MG132 and celastrol. Celastrol and arimoclomol were prepared as described in the ‘Methods’ section on ‘Induction of heat shock proteins’ in Chapter 1.

4.2.2 Western blotting

Western blotting and densitometry were performed as described in the ‘Methods’ section on ‘Western blotting’ in Chapter 1. The primary antibody for ubiquitin (ab7254) was purchased from Abcam (Toronto, ON, CA). Data was normalized to a β-tubulin control and are presented as the mean density relative to the DMSO control ± SEM for three independent replicates.

4.2.3 Viability determination

Viability was assessed as described for quantitative analysis by trypan blue staining in the ‘Methods’ section on ‘Viability determination’ in Chapter 1. Percent viability after 12 h
incubation with the indicated concentration of MG132 was normalized to 100% for the DMSO control and presented as the mean ± SEM for three independent replicates.

4.2.4 Immunofluorescence

Details pertaining to antibody staining, fluorescent image acquisition and generation of fluorescence intensity line scans can be found in the ‘Methods’ section on ‘Immunofluorescence’ in Chapter 2. For staining aggregated proteins, the Proteostat® Aggresome Detection Kit (Enzo Life Sciences) was used according to the manufacturer’s protocol. Volocity 3D image analysis software (PerkinElmer, Waltham, MA, USA) was used for image analysis, processing, generation of 3D opacity renderings from z-stack images and 3D movies.

4.2.5 Heat shock

Following 12 h treatment with 2.5 µM MG132, cells were immersed in a circulating water bath calibrated at 43°C or 44°C (± 0.2°C) for 20 min and then either fixed for immunofluorescence (20 min) or returned to 37°C until fixation at a later time point (1 or 3 h). The 0 time-point equaled the start of heat shock.

4.2.6 Statistical Analysis

Two-way ANOVA followed by Bonferroni’s test for multiple comparisons was used to test for statistical significance of Western blot densitometry and viability data using Graphpad Prism 5 software (La Jolla, CA, USA).
4.3 Results

4.3.1 Induction of HSPA6 in differentiated human neuronal cells by MG132

As shown in Figure 16, HSPA6 was induced in differentiated human SH-SY5Y neuronal cells following treatment with the proteotoxic agent MG132. Robust induction of HSPA6 was apparent at 2.5 µM MG132 compared to HSPA6 levels that were induced by treatment with celastrol, or celastrol plus arimoclomol that induces HSPA6 (Chapter 1). Classic heat shock does not induce HSPA6 in differentiated human SH-SY5Y neuronal cells (Chow et al. 2010; Deane and Brown 2016b). Interestingly, MG132 also triggered the induction of other key components of the protein disaggregation/refolding machinery, namely: DNAJB1, HSPH1 and HSPB1. The widely studied HSPA1A was also induced by MG132 whereas levels of constitutively expressed HSPA8 were not significantly affected. Induction of HSPA6 in differentiated human neuronal cells required higher concentrations of MG132 compared to HSPA1A (Figure 17a), suggesting that HSPA6 is induced by severe proteotoxic stress. Induction of HSPA6 was observed with the low dose of 0.5 µM MG132 at the longer incubation time of 24 h compared to 12 h of incubation. Viability was reduced after 24 h incubation with MG132 but was not significantly affected after 12 h over the range of 0.05 to 2.5 µM MG132 (Figure 17b).
Figure 16. Induction of HSPA6 and components of the protein disaggregation/refolding machine by proteotoxic stress

a Differentiated human SH-SY5Y neuronal cells were treated with the proteotoxic stressor MG132, or celastrol, or celastrol plus arimoclomol for 12 h, followed by Western blot analysis. β-tubulin was used as a loading control. b Change in Hsp band intensity relative to the control lane. Band densities were first normalized to the β-tubulin loading control and then expressed relative to the control lane. Induction of HSPA6, DNAJB1, HSPB1, HSPH1 and HSPA1A was significantly higher (* = p<0.01) after 2.5 µM MG132 compared to co-application of celastrol and arimoclomol (Cel + Arim). The level of constitutively expressed HSPA8 did not change significantly.
Figure a shows Western blot analysis of HSPA6, DNAJB1, HSPB1, HSPH1, HSPA1A, and HSPA8 under various conditions: Control, Cel, Cel + Arim, 0.25 μM MG132, and 2.5 μM MG132. Figure b presents bar graphs comparing the change in expression relative to control for HSPA6, DNAJB1, HSPB1, HSPH1, HSPA1A, and HSPA8 under the same conditions. The bars are shaded to indicate different treatments, and asterisks indicate statistically significant changes.
Figure 17. Effect of MG132 concentration on neuronal induction of HSPA6

a HSPA6 and HSPA1A levels were examined by Western blotting following 12 or 24 h treatment with MG132 at the indicated dosages. Induction of HSPA6 required higher doses of MG132 compared to HSPA1A. Band densities were normalized to the β-tubulin loading control and then expressed relative to the control lane (* = p<0.01; ** = p<0.05). b Effect of MG132 dosage on the viability of differentiated human neuronal cells after 12 h and 24 h (* = p<0.01).
4.3.2 Proteotoxic effects of MG132 on differentiated human neuronal cells

Ubiquitin-tagged neuronal proteins accumulated in differentiated human SH-SY5Y neuronal cells as the MG132 concentration was elevated (Figure 18a) and localized to proteostat-positive neuronal protein aggregates (Figure 18b). Misfolded, aggregation-prone proteins are targeted for proteasomal degradation by ubiquitin, and these proteins have been reported to increase after treatment with MG132 that inhibits the proteasome (Bang et al. 2014; Bang et al. 2016). Ubiquitinated proteins were detected in neuronal cytoplasmic protein aggregates identified by the proteostat marker (encircled in the magnified images on the right in Figure 18b) and confirmed by signal co-localization in the fluorescence intensity line scan of the encircled proteostat-positive protein aggregates (ubiquitin- green line, proteostat- red line). Proteostat has been employed as a marker to identify protein aggregates that form following MG132 in differentiated SH-SY5Y cells (Shen et al. 2011; Bang et al. 2014; Bang et al. 2016). It is a molecular rotor dye that is non-fluorescent in solution and becomes highly fluorescent on binding to aggregated proteins where it intercalates into the strands that hold beta sheets together (Shen et al. 2011).

4.3.3 Intracellular localization of MG132-induced HSPA6 in human neuronal cells

Immunocytochemistry revealed a cytoplasmic localization of MG132-induced HSPA6 at the cytoplasmic poles of differentiated human neuronal cells (indicated in Figure 19a top panels by arrows pointing to green HSPA6 signal). Neuronal cellular processes were detected by α-tubulin (orange signal) and neuronal nuclear DNA by DAPI (blue signal). Heat shock was applied to determine if MG132-induced HSPA6 translocates to the nucleus as observed for
**Figure 18. Elevation of neuronal poly-ubiquitinated proteins and protein aggregation following proteotoxic stress**

a Effect of MG132 dosages on poly-ubiquitinated (poly-Ub) neuronal proteins. b Co-localization of ubiquitin-tagged neuronal proteins (green) with proteostat-positive neuronal proteins (orange, encircled), confirmed by a fluorescence intensity line scan demonstrating ubiquitinated protein peaks (green) overlap with proteostat peaks (red). The line scan was generated using the ImageJ RGB (red-green-blue) Profiler plugin from a TIFF image displaying the proteostat signal in red and the ubiquitin signal in green. Scale bar represents 5 μm. Scale bar in magnified images represents 1 μm.
Figure 19. MG132-induced HSPA6 localizes to the neuronal cytoplasm

a Immunofluorescence localization of HSPA6 (green signal indicated by arrows in upper panel) and HSPA1A (green signal, lower panels) to the cytoplasm of differentiated human neuronal SH-SY5Y cells after treatment with 2.5 µM MG132. DAPI (blue) was used to visualize neuronal nuclear DNA; α-tubulin (orange) identifies neuronal processes. After MG132 treatment 66% of the cells were HSPA6 positive and 95% were HSPA1A positive. Scale bar represents 15 µm. b Effects of heat shock on the localization of MG132-induced HSPA6 and HSPA1A. HS = Heat Shock. Scale bar represents 15 µm.
celastrol and arimoclomol-induced HSPA6 (Chapter 3) and YFP-tagged HSPA6 (Khalouei et al. 2014a). However, MG132-induced HSPA6 did not translocate to the nucleus following heat shock (Figure 19b, green signal in left panels), suggesting that it is committed to proteotoxic response mechanisms in the neuronal cytoplasm. HSPA1A exhibited similar results (Figure 19a and b, bottom panels and right panels).

4.3.4 Targeting of HSPA6 and components of the disaggregation/refolding machine to the periphery of protein aggregates in the neuronal cytoplasm

Higher resolution analysis (Figure 20a, shown in boxed magnified insert) revealed that the cytoplasmic signal for MG132-induced HSPA6 (red) was localized around the periphery of proteostat-positive protein aggregates (orange). Fluorescence intensity line scans (indicated by dashed arrow in the magnified insert shown in Figure 20a) confirmed that HSPA6 fluorescence intensity (red line) was concentrated at the periphery of proteostat-positive cytoplasmic aggregates (green line). As shown in the lower panels of Figure 20a, the HSPA6 signal (red) enveloped proteostat-positive cytoplasmic protein aggregates (orange signal; indicated by top and bottom views of the cytoplasmic aggregate labelled with the white star). MG132-induced HSPA1A (red) also enveloped proteostat-positive protein aggregates (orange signal at white star), shown in Figure 20b.

As shown in Figure 21, the localization of other key components of the disaggregation/refolding machine in relation to proteostat-positive protein aggregates was investigated following treatment of the differentiated human neuronal cells with MG132. DNAJB1 (red signal in Figure 21a) and the small Hsp, HSPB1 (red signal in Figure 21b), localized at the periphery of the proteostat-positive cytoplasmic aggregates (orange signal in
Figure 20. Targeting of MG132-induced HSPA6 to the periphery of cytoplasmic protein aggregates in differentiated human neuronal cells

Cytoplasmic protein aggregates (orange signal) were detected with the proteostat marker. Fluorescence intensity line scans shown on the right demonstrated a HSPA6 and b HSPA1A fluorescence intensity peaks (red) at the periphery of proteostat peaks (green). Lower panels in a and b - The HSPA6 and HSPA1A signals (red) enveloped cytoplasmic protein aggregates (orange; indicated by white star) presented as high magnification top and bottom views. Scale bar represents 5 μm. In high magnification images the scale bar represents 1 μm.
a
Control
MG132

b
Control
MG132

Top view
Bottom view

Figure 21. Localization of components of the protein disaggregation/refolding machine to the periphery of neuronal cytoplasmic protein aggregates

a DNAJB1 (red) and b HSPB1 (red) were observed at the periphery of proteostat-positive cytoplasmic aggregates (orange), confirmed by fluorescence intensity line scans on the right. c The disaggregase HSPH1 also localized at the periphery of protein aggregates (arrows). HSPH1 signal also overlapped with the core of proteostat-positive aggregates in the cytoplasm (arrowheads), confirmed by the fluorescence intensity line scan on the right. After MG132 treatment 91% of the cells were DNAJB1 positive, 98% were HSPB1 positive and 83% were HSPH1 positive. Scale bar represents 5 μm. In high magnification images the scale bar represents 1 μm.
Figures 21a and b), as was observed for HSPA6 (Figure 20a). This was confirmed by fluorescence intensity line scans (proteostat- green line; DNAJB1 and HSPB1- red lines). Signal for HSPH1 (red signal in Figure 21c) was also observed at the periphery of the proteostat-positive cytoplasmic aggregates (orange), indicated by the arrows in the higher magnification panels, and in the fluorescence intensity line scan shown in the right panels in Figure 21c (HSPH1- red line, proteostat- green line). In addition, the HSPH1 signal overlapped with the core of proteostat-positive protein aggregates (arrowheads), confirmed by fluorescence intensity line scan (right panels in Figure 21c).

4.3.5 **Constitutively expressed HSPA8 is targeted to the periphery of cytoplasmic protein aggregates after MG132 and translocates into the nucleus after subsequent heat shock**

As shown in Figure 22, HSPA8 that is constitutively expressed in differentiated SH-SY5Y cells is localized diffusely in the neuronal cytoplasm of control cells. Interestingly, after MG132 treatment, HSPA8 (indicated by the red signal in Figure 22) also localized to the periphery of proteostat-positive cytoplasmic protein aggregates (orange signal), confirmed by fluorescence intensity line scans.

Unlike MG132-induced HSPA6 and HSPA1A (Figure 19b), constitutively expressed HSPA8 translocated after heat shock to nuclei in differentiated human neuronal cells (Figure 23a-c), in addition to localizing to the periphery of cytoplasmic protein aggregates (Figure 23d). Immediately after heat shock, HSPA8 (red), targeted nuclear speckles (arrowheads in magnified inserts of Figure 23a), that were identified by co-localization with SON (orange). The
Figure 22. Constitutively expressed HSPA8 (Hsc70) targets the periphery of cytoplasmic protein aggregates after MG132

a Control. b MG132 treatment. HSPA8 surrounded proteostat-positive cytoplasmic protein aggregates after MG132, confirmed by a fluorescence intensity line scan showing HSPA8 fluorescence peaks (red line) at the periphery of proteostat-positive protein aggregates peaks (green line). 100% of the cells were positive for constitutively expressed HSPA8 in both control and MG132-treated cells. Scale bar represents 5 μm. In high magnification images of the boxed area, the scale bar represents 1 μm.
Figure 23. HSPA8 translocates to nuclear structures after thermal stress in MG132-treated human neuronal cells

Following MG132 for 12 h, cells were subjected to heat shock for 20 min followed by recovery for 1 or 3 h. a At 20 min, HSPA8 (red) co-localized with SON (orange) at nuclear speckles (arrowheads), confirmed by the fluorescence intensity line scan on the right. b At 1 h HSPA8 (red) co-localized with the nucleolar marker nucleophosmin (orange; arrow). c Three hours post-HS HSPA8 (red) was no longer apparent at nuclear structures. The line scan on the right confirmed that HSPA8 (red line) fluorescence did not overlap with SON fluorescence (green line). d During recovery, when HSPA8 targeted the nucleolus (arrow), HSPA8 signal was also apparent at the periphery of protein aggregates (orange), confirmed by the line scan on the right. SON = nuclear speckle marker, NPM = Nucleophosmin, nucleolar marker, HS = Heat Shock. Scale bars represent 5 μm.
fluorescence intensity line scan on the right (scan line indicated by the dashed arrow in the magnified insert) confirmed that HSPA8 fluorescence peaks (red line) overlap with SON fluorescence peaks (green line). As shown in Figure 23b, later at 1 h post-heat shock, HSPA8 co-localized with nucleophosmin (NPM, orange), a label for the GC layer of the nucleolus (arrows) involved in processing of ribosomal RNA and ribosomal subunit assembly (Thiry and Lafontaine 2005; Raska et al. 2006; Hernandez-Verdun et al. 2010). Overlap of HSPA8 fluorescence (red line) and NPM fluorescence was confirmed by the fluorescence intensity line scan on the right. Later during recovery at the 3 h time-point, HSPA8 no longer targeted nuclear structures (Figure 23c), confirmed by the line scan on the right where the HSPA8 fluorescence (red line) no longer coincided with SON fluorescence peaks (green line) at nuclear speckles (arrowheads). At 1 h post-heat shock, when HSPA8 targeted the nucleolus (Figure 23d, arrow), a strong HSPA8 signal was also apparent in the cytoplasm that enveloped proteostat-positive aggregates (orange, confirmed by the line scan panel on the right).

4.3.6 Video representation of the localization of Hsps at the periphery of MG132-induced neuronal cytoplasmic protein aggregates

The localization of stress-induced HSPA6 and HSPA1A, and also constitutively expressed HSPA8, at the periphery of MG132-induced cytoplasmic protein aggregates was visualized in 3D videos presented in Supplementary Figures 1-4 (located in ‘Supplementary Materials’). The video in Supplementary Figure 1 tracks through an image stack to show HSPA6 (red signal) enveloping cytoplasmic protein aggregates (orange signal, white star). Supplementary Figure 2 is a rotational view of HSPA6 around the same protein aggregate.
Rotational views of HSPA1A and constitutively expressed HSPA8 enveloping MG132-induced cytoplasmic protein aggregates are presented in Supplementary Figures 3 and 4.

DNAJB1 (red) and HSPB1 (red) that act co-operatively with HSPA (Hsp70) proteins in the protein disaggregation/refolding machine can be seen enveloping neuronal cytoplasmic protein aggregates (orange, white star) in Supplementary Figures 5 and 6, respectively. Supplementary Figure 7 demonstrates that the disaggregase HSPH1 (red) is localized at both the periphery and the core of neuronal cytoplasmic protein aggregates after MG132 treatment. The yellow signal observed in the core of the protein aggregates represents the overlap of the HSPH1 (red) and proteostat (green) signals.
4.4 Discussion

4.4.1 Proteotoxic stress is a potent inducer of HSPA6 in differentiated human neuronal cells

Potential therapies to counter Alzheimer’s disease that appeared promising in animal models have repeatedly been found to be ineffective in human clinical trials (Dunkel et al. 2012; Cummings et al. 2014; Pratt et al. 2015; Bennett 2018; Cummings et al. 2018). This has led to the suggestion that current mouse models of the disease may not accurately reflect the full complexity of the human neurological disease (McGonigle and Ruggeri 2014; Sasaki 2015). A key feature of neurodegenerative diseases, including Alzheimer’s disease, is proteotoxic stress caused by the inability of the cellular protein quality control machinery to manage misfolded proteins and the formation of toxic oligomers (Muchowski and Wacker 2005; Westerheide and Morimoto 2005; Asea and Brown 2008; Labbadia and Morimoto 2015; Yerbury et al. 2016; Sweeney et al. 2017). There is a critical need to improve our understanding of protein quality control in human neurons to better design effective therapeutic compounds to treat neurodegenerative diseases which have been characterized as protein misfolding disorders.

The current chapter investigated HSPA6 expression and localization in differentiated human neuronal cells following exposure to MG132, a proteotoxic stress-inducing agent (Shen et al. 2011; Bang et al. 2014). The results indicate that severe proteotoxic stress triggers HSPA6 induction in differentiated human neuronal cells, whereas the widely studied HSPA1A is induced by lower dosages of MG132. Chronic proteotoxicity from persistent protein misfolding and aggregation is a prominent feature of neurodegenerative diseases. HSPA6 induction was observed following incubation with lower dosages of MG132 over a longer time frame, suggesting that chronic proteotoxicity may be a trigger for HSPA6 expression. Current animal
models of neurodegenerative diseases that lack the HSPA6 gene may be hampered in their response to severe proteotoxic stress (Chow and Brown 2007; Noonan et al. 2007a). It has been reported that severe stress induces HSPA6 protein expression in human colon cancer cells and that HSPA6 induction is transient (Noonan et al. 2007a; Deane and Brown 2016b). Knockdown of HSPA6 reduced the viability of colon cancer cells in response to MG132 (Noonan et al. 2007a). Interestingly, some human colon cancer cell lines induce HSPA6 in response to MG132 while others do not (Noonan et al. 2007a).

4.4.2 HSPA6 targets cytoplasmic protein aggregates with components of the disaggregation/refolding machine

HSPA6 and key components of the mammalian protein disaggregation/refolding machinery, namely DNAJB1, HSPB1, and HSPH1, were observed to envelope protein aggregates that form in the neuronal cytoplasm after severe proteotoxic stress induced by MG132. Interestingly, the disaggregase HSPH1, but not other Hsps, localized not only to the periphery but also the core of protein aggregates where it may facilitate the disentanglement of aggregated proteins prior to the action of the disaggregation/refolding machine at the periphery of the protein aggregate. HSPA6 has recently been reported to be upregulated in the brains of patients with Alzheimer’s disease, Parkinson’s disease, and dementia with Lewy bodies (Henderson-Smith et al. 2016; Annese et al. 2018; Santpere et al. 2018) which likely reflects a defense attempt to maintain protein homeostasis. Therapeutic enhancement of HSPA6 expression, for example by low dose co-application of celastrol and arimoclomol, could potentiate buffering capacity against protein misfolding and aggregation.
4.4.3 Constitutively expressed HSPA8 may be important for the neuronal response to multiple stressors

Chapter 3 demonstrated that heat shock induced nuclear translocation of HSPA6 in differentiated human SH-SY5Y neuronal cells that were treated with celastrol plus arimoclomol. Chapter 4 showed that MG132-induced HSPA6 is targeted to proteostat-positive cytoplasmic protein aggregates and does not translocate into the nucleus following subsequent heat stress. The lack of redistribution of HSPA6 to the nucleus following thermal stress suggests that MG132-induced HSPA6 is committed to proteotoxic response mechanisms in the neuronal cytoplasm associated with cellular reactions to protein aggregation. Thus, abnormal protein aggregation in the cytoplasm, such as that observed in neurodegenerative diseases, could hamper the ability of neuronal cells to respond effectively to additional stressors.

The present chapter demonstrates that constitutively expressed HSPA8 also localizes to the periphery of cytoplasmic protein aggregates following treatment of differentiated human neuronal cells with the proteotoxic agent MG132. Unlike MG132-induced HSPA6 and HSPA1A that are committed to cytoplasmic stress recovery mechanisms during proteotoxic stress in neuronal cells, HSPA8 is also available to translocate to the neuronal nucleus during subsequent exposure to heat stress. Following stress-induced upregulation of Hsps, levels of constitutively expressed HSPA8 in human cells exceed the level of stress-inducible HSPA proteins (Finka and Goloubinoff 2013; Finka et al. 2015). Thus, limited levels of inducible Hsps may be titrated away by an initial stress while the relative abundance of HSPA8 provides a buffer that can be mobilized in response to an additional stress. This is supported by the observation that in differentiated human neuronal cells under proteotoxic stress, HSPA8 is retained at the periphery of cytoplasmic aggregates upon subsequent exposure to heat shock, however HSPA8 was also
available to translocate to heat-sensitive nuclear structures, unlike the inducible isoforms. This observation underscores the importance of high levels of HSPA8 for neuronal protection from multiple stressors. In aging neurons that experience chronic proteotoxicity as a result of neurodegenerative disease-associated protein misfolding and aggregation, high levels of HSPA8 may be important to protect from added stressors that would otherwise exacerbate protein aggregation and accelerate disease progression.
CHAPTER 5

Knockdown of heat shock proteins HSPA6 and HSPA1A sensitizes differentiated human neuronal cells to cellular stress

5.1 Introduction

As shown in the previous chapter, HSPA6 is not detectable in differentiated human neuronal cells but is induced by proteotoxic stress resulting from treatment with the proteasome inhibitor MG132. Upregulation of HSPA6 in the brains of patients with Alzheimer’s disease, Parkinson’s disease and dementia with Lewy bodies (Henderson-Smith et al. 2016; Annese et al. 2018; Santpere et al. 2018) suggests that it may play a role in the response of human neuronal cells to the accumulation of misfolded protein aggregates. HSPA6 can be upregulated in differentiated human neuronal cells by low dose co-application of celastrol and arimoclomol that does not affect cell viability (Chapter 1). This chapter will investigate if HSPA6 upregulation by this method is protective against a subsequent heat shock stress in differentiated human neuronal SH-SY5Y cells. Following heat shock, HSPA6 and HSPA1A exhibited distinct targeting to stress-sensitive sites in the nucleus (Chapter 3) suggestive of divergent features in thermal stress recovery. HSPA6 localized to perispeckles at the periphery of nuclear speckles at early times in thermal stress recovery and later to the nucleolus with components of disaggregation/refolding machine, which was not observed for HSPA1A or HSPA8. However, it is not known if HSPA6 is protective in neuronal cells.

Tolerance of differentiated human neuronal SH-SY5Y cells to heat shock stress was evaluated with and without prior upregulation of Hsps by co-application of celastrol and arimoclomol. Expression of HSPA6 and HSPA1A was knocked down by small interfering RNA
(siRNA) technology. This chapter investigates HSPA6 and HSPA1A knockdown effects on the viability of human SH-SY5Y neuronal cells following thermal stress.

This chapter has been published in *Neurochemical Research* (2017) as “Knockdown of heat shock proteins HSPA6 (Hsp70B’) and HSPA1A (Hsp70-1) sensitizes differentiated human neuronal cells to cellular stress” by Deane CA and Brown IR. Volume 43, Issue 2, pages 340-350.
5.2 Methods

5.2.1 Cell culture and differentiation

Human SH-SY5Y neuroblastoma cells were plated onto 35 mm glass-bottom plates (for immunofluorescence) and differentiated with 10 μM all-trans-retinoic acid as described in the ‘Methods’ section on ‘Maintenance and differentiation of the SH-SY5Y cell line’ in Chapter 1.

5.2.2 siRNA treatment and Hsp induction

Differentiated neuronal cells were incubated with 1 μM Accell siRNA (Dharmacon, Lafayette, CO, USA) specific for HSPA6 (M-019455-00), HSPA1A (M-005168-01), or non-targeting siRNA (NT; D-001206-13) for 48 h prior to co-application of celastrol (0.3 μM) and arimoclomol (50 μM) for an additional 12 h. For the double knockdown, 1 μM of HSPA6-specific and HSPA1A-specific siRNAs were applied simultaneously. Target sequences of the siRNAs used can be found in Table 1 (Supplementary Material). Non-targeting siRNA tagged with a DY-547 (NT-red; #D-001960-01; Dharmacon) was used to determine the effect of celastrol and arimoclomol co-application on siRNA transfection. Celastrol and arimoclomol were prepared as described in the ‘Methods’ section on ‘Induction of heat shock proteins’ in Chapter 1. Cells were then either fixed for immunofluorescence, or harvested for Western blotting, or exposed to heat shock and subsequently assayed for cell viability.

5.2.3 Immunofluorescence

Antibody staining, fluorescent image acquisition and image analysis were carried out as described in the ‘Methods’ section on ‘Immunofluorescence’ in Chapter 2. Image processing and
fluorescence intensity measurements were performed using Volocity 3D image analysis software (PerkinElmer, Waltham, MA, USA). Images representative of three individual experiments are shown in which 50 cells were analyzed in coverslips harvested from each well of six-well culture plates. Data are presented as the mean fluorescence intensity expressed as a percent change relative to the control (labelled ‘−Cel/Arim’) ± SEM.

5.2.4 Western blotting

Western blotting and densitometry were performed as described in the ‘Methods’ section on ‘Western blotting’ in Chapter 1. Data represent the mean relative to the control (labelled ‘−Cel/ Arim’) ± SEM for three independent replicates.

5.2.5 Heat shock

Cells were subjected to heat shock by immersion in a circulating water bath calibrated at 43 ± 0.2 or 44 ± 0.2 °C for 20 min and then returned to a 37 °C humidified 5% CO₂ atmosphere for subsequent cell viability assay at the indicated time points. The start of heat shock represented the zero time-point (t = 0). For continuous heat shock, cells were immersed in a circulating water bath calibrated at 43±0.2 °C and harvested for cell viability assay at the indicated time points up to 100 min.

5.2.6 Viability determination

Viability was assessed as described for quantitative analysis by trypan blue staining in the ‘Methods’ section on ‘Viability determination’ in Chapter 1. Data was normalized to 100% at the
start of heat shock (0 min time point) and are presented as the mean±SEM for three independent replicates.

For immunofluorescence analysis of cell death, cells were treated at the indicated time points after a 20 min heat shock (44 °C) with 10 μg/mL propidium iodide (PI; #331200; Thermofisher Scientific, Burlington, ON, Canada) for 10 min and then fixed in 4% PFA for 30 min. Antibody staining for cleaved caspase-3, an apoptotic marker (#9661; Cell Signaling Technology, Danvers, MA, USA), was performed using anti-rabbit Alexafluor488® (Molecular Probes, Thermofisher Scientific, Burlington, ON, Canada) as secondary antibody. Image acquisition was carried out as described in the ‘Methods’ section on ‘Immunofluorescence’ in Chapter 1. One hundred cells per time point were scored for PI uptake and cleaved caspase-3 signal in triplicate experiments. Values are presented as a percentage of the total cell count ± SEM.
5.3 Results

5.3.1 Viability of differentiated human neuronal cells is not affected by knockdown of HSPA6 or HSPA1A

As shown in Figures 24a and b, uptake of fluorescently tagged non-targeting siRNA (NT-red) into differentiated human SH-SY5Y neuronal cells was not affected by low dose co-application of celastrol and arimoclomol, which was employed to induce Hsps. Incubation of the differentiated human neuronal cells with HSPA6 siRNA or HSPA1A siRNA did not significantly impact cell viability (Figure 24c).

5.3.2 HSPA6 knockdown does not affect the levels of other Hsps, while knockdown of HSPA1A causes upregulation of HSPA6 and downregulation of DNAJB1

Induction of HSPA6 in differentiated human neuronal cells by co-application of celastrol and arimoclomol was knocked down by HSPA6 siRNA (compare Figure 25a, top tier immunofluorescence panel labeled ‘+HSPA6 siRNA +Ce/Arim’ to top tier panel labeled ‘-siRNA +Ce/Arim’). Incubation with non-targeting (NT) siRNA did not affect the induction of HSPA6 (Figure 25a, top tier panel labeled ‘+NT siRNA +Ce/Arim’). siRNA knockdown of HSPA6 did not impact the induction of HSPA1A (Figure 25a, second tier panels). Expression of DNAJB1, HSPB1, HSPH1 and HSPA8 was also not affected by knockdown of HSPA6 (Figures 25a, bottom panels). As shown in Figure 25b, quantitation of the immunofluorescence signals revealed that HSPA6 siRNA knocked down expression of HSPA6 in the differentiated human neuronal cells by 98.7% ±4.8 while the expression of other Hsps was not affected.

As shown in Figure 26a, HSPA1A siRNA knocked down the induction of HSPA1A in
Figure 24. Viability of differentiated human neuronal cells is not affected by HSPA6 and HSPA1A siRNAs

a Differentiated human SH-SY5Y neuronal cells were incubated with fluorescently tagged non-targeting siRNA (NT-red) in the presence or absence of celastrol/arimoclomol. Neuronal uptake of NT-red was not affected by the presence of celastrol/arimoclomol which was employed to induce Hsps. Neuronal nuclei were identified by DAPI (blue). Scale bar = 10 μM. b Quantitation of NT-red fluorescence signal shown in panel a. c Neuronal viability was not affected by incubation with HSPA6 siRNA or HSPA1A siRNA.
Figure 25. siRNA knockdown of HSPA6 in differentiated human neuronal cells

a Immunofluorescent neuronal images visualizing the effect of HSPA6 siRNA on Hsp induction by celastrol/arimoclomol. HSPA6 siRNA knocked down HSPA6 and expression of other Hsps was not affected. NT siRNA = non-targeting siRNA. Scale bar = 15 µM. b Quantitation of neuronal Hsp fluorescence signals shown in panel a. FI = Fluorescence Intensity; * = p<0.05 (significant difference relative to ‘-Cel/Arim’ treatment); # = p<0.05 (significant difference relative to ‘+Cel/Arim’ treatment).
differentiated human neuronal cells (compare top tier immunofluorescence panel labeled ‘+HSPA1A siRNA +Ce/Arim’ to top tier panel labeled ‘-siRNA +Ce/Arim’). Interestingly, HSPA1A knockdown resulted in the upregulation of HSPA6 and the downregulation of DNAJB1 (Figure 26a, second and third tier panels respectively). HSPA1A knockdown did not affect the expression of HSPB1, HSPH1 and HSPA8 (Figure 26a, lower panels).

The comparative effects of HSPA6 and HSPA1A siRNA on the expression of Hsps in differentiated human neuronal cells is shown in Figure 27. HSPA6 siRNA dramatically knocked down neuronal expression of HSPA6 by 94.2% ±5.2, while HSPA1A and the other Hsps were not comparably affected (compare lane labeled ‘HSPA6 siRNA +Ce/Arim’ to lane labeled ‘+Ce/Arim’). HSPA1A siRNA knocked down expression of HSPA1A in differentiated human neuronal cells by 79.1% ±5.7, downregulated DNAJB1 by 52.8% ±15.6 and upregulated HSPA6 by 125.5% ±39.7 (compare lane labeled ‘HSPA1A siRNA +Ce/Arim’ to lane labeled ‘+Ce/Arim’). Levels of HSPB1, HSPA8 and HSPH1 were not affected. To verify that DNAJB1 knockdown in the HSPA1A siRNA condition was not due to off-target effects, sequence comparison of the HSPA1A- and HSPA6-specific siRNAs with the DNAJB1 mRNA sequence (NM_006145.1) was performed. No sequence overlap was found.
Figure 26. Effect of HSPA1A siRNA on expression of neuronal Hsp

a HSPA1A siRNA knocked down HSPA1A and DNAJB1 and upregulated HSPA6 in differentiated human neuronal cells. Scale bar = 15 µM. b Quantitation of neuronal Hsp fluorescence signals shown in panel a. * = p<0.05 (significant difference relative to ‘-Cel/Arim’ treatment); # = p<0.05 (significant difference relative to ‘+Cel/Arim’ treatment).
Figure 27. Comparison of the knockdown effects of HSPA6 siRNA vs HSPA1A siRNA on differentiated human neuronal cells

a Western blot analysis of the effect of HSPA6 siRNA and HSPA1A siRNA on levels of neuronal Hsps following co-application of celastrol/arimoclomol. β-tubulin was employed as a loading control. b Quantitation of Western blot Hsp levels. * = p<0.01 (significant difference relative to ‘-Cel/Arim’ treatment); # = p<0.01 (significant difference relative to ‘+Cel/Arim’ treatment).
5.3.3 HSPA6 and HSPA1A are required for cellular protection against thermal stress in differentiated human neuronal SH-SY5Y cells

Differentiated human neuronal cells were subjected to heat shock at 43°C for 20 min followed by recovery at 37°C. Cell viability was then measured at time points up to 180 min. As shown in Figure 28a, differentiated human neuronal cells were better able to tolerate thermal stress following co-application of celastrol and arimoclomol which induced Hsps, as evidenced by comparing the ‘+Ce/Arim’ treatment (blue squares; 82.1% ±2.3 at 180 min) with the ‘-Ce/Arim’ treatment (black dots; 67.4% ±3.3 at 180 min). This protective effect was lost if induction of either HSPA6 (orange triangles; 71.6% ±1.2 at 180 min) or HSPA1A (green triangles; 62.9% ±5.8 at 180 min) was knocked down by treatment with siRNA.

More pronounced effects on neuronal cell viability were observed when thermal stress was increased from 43°C to 44°C (Figure 28b). Cell viability was 39.8% ±6.0 at the 180 min recovery point after 44°C heat shock (‘-Ce/Arim’, black dots) and this was increased to 59.5% ±4.2 in the celastrol/arimoclomol co-application treatment (‘+Ce/Arim’, blue squares). Knockdown of HSPA6 by siRNA treatment (‘A6 siRNA +Ce/Arim’, orange triangles) resulted in a loss of the protective effect (cell viability 38.2% ±5.8). An increased negative impact on neuronal cell viability was apparent when HSPA1A was knocked down by siRNA (21.9% ±7.8 cell viability at the 180 min time point, ‘A1A siRNA +Ce/Arim’, green triangles).

Neuronal cells were also exposed to continuous 43°C heat shock for time points up to 100 min (Figure 28c). Protection against thermal stress derived from celastrol/arimoclomol treatment was diminished by HSPA6 siRNA (‘A6 siRNA +Ce/Arim’, orange triangles) with a more pronounced effect observed for HSPA1A knockdown (‘A1A siRNA +Ce/Arim’, green triangles).
Figure 28. siRNA knockdown of HSPA6 and HSPA1A sensitizes differentiated human neuronal cells to thermal stress

a Subsequent to +/- siRNA and +/- celastrol/arimoclomol treatments, neuronal cells were heat shocked at 43°C for 20 min followed by recovery at 37°C. Cell viability was then measured to time points up to 180 min. b Severity of heat shock increased from 43°C to 44°C for 20 min. c Effect of continuous heat shock at 43°C for time points up to 100 min. Symbols: -Cel/Arim (black dots) = neuronal cells not treated with celastrol/arimoclomol to induce Hsps; +Cel/Arim (blue squares) = Hsps induced by co-application of celastrol/arimoclomol; A1A siRNA + Cel/Arim (green triangles) = HSPA1A siRNA coupled with Cel/Arim co-application; A6 siRNA + Cel/Arim (orange triangles) = HSPA6 siRNA coupled with Cel/Arim; NT siRNA + Cel/Arim (red diamonds) = non-targeting siRNA with Cel/Arim. * = p<0.05; ** = p<0.01.
A double knockdown was performed by co-application of HSPA6 and HSPA1A siRNAs. As previously shown in Figure 24c for single addition of the siRNA, Figure 29a demonstrated that addition of the two siRNAs did not significantly impact cell viability. Following induction of Hsps by celastrol/arimoclomol, Western blotting demonstrated an 89.2% ±1.8 reduction of HSPA6 and a 74.0% ±5.8 reduction of HSPA1A by co-application of HSPA6 and HSPA1A siRNAs (Figure 29b). DNAJB1 was 40.6% ±5.6 downregulated in the HSPA6 and HSPA1A double knockdown condition, which was observed previously for HSPA1A knockdown, but not HSPA6 knockdown (Figure 27). Levels of HSPH1, HSPA8, and HSPB1 were unchanged. As shown in Figure 29c, following heat shock at 44 °C and subsequent recovery at 37 °C, similar reduction of neuronal viability was observed in the double knockdown of HSPA6 and HSPA1A (23.4% ±2.9 viability at the 180 min time point, purple triangles; ‘A1A&A6 siRNA +Cel/Arim’) compared with single knockdown of HSPA1A (Figure 28b, 21.9% ±7.8 viability at the 180 min time point).

5.3.4 Effect of HSPA1A and HSPA6 knockdown on neuronal cell death after thermal stress

At the indicated time points after 44 °C heat shock, cells were assayed for propidium iodide (PI) uptake and cleaved caspase-3, an apoptotic marker (Figure 30a). When HSPA1A was knocked down by siRNA, cells were increasingly positive for cleaved caspase-3 at the 20, 60 and 180 min recovery time points (Figure 30b, p<0.05). Interestingly, HSPA6 knockdown resulted in an increasing number of cells that were propidium iodide (PI) positive, as was also observed in the double knockdown treatment (Fig. 7b, p<0.05).
Figure 29. Double knockdown of HSPA1A and HSPA6

**a** Cell viability was not affected by co-application of HSPA1A and HSPA6 siRNAs. **b** HSPA6 and HSPA1A induction that was observed following celastrol/arimoclomol was inhibited by co-application of HSPA6 and HSPA1A siRNAs, but not by non-targeting (NT) siRNA. * = p<0.05; ** = p<0.001. **c** Following siRNA and Cel/Arim treatments, heat shock was applied at 44°C for 20 min followed by recovery at 37°C. Cell viability was then measured to time points up to 180 min. * = p<0.05
Figure 30. Effect of HSPA6 and HSPA1A knockdown on neuronal cell death after thermal stress

a Cells were assayed for uptake of propidium iodide (PI; pink) and signal for cleaved caspase-3 (green), an apoptotic marker. DAPI (blue) was employed to identify nuclei. Scale bar = 20 μm. b Following knockdown of HSPA1A, neuronal cells were increasingly positive for the apoptosis marker cleaved caspase-3 at time points following heat shock, whereas HSPA6 knockdown resulted in an increasing number of PI positive cells.
a

<table>
<thead>
<tr>
<th>Condition</th>
<th>No HS</th>
<th>20 min</th>
<th>60 min</th>
<th>180 min HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ A1A siRNA + Cel/Arim</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ A6 siRNA + Cel/Arim</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ A1A siRNA + A6 siRNA +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cel/Arim</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b

![Graph showing percentage of total cell count for different conditions and time points.](image)
5.4 Discussion

5.4.1 Upregulation of Hsps by celastrol and arimoclomol enhances neuronal tolerance to thermal stress

Low dose co-application of celastrol/arimoclomol that induces Hsps (Chapter 1) enhanced the ability of differentiated human neuronal cells to tolerate thermal stress. Viability was higher in the celastrol/arimoclomol-treated cells compared to control cells at the end of a 20 min period at elevated temperature suggesting that upregulation of Hsps enhanced the ability of cells to survive a sudden change in temperature. At the end of recovery (180 min time point), celastrol/arimoclomol-treated cells maintained 59.5% ±4.2 cell viability compared to 39.8% ±6.0 in control cells. Neuronal cells were unable to survive a continuous heat shock at 44°C for longer than 100 min although viability was improved at individual time points in cells treated with celastrol/arimoclomol relative to control. As a therapeutic strategy to combat neurodegenerative protein misfolding disorders, upregulation of Hsps by co-application of celastrol and arimoclomol could be a protective strategy to enhance stress tolerance and prevent loss of viability in differentiated neuronal cells in vivo.

5.4.2 Effects of HSPA6 and HSPA1A knockdown

Knockdown of HSPA6 did not affect levels of HSPA1A. Hence HSPA1A is not upregulated to compensate for the loss of HSPA6 in differentiated human neurons. siRNA knockdown of HSPA6 did not influence levels of DNAJB1, HSPH1, HSPB1 and HSPA8. In contrast, knockdown of HSPA1A resulted in increased levels of HSPA6 in differentiated human neuronal cells. Interestingly, it has been reported that knockdown of HSPA1A in human cancer
cells lines increased levels of HSPA6 and that knockdown of HSPA6 did not elevate levels of HSPA1A (Noonan et al. 2007a). Knockdown of HSPA1A could be stressful to differentiated human neurons, leading to enhanced induction of HSPA6. Knockdown of HSPA1A in human neuronal cells also caused downregulation of DNAJB1. Their expression may be linked as they work together in an Hsp70/40 protein disaggregation/refolding complex (Nillegoda and Bukau 2015; Nillegoda et al. 2015). Knockdown of HSPA6 and HSPA1A did not result in upregulation of the constitutively expressed Hsp70 family member, HSPA8 (Hsc70).

Knockdown of HSPA6 and HSPA1A resulted in loss of the protective effects of co-application of celastrol/arimoclomol and sensitized human neuronal cells to thermal stress. This suggests that HSPA6 and HSPA1A contribute to the protective effects of celastrol and arimoclomol co-application. More pronounced effects were observed at 44 °C heat shock compared to 43 °C. Knockdown of HSPA1A had a stronger negative impact, compared to HSPA6 knockdown, on the ability of differentiated human neuronal cells to survive heat shock, even though HSPA1A knockdown increased levels of HSPA6. The elevated levels of HSPA6 did not compensate for the loss of HSPA1A with regard to neuronal viability following heat shock. This could be due to different effects on thermal stress recovery which is supported by differential targeting of HSPA6 and HSPA1A to nuclear structures following heat shock that was demonstrated in Chapter 3.

5.4.3 Different effects of HSPA6 and HSPA1A on neuronal cell death following thermal stress

HSPAs have anti-apoptotic properties and are known to influence cell death in a number of different ways (Rerole et al. 2011). When HSPA1A was knocked down, neuronal cells
became increasingly positive for cleaved caspase-3, an apoptotic marker, at time points following heat shock. Knockdown of HSPA6 resulted in a different effect on cell viability in that cells were increasingly positive for propidium iodide uptake, hence HSPA6 may have different effects on the mode of neuronal cell death compared to HSPA1A. Propidium iodide is taken up by cells with a compromised membrane which occurs during late stages of apoptosis or in necrotic cells (Rieger et al. 2011).
CHAPTER 6

Knockdown of HSPA family members enhances the killing effect of the chemotherapeutic agent cisplatin on human neuroblastoma cells

6.1 Introduction

Unlike neurodegenerative diseases in which disease onset is associated with declining Hsp levels and a reduced capacity to mount a heat shock response, cancer cells express elevated levels of Hsps that contribute to their ability to resist cell death and stabilize oncogenic proteins that are prone to misfolding and aggregation (Yaglom et al. 2007; Calderwood and Ciocca 2008; Gabai et al. 2009; Rerole et al. 2011; Calderwood and Gong 2016; Chatterjee and Burns 2017). Elevated levels of HSPA proteins in cancer cells correlates with a high metastatic potential and chemoresistance (Ciocca et al. 2013; Chatterjee and Burns 2017). To compound the problem, treatment of cancer patients with chemotherapeutics such as cisplatin further enhances Hsp levels which can lead to the development of chemoresistance with repeated treatments (Wachsberger et al. 1997; Vargas-Roig et al. 1998; Calderwood and Gong 2016). This chapter investigates levels of HSPA, DNAJ, and HSPH components of the disaggregation/refolding machine following cisplatin treatment in human neuroblastoma cells and the effect of knockdown of HSPA family members on the killing effect of cisplatin. Neuroblastoma is the third most common pediatric cancer and exhibits a high frequency of metastatic disease.

Cisplatin was approved for clinical use in 1978 and is employed to treat a variety of tumor types including neuroblastoma (Dasari and Tchounwou 2014). Administration of cisplatin in vivo results in exposure to both cancerous and non-cancerous cells. A common negative side effect of cisplatin chemotherapy is peripheral neuropathy in which nerve terminals in
differentiated neurons are impacted (Stewart et al. 1982; Gietema et al. 2000; Ruggiero et al. 2013; Starobova and Vetter 2017). Knockdown of HSPA proteins could sensitize cells to cisplatin-induced apoptosis and permit the use of lower therapeutic dosages to limit adverse side effects. Since a major side effect of cisplatin is peripheral neuropathy which results from damage to differentiated neurons, this chapter also investigates the effects of HSPA knockdown on differentiated human neuronal cells.

Chapter 6 was presented at the International Society for Neuroscience (SfN) conference in San Diego (November 3-7th 2018). Presentation Title: Knockdown of Hsp70 heat shock proteins enhances the sensitivity of human neuroblastoma cells to the chemotherapeutic agent cisplatin.
### 6.2 Methods

#### 6.2.1 Cell culture and differentiation

Human SH-SY5Y neuroblastoma cells were plated onto polystyrene tissue culture plates (for Western blotting and viability assays) or 35 mm glass-bottom plates (for immunofluorescence). Dividing neuroblastoma cells were allowed to settle and adhere to the growth surface for 24 h prior to experimental use. Differentiated neuronal cells were generated by incubation with 10 μM all-trans-retinoic acid as described in the ‘Methods’ section on ‘Maintenance and differentiation of the SH-SY5Y cell line’ in Chapter 1 to inhibit cell division and induce the extension of neuronal cellular processes (Jacobs et al. 2006; Cheung et al. 2009).

#### 6.2.2 Treatment with cisplatin and siRNAs

Cisplatin (479306; Sigma Aldrich) was added to the media at the indicated concentrations which have previously been used in human SH-SY5Y cells (Lasorella et al. 1995; Tieu et al. 1999; Wang et al. 2006; Florea et al. 2017). Cells were incubated with the indicated concentrations of cisplatin for 48 h unless otherwise specified. Cisplatin stock was prepared at 0.5 mg/mL dissolved in 0.9% NaCl and stored at -20°C until use. For knockdown experiments, cells were incubated with 1 μM of the indicated Accell siRNAs for HSPA1A (M-005168-01), HSPA8 (E-017609-00), or non-targeting (NT; D-001206-13), from Dharmacon (Lafayette, CO, USA) for 48 h prior to the addition of cisplatin. For double knockdown, 1 μM each of HSPA1A and HSPA8 siRNAs were added. Target sequences of the siRNAs used can be found in Table 1 (Supplementary Material).
6.2.3 Western blotting

Western blotting and densitometry were performed as described in the ‘Methods’ section on ‘Western blotting’ in Chapter 1. Secondary antibodies conjugated to horseradish peroxidase (Sigma Aldrich, St. Louis, MO, USA) were detected using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA) on a Chemidoc XRS+ Gel Imager (Bio-Rad). Data are presented as mean density relative to control ± SEM for three independent blots. Statistical significance was calculated by two-way ANOVA followed by Bonferroni’s test for multiple comparisons using GraphPad Prism 5 software (La Jolla, CA, USA).

6.2.4 Viability assay

Viability was assessed at the end of cisplatin incubation as described for quantitative analysis by trypan blue staining in the ‘Methods’ section on ‘Viability determination’ in Chapter 1. Immunofluorescence time-course analysis of cell death after cisplatin was carried out as described in the ‘Methods’ section on ‘Viability determination’ in Chapter 5.

6.2.5 Immunofluorescence

Antibody staining, fluorescent image acquisition and image analysis were carried out as described in the ‘Methods’ section on ‘Immunofluorescence’ in Chapter 2.
6.3 Results

6.3.1 Induction of Hsps by the chemotherapeutic agent cisplatin

As shown in Figure 31 by Western blot, and quantified in the bar graphs, cisplatin induced heat shock protein HSPA1A in human SH-SY5Y neuroblastoma (NB) cells. Upregulation of DNAJB1 and HSPH1 was also detected. Cisplatin did not induce HSPA6 or alter levels of constitutively expressed HSPA8 in neuroblastoma cells. Treatment with the proteasome inhibitor MG132 was included as a positive control for HSPA6.

Cisplatin also induced HSPA1A and DNAJB1 in differentiated (DIFF) neuronal cells (Figure 31). HSPH1 was upregulated, although the level of induction was lower compared to that observed in neuroblastoma cells. Induction of HSPA6 was not observed and no change was detected in the level of constitutively expressed HSPA8. Differentiated human neuronal cells were obtained by treatment of neuroblastoma cells with retinoic acid which inhibits cell division and stimulates the growth of neuronal processes (Jacobs et al. 2006; Cheung et al. 2009). The time course of Hsp induction following cisplatin is shown in Figure 32. Induction of HSPA1A preceded upregulation of DNAJB1 and HSPH1 in both neuroblastoma and differentiated neuronal cells.

6.3.2 siRNA knockdown of HSPA1A and HSPA8 protein levels

Knockdown of stress-inducible HSPA1A and constitutively expressed HSPA8 was evaluated as shown in Figure 33. HSPA1A siRNA knocked down HSPA1A by 73.5% ±1.1 in neuroblastoma cells and 80.7% ±5.0 in differentiated neuronal cells. HSPA8 siRNA knocked
Figure 31. Induction of Hsps by cisplatin in human SH-SY5Y neuroblastoma cells

Western blot of heat shock proteins HSPA1A, DNAJB1, HSPH1, HSPA6, and HSPA8 following incubation of neuroblastoma (NB) and differentiated (DIFF) neuronal cells with the indicated concentrations of cisplatin for 48 h. Positive control (+ Control) - incubation with 10 µM MG132 which induces Hsps including HSPA6. * = p<0.01; # = p<0.05
Figure 32. Time course of Hsp induction following cisplatin

Hsp levels in human neuroblastoma (NB) and differentiated (DIFF) neuronal cells were determined at time points after incubation with 10 µM cisplatin. HSPA1A upregulation preceded induction of DNAJB1 or HSPH1 in both cell types. * = p<0.01
Figure 33. siRNA knockdown of stress-inducible HSPA1A and constitutively expressed HSPA8

Effect of HSPA1A and HSPA8 siRNAs on Hsp protein levels in neuroblastoma (NB) and differentiated (DIFF) neuronal cells was assessed by Western blotting after incubation with 10 μM cisplatin (CIS) for 48 h. Percent change in Hsp band intensity relative to the control is shown quantitatively in the bar graphs for three independent blots. NT = non-targeting siRNA. CIS = Cisplatin. * = p<0.01
down HSPA8 by 57.1% ±3.6 in neuroblastoma cells and 72.9% ±1.0 in differentiated neuronal cells compared to levels observed in cells treated with cisplatin (CIS) only. HSPA8 siRNA did not affect levels of HSPA1A, and HSPA1A siRNA did not affect levels of HSPA8. Co-addition of HSPA1A and HSPA8 siRNAs resulted in knockdown levels similar to that seen for individual knockdowns (55.6% ±4.6 knockdown of HSPA8 and 74.0% ±1.7 knockdown of HSPA1A in neuroblastoma cells; 76.2% ±3.8 knockdown of HSPA8 and 83.1% ±3.4 knockdown of HSPA1A in differentiated neuronal cells). HSPA1A and HSPA8 siRNAs, alone or in combination, did not affect levels of HSPH1 or DNAJB1. Knockdown of HSPA6 was not examined as it was not induced by cisplatin as shown in Figures 31 and 32.

6.3.3 Knockdown of inducible HSPA1A or constitutively expressed HSPA8 enhances the killing effect of cisplatin on human neuroblastoma cells

The effect of cisplatin treatment on the viability of dividing human neuroblastoma cells is shown in Figure 34, left panel (‘no siRNA’ black line). The killing effect of cisplatin was enhanced in neuroblastoma cells treated with inducible HSPA1A siRNA (red line), but not in cells treated with non-targeting siRNA (yellow line). Knockdown of constitutively expressed HSPA8 (blue line) caused a more pronounced reduction of neuroblastoma cell viability. A decrease in viability was observed in cells treated with HSPA8 siRNA without cisplatin (0 µM cisplatin), suggesting that constitutively expressed HSPA8 is required to maintain cell viability in neuroblastoma cells. HSPA1A siRNA and non-targeting siRNA in the absence of cisplatin did not affect cell viability. Co-application of HSPA8 and HSPA1A siRNAs (green line) resulted in a similar viability effect as individual knockdown of HSPA8.
Knockdown of inducible HSPA1A enhanced the killing effect of cisplatin on human neuroblastoma cells (left panel). HSPA8 knockdown exhibited a more pronounced effect. Dual HSPA1A and HSPA8 knockdown produced a similar viability effect as individual knockdown of HSPA8. Differentiated neuronal cells also displayed enhanced sensitivity to cisplatin when HSPA8 was knocked down. However, the effect was less pronounced compared to that observed for HSPA8 knockdown in dividing neuroblastoma cells. C = control (no siRNA and no cisplatin).

* = p<0.05, ** = p<0.01, *** = p<0.001
Differentiated human neuronal cells were better able to tolerate cisplatin compared to dividing neuroblastoma cells (compare ‘no siRNA’ black line viability curves for neuroblastoma and differentiated neurons in Figure 34). Knockdown of HSPA8 (blue line) reduced cell viability in differentiated neuronal cells after cisplatin treatment compared to no siRNA (black line as shown in Figure 34, right panel). However, the effects were less pronounced compared to the knockdown effects of HSPA8 in neuroblastoma cells. Reduced neuronal viability after cisplatin was also observed following co-application of HSPA8 and HSPA1A siRNAs (green line), whereas a lesser effect was observed for individual knockdown of inducible HSPA1A (red line). As was observed in dividing cells, an impact of HSPA8 siRNA knockdown on viability was also observed in differentiated neuronal cells in the absence of cisplatin (0 μM cisplatin).

6.3.4 Cisplatin triggers cell death earlier in neuroblastoma cells compared to differentiated human neuronal cells

The time-course of cell death was assessed by fluorescence immunocytochemistry employing an antibody against the apoptotic marker cleaved caspase-3 (Cl Casp3; green), and propidium iodide (PI; red), a cell death indicator that is taken up by late apoptotic and necrotic cells having a compromised membrane, but excluded from healthy cells (Rieger et al. 2011). Signals for the cell death markers propidium iodide (PI) and cleaved caspase-3 were detected 2 h after addition of cisplatin to neuroblastoma cells (Figure 35a). These cell death markers were detected later at 12 h in differentiated neurons (Figure 35b). This suggests that differentiated neuronal cells are able to tolerate cisplatin for longer periods of time before being triggered into cell death in comparison to neuroblastoma cells.
Figure 35. Time course of the effect of cisplatin on cell death in neuroblastoma and differentiated neuronal cells

a Fluorescent signal for propidium iodide (PI, red signal) uptake, cleaved caspase-3 (Cl casp3, green signal) and nuclear DNA (DAPI, blue signal) in neuroblastoma cells following incubation with 10 µM cisplatin; b Differentiated neuronal cells following incubation with 10 µM cisplatin. Scale bar represents 15 µm.
6.3.5 Localization of Hsps in cisplatin-treated differentiated human neuronal cells

Cisplatin chemotherapy triggers a negative side effect on differentiated neurons that results in a clinical condition termed peripheral neuropathy (Stewart et al. 1982; Gietema et al. 2000; Ruggiero et al. 2013; Starobova and Vetter 2017). The intracellular localization of HSPA1A and HSPA8 was evaluated to identify cisplatin-sensitive sites in differentiated human neuronal cells. Following cisplatin treatment, HSPA1A and HSPA8 localized to the cytoplasmic poles of differentiated neuronal cells (indicated by arrows in Figure 36, upper panel). Components of the disaggregation/refolding machine DNAJB1 and HSPH1, also localized to the cytoplasmic poles (arrows in Figure 36). As shown at higher magnification in Figure 37, after cisplatin treatment, the signals for HSPA1A and HSPA8 increased in neuronal processes relative to controls, particularly at the distal tip (indicated by arrows in Figure 37a). The increased HSPA1A and HSPA8 signal was accompanied by an increase in the signal for DNAJB1 (figure 37a, upper panel) and HSPH1 (Figure 37a, lower panel), which are components of the disaggregation/refolding machine. As shown in Figure 37b, high resolution fluorescence intensity line scans confirmed co-localization of HSPA1A and HSPA8 with machine components at the distal tip of differentiated neuronal processes.
Figure 36. Localization of Hsps in cisplatin-treated differentiated human neuronal cells

Cells were incubated with 10 µM cisplatin for the indicated time points. HSPA1A, HSPA8, DNAJB1, and HSPH1 localized to the cytoplasmic poles of the differentiated neuronal cells (indicated by arrows). Nuclear DNA was labelled with DAPI (blue). Scale bar represents 15 µm.
**Figure 37. Hsp localization in the distal ends of differentiated neuronal cellular processes after cisplatin**

**a** At higher magnification, immunofluorescent signals for HSPA1A, HSPA8, DNAJB1, and HSPH1 were observed to increase after cisplatin in neuronal cellular processes, particularly at the distal tip (indicated by arrows and confirmed by the fluorescence intensity line scans on the right). Cyto = cytoplasmic pole. Scale bar represents 10 µm. **b** Hsp localization at the distal tip of neuronal processes (boxed area) is shown in magnified images on the right. The line scans demonstrate HSPA1A, HSPA8, DNAJB1, and HSPH1 co-localization after cisplatin treatment at the distal tip of cellular processes of differentiated neuronal cells. The scanned regions are indicated by the dashed arrow in the merge panels. Nuclear DNA was labelled with DAPI (blue). Scale bar represents 2 µm.
6.4 Discussion

6.4.1 Hsps and cancer

Neuroblastoma is a common pediatric cancer that develops from neural crest-derived cells of the sympathoadrenal lineage and exhibits a high frequency of metastatic disease (Davidoff 2012; Ratner et al. 2016; Nakagawara et al. 2018). Cancer cells express elevated levels of Hsps that contribute to the cancer phenotype by inhibiting cancer cell death and stabilizing oncogenic proteins involved in promoting cancer cell proliferation and survival (Yaglom et al. 2007; Calderwood and Ciocca 2008; Gabai et al. 2009; Rerole et al. 2011; Calderwood and Gong 2016; Chatterjee and Burns 2017). Elevated levels of Hsps have been observed in a range of malignant tumor types, correlating with tumor metastasis and associated with a poor prognosis for cancer patients (Ciocca et al. 2013; Chatterjee and Burns 2017).

6.4.2 Cisplatin induces components of a mammalian disaggregation/refolding machine

Cancer cells are dependent on Hsps to stabilize oncogenic proteins that are prone to misfolding and aggregation (Chatterjee and Burns 2017). To compound the problem, treatment of cancer patients with chemotherapeutics, such as cisplatin, further enhances Hsp levels (Ciocca et al. 2013; Calderwood and Gong 2016). Chapter 6 demonstrated that cisplatin triggers induction of HSPA1A in human SH-SY5Y neuroblastoma cells, but not HSPA6 suggesting that HSPA6 is not involved in the response of neuroblastoma cells to cisplatin damage. Cisplatin induced upregulation of DNAJB1 and HSPH1 that represent key components of the protein disaggregation/refolding machine that maintains protein homeostasis in mammalian cells (Gao et al. 2015; Nillegoda and Bukau 2015). The induction of this set of Hsps suggests that
neuroblastoma cells respond to cisplatin by increasing their capacity to counter protein misfolding and aggregation. Upregulation of Hsps impedes the ability of cisplatin to trigger cell death and contributes to the development of drug resistant cells that limit successful cancer therapy (Wachsberger et al. 1997; Vargas-Roig et al. 1998; Calderwood and Gong 2016). In differentiated human neuronal cells, cisplatin induced HSPA1A, DNAJB1, and HSPH1 components of the disaggregation/refolding machine, although the disaggregase protein HSPH1 showed less increase compared to that observed in neuroblastoma cells. This could reflect the inherent proteostasis imbalance of neuroblastoma cells and enhanced susceptibility to protein aggregation due to the high amounts of aggregation-prone oncogenic proteins (Calderwood and Gong 2016; Chatterjee and Burns 2017).

6.4.3 **Knockdown of inducible HSPA1A or constitutively expressed HSPA8 enhances the killing effect of cisplatin on human neuroblastoma cells**

Enhanced sensitivity of human neuroblastoma cells to cisplatin was observed following siRNA knockdown of inducible HSPA1A. This could represent a strategy to reduce therapeutic concentrations of cisplatin needed to trigger apoptosis in neuroblastoma cells and thereby limit adverse side-effects including peripheral neuropathy. Knockdown of inducible HSPA1A has been investigated as a strategy to enhance the killing effect of cisplatin in human osteosarcoma cells (Mori et al. 2017) and cervical squamous cell carcinoma cells (Yoshidomi et al. 2014). Constitutively expressed HSPA8 has received less attention compared to stress-inducible HSPA1A in the cancer literature (Calderwood and Ciocca 2008; Ciocca et al. 2013; Murphy 2013; Sherman and Gabai 2015; Calderwood and Gong 2016). HSPA8 has been recently reported to act more efficiently than stress-inducible HSPA1A as a key component of the
mammalian protein disaggregation/refolding machine (Gao et al. 2015; Kirstein et al. 2017). Furthermore, HSPA8 provides a rapid response mechanism to cellular stress that circumvents the time lag required to upregulate inducible Hsps (Chapters 2, 3 and 4). Hence the knockdown effect of HSPA8 on the sensitivity of human neuroblastoma cells to cisplatin merits investigation.

The effect of HSPA8 knockdown on neuroblastoma cell viability after cisplatin was more pronounced compared to knockdown of inducible HSPA1A. Interestingly, the viability of neuroblastoma cells was reduced by HSPA8 knockdown in cells that did not receive cisplatin. This impact was not observed for cells treated with inducible HSPA1A siRNA, suggesting that knockdown of HSPA8 levels without cisplatin could be a potential therapeutic approach to trigger neuroblastoma cell death. However, knockdown of HSPA8 also had adverse effects on differentiated neuronal cell viability that was not observed for HSPA1A knockdown suggesting that knockdown of the constitutively expressed HSPA8 could have adverse effects on healthy cells and exacerbate side effect damage by cisplatin. Thus, HSPA8 knockdown would require very specific targeting of HSPA8 siRNAs and cisplatin to tumor cells in order to avoid exposure to healthy cells. Targeted delivery mechanisms including nanoparticle carriers that are under development could be a strategy to reduce exposure to healthy cells (MacDiarmid et al. 2009; Creixell and Peppas 2012; Xu et al. 2013; Pina et al. 2017). While specificity of such targeting strategies are improving (Rosenblum et al. 2018), due to potential off-target effects of HSPA8 siRNAs on differentiated neuronal cells, HSPA8 knockdown should be postponed as a therapeutic strategy until targeting specificity is enhanced.
6.4.4 Localization of cisplatin-induced Hsps to the distal ends of differentiated neuronal processes

Both cancerous and non-cancerous cells are exposed to chemotherapeutic agents following in vivo administration (Stewart et al. 1982; Gietema et al. 2000; Ruggiero et al. 2013; Starobova and Vetter 2017). Cisplatin chemotherapy results in toxic side effects such as peripheral neuropathy in which the nerve terminals of differentiated neurons are impacted (Stewart et al. 1982; Gietema et al. 2000; Ruggiero et al. 2013; Starobova and Vetter 2017). An increased signal for both HSPA1A and HSPA8 was observed in neuronal processes after cisplatin treatment of differentiated human neuronal cells. High resolution analysis revealed that HSPA1A and HSPA8 targeted the distal tips of neuronal cellular processes suggesting these sites are cisplatin-sensitive. Components of the disaggregation/refolding machine including the holdase DNAJB1 and the disaggregase HSPH1, co-localized with HSPA1A and HSPA8 at the distal tip of neuronal processes. This suggests that the disaggregation/refolding machine may be involved in protecting neuronal processes in differentiated neuronal cells from cisplatin damage.

In differentiated neurons, knockdown of HSPA8 reduced viability and therefore this strategy could interfere with protective mechanisms at the distal ends of processes, elevating cisplatin side effect damage and increasing symptoms of peripheral neuropathy. A solution to reduce exposure to differentiated neurons is the targeted delivery of cisplatin and HSPA knockdown siRNA to the tumor microenvironment by encasement in nanoparticle carriers (MacDiarmid et al. 2009; Creixell and Peppas 2012; Xu et al. 2013; Pina et al. 2017). While research and development of targeted nanoparticle carriers has exploded in the past few years, none of these strategies have yet advanced beyond clinical trials (Rosenblum et al. 2018). Therefore, until targeting specificity is greatly enhanced to avoid off-target effects of HSPA8 on
healthy cells, HSPA1A represents a safer knockdown target to enhance the killing effect of cisplatin on neuroblastoma cells.
7 CONCLUSION

7.1 Overview

The heat shock response (HSR) is an evolutionarily conserved response to changes in the cellular environment that threaten protein homeostasis and results in the upregulation of a set of heat shock proteins (Hsps) that aid in stress recovery (Morimoto 1993; Morimoto 2011). Transcription of Hsps is controlled by heat shock transcription factor 1 (HSF1) (Morimoto 1993; Morimoto 2011). Activities of Hsps, such as protein refolding (Fan et al. 2003; Goloubinoff and De Los Rios 2007; Mayer 2013; Mattoo and Goloubinoff 2014; Dekker et al. 2015) and disruption of protein aggregates (Nillegoda and Bukau 2015; Nillegoda et al. 2015), require cooperation between several different classes of Hsps. The HSPA (Hsp70) family is a multigene family composed of constitutively expressed and stress-inducible members that are involved in folding proteins to the correct native conformation (Morimoto 2008; Clerico et al. 2015). Members of the DNAJ family deliver misfolded proteins to HSPA and stimulate its ATPase activity for active refolding (Kampinga et al. 2009; Kampinga and Craig 2010; Mattoo and Goloubinoff 2014). HSPH family proteins act as nucleotide exchange factors (NEFs) for HSPA and play a role in directing the fate of misfolded protein substrates for disaggregation and refolding, as opposed to BAG family NEFs that promote proteasomal degradation of HSPA substrates (Vos et al. 2008; Bracher and Verghese 2015b). In mammalian cells, HSPH cooperates with HSPA and DNAJ as components of a protein disaggregation/refolding machine that facilitates dissociation of aggregated proteins (Rampelt et al. 2012; Nillegoda and Bukau 2015; Nillegoda et al. 2015).

To date, key investigations of the mammalian protein disaggregation/refolding machine composed of HSPA, DNAJ and HSPH family members have been carried out by mixing
components with pre-formed aggregates in vitro (Rampelt et al. 2012; Gao et al. 2015; Nillegoda and Bukau 2015; Nillegoda et al. 2015; Kirstein et al. 2017). The present thesis advances knowledge of the machine by identifying subcellular sites where machine components co-localize after stress in differentiated human neuronal SH-SY5Y cells to provide insight into mechanisms that may require protection by the disaggregation/refolding machine in living cells.

The thesis explores three members of the human HSPA family in neuronal cells, namely inducible HSPA6 (Hsp70B’) that has received little attention in the literature, stress inducible HSPA1A (Hsp70-1) and constitutively expressed HSPA8 (Hsc70). The little studied HSPA6 is a strictly inducible member that is found in the human genome but is absent in rat and mouse genomes. Current rodent models of neurodegenerative diseases have been ineffective for the development of human disease-modifying drugs (Dunkel et al. 2012; Cummings et al. 2014; Pratt et al. 2015; Bennett 2018; Cummings et al. 2018). Such treatments have failed in human clinical trials, despite positive preclinical studies (Lang 2010; t Hart et al. 2012; McGonigle and Ruggeri 2014; Sasaki 2015). Rat and mouse genomes are missing the HSPA6 gene that is present in the human genome (Noonan et al. 2007b). A better understanding of differing features between HSPA family members could help to improve our understanding of the stress response in human cells. This could aid in the development of more effective therapies for a wide variety of human diseases in which Hsps influence disease pathology (See ‘Conclusion’ section on ‘Therapeutic Implications’).

The diversity of Hsp multigene families in mammals is thought to have facilitated the specialization of Hsp responses to a variety of stresses and for protection of different subcellular structures and biological processes (Boorstein et al. 1994; Vos et al. 2008; Hageman et al. 2011). The present results advance knowledge of the little studied human HSPA6 and highlight features
that are not observed for the more widely studied members, namely inducible HSPA1A and constitutively expressed HSPA8, in the neuronal stress response. Specifically, HSPA6 exhibited targeting to perispeckles, located at the periphery of nuclear speckles, that have been characterized as transcription sites (Bregman et al. 1995; Mortillaro et al. 1996; Hall et al. 2006; Ghamari et al. 2013). Furthermore, HSPA6 localized to the GC layer of the nucleolus with components of a disaggregation/refolding machine 3 h after heat shock compared to HSPA1A and HSPA8 that targeted the nucleolus earlier in thermal stress recovery at 1 h, and returned to the cytoplasm by 3 h. Low dose co-application of celastrol and arimoclomol that induces HSPA6 improved neuronal viability following exposure to heat shock in differentiated human neuronal cells and siRNA knockdown of HSPA6 increased neuronal sensitivity to heat shock, suggesting that it is involved in neuronal protection against thermal stress.

Additionally, this thesis supports a role for HSPA6 in the response to abnormal protein misfolding and aggregation in differentiated human neuronal cells. Treatment with the proteasome inhibitor MG132 that induces protein aggregation in the cytoplasm is a stronger inducer of HSPA6, compared to low dose co-application of celastrol and arimoclomol. Interestingly, HSPA6 is upregulated in the brains of patients with Alzheimer’s disease, Parkinson’s disease and dementia with Lewy bodies (Henderson-Smith et al. 2016; Annese et al. 2018; Santpere et al. 2018). Under conditions of MG132 stress, HSPA6 localized to the periphery of cytoplasmic aggregates with components of a mammalian protein disaggregation/refolding machine. HSPA6 could play a role in combating abnormal protein aggregation in the neuronal cytoplasm.

The importance of constitutively expressed HSPA8 in the neuronal stress response is also highlighted. HSPA8 is expressed at high levels in neurons relative to other cells types and may
pre-protect neurons from stress (Manzerra et al. 1997; Chen and Brown 2007a). Following thermal stress, HSPA8 translocated to the nucleus and targeted nuclear speckles and the GC layer of the nucleolus that was also observed for inducible HSPA1A. HSPA8 could act as a rapid responder to stress in neuronal cells that circumvents the time lag required to upregulate inducible HSPA1A. Furthermore, HSPA8 but not HSPA6 or HSPA1A translocated into the nucleus and targeted stress-sensitive nuclear structures in differentiated neuronal cells previously treated with MG132 to induce protein misfolding and aggregation in the cytoplasm. Hence HSPA8 may be important in neuronal cells to respond to multiple stressors whereas inducible isoforms that are expressed at lower levels may be titrated by an initial stress (Finka and Goloubinoff 2013; Finka et al. 2015).

Lastly, knockdown of inducible HSPA1A and constitutively expressed HSPA8 was shown to enhance the killing effect of cisplatin in human neuroblastoma cells. HSPA8 knockdown had a more pronounced effect. However, the data also revealed that HSPA8 knockdown enhanced cell death in differentiated human neuronal cells and may hamper their ability to mount an Hsp protective response at the distal ends of neuronal processes. Targeted delivery of cisplatin and HSPA8 siRNAs to the tumor microenvironment in nanoparticle carriers could reduce exposure to differentiated neurons and other healthy cells and thereby minimize potential side effects such as peripheral neuropathy (MacDiarmid et al. 2009; Creixell and Peppas 2012; Xu et al. 2013; Pina et al. 2017). Until specificity of targeting strategies can ensure that off-target effects of HSPA8 knockdown on healthy neurons are avoided, HSPA1A is a safer knockdown target.
7.1.1 HSPA6 (Hsp70B’) and the stress response of human neuronal cells

Expression of HSPA6 was investigated by Western blotting to compare its induction with the more widely studied inducible HSPA1A. In addition, immunofluorescent localization was employed to identify potential differences in subcellular stress-targeting relative to HSPA1A and constitutively expressed HSPA8 in differentiated human neuronal SH-SY5Y cells. Limited data on HSPA6 are available to establish whether its role in the stress response of human cells is similar or divergent from HSPA1A or HSPA8. Unlike HSPA1A that is expressed at low basal levels in most cells, HSPA6 has not been detected in unstressed cells and is thus strictly inducible (Noonan et al. 2007a).

Chapter 3 demonstrated differential targeting of HSPA6 compared to HSPA1A or HSPA8 following heat shock which suggests divergent features in the neuronal response to thermal stress. Figure 40 displays a schematic diagram summarizing the temporal sequence of Hsp targeting that was observed following heat shock in Chapters 2 and 3. HSPA6 exhibited features that were not observed for HSPA1A or HSPA8, including (i) targeting to perispeckles (previously characterized as transcription sites) at the periphery of nuclear speckles, and (ii) targeting to the GC layer of the nucleolus with components of a protein disaggregation/refolding machine 3 h after heat shock. This suggests that HSPA6 could be involved in the recovery of transcription following stress-induced inhibition. Activation of the HSR involves massive transcriptional re-organization to inhibit housekeeping gene transcription and upregulate Hsp gene transcription (Neef et al. 2011; Vihervaara and Sistonen 2014). Dynamic gene transcription is essential to maintain neuronal functions including signal transmission and synaptic plasticity (Qiu and Ghosh 2008; West and Greenberg 2011). Neuronal cells could benefit from rapid transcriptional recovery following stress-induced inhibition.
Figure 38. Schematic of sequential Hsp targeting to nuclear structures and transient co-localization of components of the disaggregation/refolding machine during heat shock recovery in differentiated human neuronal cells

(1) Low dose co-application of celastrol and arimoclomol was employed to upregulate Hsps in differentiated human neuronal cells including HSPA1A (red), HSPA6 (blue), DNAJB1 and HSPB1 (induction indicated by red arrows). (2) Subsequent application of heat shock (43°C) resulted in translocation of Hsps into the nucleus and localization to stress-sensitive sites. At the 20 min and 1 h, nuclear speckles were targeted by HSPA1A and constitutively expressed HSPA8 (orange) along with components of a disaggregation/refolding machine (HSPH1, DNAJB1, DNAJA1, and HSPB1). HSPA1A and HSPA8 also targeted the GC layer of the nucleolus at 1 h with DNAJA1 and BAG-1 that targets HSPA substrates for degradation. HSPA6 targeted perispeckles at 20 min and 1 h which was not observed for other Hsps. (Hsp movement is indicated by green arrows). (3) At 3 h after heat shock, HSPA6 targeted the GC layer of the nucleolus with components of a disaggregation/refolding machine, DNAJB1 and HSPH1, whereas HSPA1A and HSPA8 returned to the cytoplasm. Hence differential targeting of HSPA6 was observed compared with the more widely studied HSPA family members, HSPA1A and HSPA8, suggesting that it plays a unique role in thermal stress recovery of differentiated human neuronal cells. The nucleolus is composed of 3 layers: GC = granular component; FC = fibrillar center; DFC = dense fibrillar component.
Members of the protein disaggregation/refolding machine do not target perispeckles with HSPA6 suggesting its function at transcription sites does not involve dissociation and refolding of proteins. However, HSPA6 has been reported to have high intrinsic ATPase activity and is capable of refolding heat-denatured p53 in the absence of DNAJ proteins (Hageman et al. 2011). Therefore, it is possible that HSPA6 could perform a refolding function at transcription sites that does not require DNAJ or HSPH co-chaperones. High intrinsic ATPase activity in the absence of stimulation by co-chaperones could potentially speed up refolding of substrates which may be beneficial in times of severe proteotoxic stress. However, the lack of regulation by co-chaperones could be dangerous under normal conditions, thus it would require more stringent conditions for expression, consistent with the lack of HSPA6 expression in unstressed cells and upregulation by severe proteotoxic stress, but not other stresses (Noonan et al. 2007a; Deane and Brown 2016b).

Another possibility could be that HSPA6 is involved in stabilizing important neuronal mRNAs at transcription sites during stress-induced transcriptional disruption in preparation for rapid recovery. HSPA proteins play a role in stabilizing housekeeping mRNAs that code for signaling proteins and transcription factors by binding to AU-rich elements in the 3’ untranslated regions (3’UTR) of these mRNAs (Kishor et al. 2013; Kishor et al. 2017). This may be particularly important in neurons that have high energetic demands (Pissadaki and Bolam 2013; Safiulina and Kaasik 2013) and thus would benefit from stabilizing certain mRNAs during temporary, stress-related re-organization of gene expression. This would allow rapid recovery of normal functions and reduce energy requirements associated with the degradation and re-synthesizing of important mRNAs. HSPA binding to RNA is independent of its chaperone
activity (Kishor et al. 2017). Thus, HSPA6 could perform this function despite the lack of co-localization with co-chaperones at perispeckles.

Noncoding RNAs are emerging as key regulators of the stress response in mammals, including the regulation of HSPAs (Mariner et al. 2008; Place and Noonan 2014; Larsen et al. 2017). For example, binding to long non-coding RNAs (lncRNAs) is thought to sequester HSPAs in the nucleolus under some conditions (Audas et al. 2012). In the primate lineage, lncRNAs are highly expressed in the central nervous system and are hypothesized to underlie the origin of higher cognitive function (Baillie et al. 2011; Friedli and Trono 2015; Trono 2015; Larsen et al. 2017). Interestingly, Alu RNA is a primate-specific lncRNA that is transcribed from a retrotransposon and localizes to sites of transcription where it inhibits RNA polymerase during heat shock in human cells (Mariner et al. 2008) and has recently been linked to mitochondrial dysfunction in neurodegenerative diseases (Larsen et al. 2017). Perhaps the evolution of novel regulatory RNAs in the neuronal stress response could have coincided with the evolution of key stress response mediators, for example HSPA family members (i.e. HSPA6), to facilitate the interaction between RNA polymerase and Alu RNA that is not present in rat or mouse cells. Further research is needed to understand the precise functional role of HSPA6 at transcription sites in stressed human neuronal cells.

The data suggest that HSPA6 could also be involved in facilitating nucleolar recovery in the granular component (GC) layer that is involved in ribosomal RNA processing and ribosomal subunit assembly (Thiry and Lafontaine 2005; Raska et al. 2006; Hernandez-Verdun et al. 2010). HSPA6 targeting to the GC layer of the nucleolus was observed later during thermal stress recovery, at 3 h, compared with HSPA1A and HSPA8 that targeted the nucleolus 1 h after heat shock. HSPA6 co-localized with DNAJB1 and HSPH1 components of the
disaggregation/refolding machine suggesting a potentially different role in nucleolar recovery compared with HSPA1A and HSPA8 that co-localized with DNAJA1, and BAG-1 that is associated with targeting of HSPA substrates for proteasomal degradation (Bracher and Verghese 2015a; Bracher and Verghese 2015b). This could indicate a switch from degradation at early stages of nucleolar recovery to disaggregation/refolding of damaged nucleolar proteins at later times of thermal stress recovery in differentiated human neuronal cells. The latter recovery strategy employing HSPA6, DNAJB1, and HSPH1 components of the disaggregation/refolding machine would appear to be missing in rodent animal models of neurodegenerative diseases.

Knockdown studies presented in Chapter 5 showed that upregulation of HSPA6 by low dose co-application of celastrol and arimoclomol protected differentiated human neuronal cells from a subsequent exposure to thermal stress. In vivo, HSPA6 is upregulated in the brains of patients with Alzheimer’s disease, Parkinson’s disease and dementia with Lewy bodies (Henderson-Smith et al. 2016; Annese et al. 2018; Santpere et al. 2018). Additionally, proteotoxic stress by MG132 that induces the accumulation of protein aggregates in the cytoplasm of differentiated human neuronal SH-SY5Y cells is a potent inducer of HSPA6. Following MG132, HSPA6 targeted the periphery of neuronal cytoplasmic protein aggregates with components of a protein disaggregation/refolding machine including DNAJB1 and HSPH1 (Chapter 4). Taken together these data suggest that HSPA6 could be involved in combating protein misfolding and aggregation stress in differentiated human neuronal cells and thus represent a novel target for the treatment of neurodegenerative diseases. Hence upregulation of a set of Hsps, that includes HSPA6 by low dose co-application of celastrol and arimoclomol, could be a promising therapeutic strategy (Discussed further in the ‘Conclusion’ section on ‘Therapeutic Implications’).
7.1.2 Constitutively expressed HSPA8: a rapid responder to neuronal stress

Studies on the housekeeping functions of HSPAs typically focus on the constitutively expressed HSPA8 (Hsc70). While this is a reasonable strategy given the abundance of HSPA8 and that it is expressed in all cells (Finka et al. 2015), HSPA8 also plays a role in stress recovery. Studies investigating the role of HSPAs in stress recovery typically focus on HSPA1A, also a reasonable strategy given that it is strongly upregulated by stress (Brocchieri et al. 2008). However, HSPA8 is expressed at high levels in neuronal cells relative to other cell types and may pre-protect neurons from cellular stress (Manzerra et al. 1997; Chen and Brown 2007a). The thesis highlights the importance of HSPA8 for neuronal protection from thermal stress, suggesting that it could act as a rapid responder to stress in neuronal cells without the time-lag that is required to upregulate inducible HSPA1A and that it may be required for neurons to respond to multiple stressors.

The stress-induced intracellular targeting pattern observed for HSPA8 in differentiated human neuronal cells was similar to that of HSPA1A following heat shock. Specifically, HSPA1A and HSPA8 translocated into the nucleus and targeted nuclear speckles at 20 min (immediately after heat shock), and later to the GC layer of the nucleolus at 1 h. Nuclear speckles are rich in RNA splicing factors (Corell and Gross 1992; Biamonti and Caceres 2009). Splicing of pre-mRNA in the nucleus is disrupted by heat stress and Hsps are required for splicing recovery (Yost and Lindquist 1986; Yost and Lindquist 1991; Corell and Gross 1992; Biamonti and Caceres 2009). More recently it has been recognized that nuclear speckles also contain other factors involved in RNA metabolism, DNA repair, and epigenetic modifications, and act as a central hub for the co-ordination of gene expression (Glaganski et al. 2017). The importance of nuclear speckles is highlighted by the observation that HSPA8 and HSPA1A
target these structures very rapidly after heat shock, even faster than targeting to the GC layer of the nucleolus that is involved in ribosomal RNA processing and ribosomal subunit assembly (Thiry and Lafontaine 2005; Raska et al. 2006; Hernandez-Verdun et al. 2010). Targeting of HSPA8 was also observed to nuclear speckles and the GC layer of the nucleolus in cells that did not receive celastrol and arimoclomol co-application to induce HSPA1A. This suggests that in neuronal cells, HSPA8 is a rapid stress responder that can be mobilized to stress-sensitive sites without the time-lag that is required to upregulate levels of inducible HSPA1A. Furthermore, HSPA8 localization mimicked that of HSPA1A following proteotoxic stress by MG132 treatment (Chapter 4) and damage caused by treatment with the chemotherapeutic agent cisplatin (Chapter 5) in differentiated human neuronal cells.

Further underscoring the importance of HSPA8 in neuronal cells is the observation that following treatment with MG132, HSPA8, but not HSPA1A or HSPA6, translocated after a subsequent heat shock to the nucleus and targeted nuclear speckles and the nucleolus. Unlike inducible HSPA isoforms that may be ‘tied up’ by an initial stressor, HSPA8 that is expressed at higher levels (Finka and Goloubinoff 2013; Finka et al. 2015) may be important for neurons to respond to multiple stressors. Hence in aging neurons that experience chronic proteotoxicity as a result of neurodegenerative disease-associated protein misfolding and aggregation, high levels of HSPA8 may be important to protect from added stressors that would otherwise exacerbate protein aggregation and accelerate disease progression.

Interestingly, levels of the constitutively expressed HSPA8 in different neuronal subtypes correlates with the frequency of neurodegenerative diseases that affect those neuronal subpopulations (Chen and Brown 2007a). For example, cortical neurons, associated with Alzheimer’s disease and high disease frequency, exhibit low levels of cortical HSPA8, and hence
reduced buffering capacity against protein misfolding and aggregation. Whereas, motor neurons, associated with ALS and low disease frequency, demonstrate high levels of constitutively expressed HSPA8, and hence increased buffering capacity against protein misfolding and aggregation. Thus, enhancing HSPA8 levels could be a promising approach to improve neuronal buffering capacity, particularly for Alzheimer’s disease patients in which HSPA8 pre-protection of affected neurons may be lower.

### 7.2 Therapeutic Implications

As key components of the cellular stress response, Hsps are essential to maintain the proteostatic health of a cell (Morimoto 2011; Clerico et al. 2015). As a result, they are implicated in a wide range of human diseases, including neurodegenerative diseases and cancer which are explored in the present thesis (Muchowski and Wacker 2005; Westerheide and Morimoto 2005; Asea and Brown 2008; Sherman and Gabai 2015; Chatterjee and Burns 2017). Declining Hsp levels and reduced capacity to mount a heat shock response with age are associated with late onset neurodegenerative diseases, also referred to as ‘protein misfolding disorders’ (Mattson and Magnus 2006; Asea and Brown 2008). Because of their role in maintaining protein homeostasis, upregulation of Hsps has been proposed as a strategy to combat protein misfolding and aggregation in neurodegenerative diseases (Muchowski and Wacker 2005; Westerheide and Morimoto 2005; Asea and Brown 2008). Conversely, mutations that cause increased expression of Hsps are associated with a greater likelihood of developing cancer (Calderwood and Ciocca 2008; Calderwood and Gong 2016). Hsps act co-operatively in a number of signaling networks that control key features of the cancer phenotype (Calderwood and Ciocca 2008; Calderwood and Gong 2016; Wu et al. 2017). Elevated levels of Hsps contribute to the ability of cancer cells to evade
cell death mechanisms and help to stabilize oncogenic proteins that promote cancer cell survival and proliferation (Calderwood and Ciocca 2008; Ciocca et al. 2013). Furthermore, Hsps contribute to chemotherapy-resistant phenotypes in many types of cancer, including neuroblastoma (D’Aguanno et al. 2011; Wu et al. 2017). Thus, downregulation of Hsps in cancer cells has been proposed as a strategy to enhance the killing effect of chemotherapeutic agents such as cisplatin (Chatterjee and Burns 2017).

A better understanding of the proteostasis machinery in human cells is required to design more effective Hsp-based therapies for human diseases. This thesis advances knowledge of members of the human HSPA multigene family in human neuronal cells, demonstrating novel results on their intracellular localization. Upregulation of HSPA6 by MG132 that triggers accumulation of protein aggregates in human neuronal cells, and its upregulation in the brains of patients with Alzheimer’s disease, Parkinson’s disease or dementia with Lewy bodies (Henderson-Smith et al. 2016; Annese et al. 2018; Santpere et al. 2018), suggests that HSPA6 could play a role in the neuronal response to protein aggregation. HSPA6 is not expressed in dividing or differentiated SH-SY5Y cells treated with the chemotherapeutic agent cisplatin suggesting that it likely is not involved in the response to cisplatin damage. On the other hand, inducible HSPA1A and constitutively expressed HSPA8 may be effective knockdown targets to enhance the killing effect of cisplatin in human neuroblastoma cells. However, due to adverse effects of HSPA8 knockdown in differentiated human neuronal cells, HSPA8 knockdown would require specific targeting to tumor cells in nanoparticle carriers (MacDiarmid et al. 2009; Creixell and Peppas 2012; Xu et al. 2013; Pina et al. 2017). At present HSPA1A is a safer knockdown target until such strategies have shown specificity to avoid off-target effects of HSPA8 siRNA on healthy cells.
7.2.1 HSPA6 as a therapeutic target for the treatment of neurodegenerative diseases

Treatment with the proteotoxic stress-inducing agent MG132 that increases protein aggregation in the cytoplasm is a potent inducer of HSPA6 in differentiated human neuronal SH-SY5Y cells. Furthermore, HSPA6 targets the periphery of misfolded protein aggregates in the neuronal cytoplasm with DNAJB1 and HSPH1 components of the mammalian protein disaggregation/refolding machine that can restore biological function to aggregated proteins (Nillegoda et al. 2015). A 6.3-fold increase of HSPA6 expression in the brains of Alzheimer’s disease patients and a 30.4-fold increase in HSPA6 expression in Parkinson’s disease patients has been reported (Annese et al. 2018). In the brains of patients with Parkinson’s disease with dementia HSPA6 is upregulated 9.4-fold compared with a 3.4-fold increase of HSPA1A (Henderson-Smith et al. 2016). Taken together these data suggest that HSPA6 may be involved in the response of human neuronal cells to abnormal protein misfolding and aggregation.

When HSPA1A was knocked down by siRNA, an upregulation of HSPA6 was observed in human neuronal cells (Chapter 5) and in human colon cancer cell lines (Noonan et al. 2007a). HSPA6 exhibits divergent features relative to HSPA1A in the response of human neuronal cells to thermal stress, as determined by non-overlapping localization (Chapter 3), thus a compensatory viability effect was not observed despite enhanced levels of HSPA6 following HSPA1A knockdown. However, immunofluorescent analysis of HSPA6 and HSPA1A following MG132 revealed similar targeting with DNAJB1 and HSPH1 components of the disaggregation/refolding machine to the periphery of cytoplasmic protein aggregates (Chapter 4) suggesting that both HSPA family members could play a role in combating abnormal protein aggregation in differentiated human neuronal cells.
Therapies that have shown beneficial effects in rat and mouse models of neurodegenerative diseases have failed to translate into effective therapies following advancement to human clinical trials (Lang 2010; t Hart et al. 2012; McGonigle and Ruggeri 2014; Sasaki 2015). Evolution of higher cognitive function is thought to be a major event in primate evolution (Friedli and Trono 2015; Trono 2015). Hence, rat and mouse models may fail to encompass all aspects of human neurological diseases. This is being addressed by the generation of primate animal models using the marmoset (Lang 2010; t Hart et al. 2012; McGonigle and Ruggeri 2014; Sasaki 2015). Interestingly, the HSPA6 gene is found in the marmoset genome (NCBI gene ID: 100411854). However, since marmoset models are in early testing stages, it is important to investigate the effects of potential therapeutic compounds in differentiated human neuronal cells. Low dose co-application of celastrol and arimoclomol is a promising strategy to boost HSPA6 levels in differentiated human neuronal cells without significant adverse effects on neuronal viability or morphology (Chapter 1). This strategy also has the potential to combat additional pathological features of these diseases including inflammatory and oxidative stress (Allison et al. 2001; Venkatesha et al. 2012; Ciocca et al. 2013; Amor et al. 2014; Sharma et al. 2015).

7.2.2 The mammalian protein disaggregation/refolding machine in human neuronal cells: therapeutic potential for neurodegenerative diseases

HSPH1 (Hsp105α), a member of the mammalian HSPH (Hsp110) family, acts co-operatively with HSPA/DNAJ as a disaggregase to dissociate aggregated proteins in vitro (Rampelt et al. 2012; Gao et al. 2015; Nillegoda and Bukau 2015; Nillegoda et al. 2015). The small heat shock protein, HSPB1 (Hsp27), is not an essential member of the
disaggregation/refolding machine, however, it has been shown to enhance protein disaggregation by the machine (Mogk et al. 2003; Duennwald et al. 2012; Rampelt et al. 2012; Nillegoda and Bukau 2015; Nillegoda et al. 2015). Small Hsps (sHsps) form assemblies with protein aggregates making them accessible to HSPA proteins, which subsequently displace sHsps to initiate protein refolding (Zwirowski et al. 2017).

These prior studies on the machine have been carried out *in vitro* via biochemical reconstitution of the disaggregation/refolding reaction by mixing Hsp components with pre-formed protein aggregates (Rampelt et al. 2012; Gao et al. 2015; Nillegoda and Bukau 2015; Nillegoda et al. 2015). Recent studies demonstrated that knockdown of the *C. elegans* homologs of the HSPH and DNAJ components of the disaggregation/refolding machine prevented efficient clearance of GFP aggregates in the nervous system (Kirstein et al. 2017). Furthermore, the machine has been reported to dissolve amyloid fibrils of α-synuclein that are associated with Parkinson’s disease (Gao et al. 2015). To target the disaggregation/refolding machine for the treatment of neurodegenerative diseases, a better characterization of machine components in human neuronal cells is required. To my knowledge, the data presented here are the first to investigate the components of the mammalian protein disaggregation/refolding machine in intact human neuronal cells. Thermal and proteotoxic stress induced co-localization of machine components at stress-sensitive sites in differentiated human neuronal cells. Interestingly, different combinations of HSPA and DNAJ members assembled at nuclear speckles and the nucleolus with the HSPH1 disaggregase, and also at different time points during thermal stress recovery in the nucleolus (Figure 38). This suggests that the machine may recruit different components to optimize the machine at different subcellular sites or during different phases of stress recovery.
7.2.3 Knockdown of HSPA family members as a strategy to enhance the killing effect of cisplatin on human neuroblastoma cells

Knockdown of inducible HSPA1A has been reported to enhance the killing effect of the chemotherapeutic agent cisplatin in human osteosarcoma cells (Mori et al. 2017) and cervical squamous cell carcinoma cells (Yoshidomi et al. 2014). Other members of the human HSPA family have received less attention in the cancer literature. HSPA6 was not induced by cisplatin suggesting that it likely does not play a role in the response to cisplatin in neuroblastoma or differentiated neuronal cells. However, Chapter 6 demonstrated that siRNA knockdown of either inducible HSPA1A or constitutively expressed HSPA8 enhanced the killing effect of cisplatin on dividing human SH-SY5Y neuroblastoma cells. Interestingly, Accell siRNAs employed for the present investigation have been utilized for in vivo animal administration and have potential clinical utility (Hickerson et al. 2011; Nakajima et al. 2012; Gherardini et al. 2013; Hegde et al. 2014). Intracerebroventricular injection of Accell siRNAs into the adult rat brain resulted in successful uptake by neuronal cell types suggesting that these knockdown siRNAs could be effective for adult neurons (Nakajima et al. 2012).

Knockdown of HSPA8 alone reduced the viability of neuroblastoma cells without cisplatin treatment, highlighting the importance of HSPA8 for the viability of neuroblastoma cells. This suggests that HSPA8 knockdown alone, without cisplatin treatment, could be a strategy for tumor cell killing. A caveat is that HSPA8 is essential for the viability of differentiated neuronal cells as a decrease in viability was observed following HSPA8 knockdown in the absence of cisplatin. HSPA8 targeted the distal tips of differentiated neuronal processes with components of a disaggregation/refolding machine following treatment with cisplatin suggesting that HSPA8 is needed to protect the ends of neuronal processes. Damage to
peripheral nerves is a common adverse side effect of cisplatin and causes peripheral neuropathy in patients (Stewart et al. 1982; Gietema et al. 2000; Ruggiero et al. 2013; Starobova and Vetter 2017). Knockdown of HSPA8 in differentiated neuronal cells could increase cisplatin damage to process ends and exacerbate side effects of peripheral neuropathy. A solution to reduce exposure to differentiated neurons is the targeted delivery of cisplatin and HSPA8 knockdown siRNA to the tumor microenvironment by encasement in nanoparticle carriers (MacDiarmid et al. 2009; Creixell and Peppas 2012; Xu et al. 2013; Pina et al. 2017). Strategies for co-targeting cisplatin and siRNAs have been developed that allow sequential release of siRNAs followed by cisplatin for maximal effectiveness (Creixell and Peppas 2012). However, since none of these strategies involving active targeting of nanoparticles to tumor sites and specific uptake by tumor cells have yet completed human clinical trials (Rosenblum et al. 2018), HSPA1A may be a safer knockdown target. Since HSPA8 is ubiquitously expressed in healthy cells, in vivo HSPA8 knockdown could impede protective mechanisms not only in differentiated neurons but also healthy cells surrounding the tumor site and therefore should be avoided until targeting specificity can prevent off-target effects.
REFERENCES

doi:10.1038/417109a

doi:10.1038/nsmb813

lastrol, a potent
antioxidant and anti-inflammatory drug, as a possible treatment for Alzheimer's disease.
Prog Neuropsychopharmacol Biol Psychiatry 25:1341-1357 doi:S0278-5846(01)00192-0

Inflammation in neurodegenerative diseases--an update. Immunology 142:151-166
doi:10.1111/imm.12233

Immunology 129:154-169 doi:10.1111/j.1365-2567.2009.03225.x

Annese A, Manzari C, Lionetti C, Picardi E, Horner DS, Chiara M, Caratozzolo MF, Tullo
A, Fosso B, Pesole G, D’Erchia AM (2018) Whole transcriptome profiling of Late-
Onset Alzheimer's Disease patients provides insights into the molecular changes involved

Neurodegenerative Diseases and Neuroprotection. Springer Science+Business Media
B.V. pp. 1-373,

Audas TE, Jacob MD, Lee S (2012) Immobilization of proteins in the nucleolus by ribosomal
intergenic spacer noncoding RNA. Mol Cell 45:147-157

PM, Rizzi P, Smith S, Fell M, Talbot RT, Gustinich S, Freeman TC, Mattick
retrotransposition alters the genetic landscape of the human brain. Nature 479:534-537
doi:10.1038/nature10531

aggresome formation and autophagy in differentiated SH-SY5Y cells. Neurosci Lett

autophagic clearance. Mol Cell Neurosci 75:71-80 doi:10.1016/j.mcn.2016.06.007

Batulan Z, Shinder GA, Minotti S, He BP, Doroudchi MM, Nalbantoglu J, Strong MJ, Durham
HD (2003) High threshold for induction of the stress response in motor neurons is
associated with failure to activate HSF1. J Neurosci 23:5789-5798 doi:
10.1523/JNEUROSCI.23-13-05789.2003

in the rat cerebellum following hyperthermia. Brain Res Mol Brain Res 75:309-320
doi:S0169328X9900323X

synapse following hyperthermic stress in the brain. J Neurochem 74:641-646 doi:
10.1046/j.1471-4159.2000.740641.x


178


doi:10.1016/j.bbrc.2010.03.050


doi:10.1101/cshperspect.a000646


188


SUPPLEMENTARY MATERIAL

Supplementary Figure 1 Video representation of HSPA6 (Hsp70B’) surrounding cytoplasmic protein aggregates after MG132 treatment of differentiated human SH-SY5Y neuronal cells. The video tracks through an image stack demonstrating HSPA6 (red signal) at the periphery of cytoplasmic protein aggregates (orange signal, indicated by the white star). Nuclear DNA is labelled with DAPI (blue).

Link to Windows Media Video (.wmv) video file of Supplementary Figure 1:

Supplementary Figure 2 Rotational view of HSPA6 surrounding neuronal cytoplasmic protein aggregates. 3D visualization of a differentiated human SH-SY5Y neuronal cell after MG132 showing a rotational view of HSPA6 (red signal) surrounding proteostat-positive protein aggregates (orange signal; indicated by the white star). The nucleus is labelled with DAPI (blue).

Link to Windows Media Video (.wmv) video file of Supplementary Figure 2:

Supplementary Figure 3 Rotational view of HSPA1A (Hsp70-1) localization around the periphery of MG132-induced neuronal cytoplasmic protein aggregates. Rotational view of
HSPA1A (red signal) surrounding proteostat-positive protein aggregates (orange signal, indicated by the white star) in the neuronal cytoplasm. The nucleus is labelled with DAPI (blue).

**Link to Windows Media Video (.wmv) video file of Supplementary Figure 3:**

![Supplementary Figure 3.wmv](attachment://Supplementary Figure 3.wmv)

**Supplementary Figure 4** Video presentation of constitutively expressed HSPA8 (Hsc70) localized at the periphery of cytoplasmic protein aggregates following MG132 treatment. Rotational video of HSPA8 (red) surrounding proteostat-positive neuronal cytoplasmic protein aggregates (orange, indicated by the white star) in a differentiated human SH-SY5Y neuronal cell. The nucleus is labelled with DAPI (blue).

**Link to Windows Media Video (.wmv) video file of Supplementary Figure 4:**

![Supplementary Figure 4.wmv](attachment://Supplementary Figure 4.wmv)

**Supplementary Figure 5** Video representation of DNAJB1 (Hsp40-1) at the periphery of cytoplasmic protein aggregates. Rotational 3D view that shows DNAJB1 (red) around the periphery of cytoplasmic protein aggregates (orange, indicated by the white star) following MG132 treatment. The nucleus is labelled with DAPI (blue).
Supplementary Figure 6 Rotational video of HSPB1 (Hsp27) surrounding neuronal cytoplasmic protein aggregates after MG132 treatment. Rotational view of HSPB1 (red) around the periphery of proteostat-positive protein aggregates (orange, indicated by the white star) in the neuronal cytoplasm. The nucleus is labelled with DAPI (blue).

Supplementary Figure 7 Video representation of HSPH1 (Hsp105α) localized to both the periphery and the core of proteostat-positive protein aggregates after MG132 treatment. The video tracks through image stacks demonstrating the localization of HSPH1 signal (red) at the periphery (arrows) and at the core (arrowheads) of neuronal cytoplasmic protein aggregates (green). The nucleus is labelled with DAPI (blue).
## Table 1. List of siRNA target sequences

Accell SMARTpool siRNAs specific for HSPA1A, HSPA6, HSPA8 and control (non-targeting) were purchased from Dharmacon (Lafayette, CO, USA). Each SMARTpool contains a mixture of 4 Accell siRNAs specific for the target gene. Catalog codes and target sequences for each siRNA are listed in the table. Accell siRNAs contain modifications for self-delivery into difficult-to-transfect cell types without the need for traditional transfection reagents, viruses or electroporation. Accell siRNAs have been used for *in vivo* administration (Hickerson et al. 2011; Hegde et al. 2014) including the central nervous system (Nakajima et al. 2012; Gherardini et al. 2013).

<table>
<thead>
<tr>
<th>Target Gene (Accell SMARTpool)</th>
<th>NCBI Gene ID</th>
<th>Accell siRNA (4 per pool)</th>
<th>siRNA Target Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPA1A (E-005168-01)</td>
<td>3303</td>
<td>A-005168-14</td>
<td>CUAGUAUUUCUGUUUGUCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A-005168-15</td>
<td>CUGCCAUCUUACGACUAUU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A-005168-17</td>
<td>CCUGUGUUUGCAAUGUUGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A-005168-18</td>
<td>CCAUUGAGGAGGUAGAUA</td>
</tr>
<tr>
<td>HSPA6 (E-019455-00)</td>
<td>3310</td>
<td>A-019455-13</td>
<td>CCUUUGACAUUGAUGCUAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A-019455-14</td>
<td>GUUUUGAACUCAGUGCAU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A-019455-15</td>
<td>CUUGCAUGUAUGAAUUUG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A-019455-16</td>
<td>GCUUUCACCUAUUUUUG</td>
</tr>
<tr>
<td>HSPA8 (E-017609-00)</td>
<td>3312</td>
<td>A-017609-14</td>
<td>GAAUCAAGUGCGAAUGAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A-017609-15</td>
<td>CUGAGAUGUUUCAAGAUAU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A-017609-16</td>
<td>UCAAUGUACUUAGAAUAU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A-017609-17</td>
<td>GAGGUGUCUUCUAUGGUUC</td>
</tr>
<tr>
<td>Non-targeting (D-001910-10)</td>
<td>N/A</td>
<td>D-001910-01</td>
<td>UGGUUUACAUUGUGACUA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D-001910-02</td>
<td>UGGUUUACAUUGUUUCUGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D-001910-03</td>
<td>UGGUUUACAUUUUUCUA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D-001910-04</td>
<td>UGGUUUACAUUGUGUGA</td>
</tr>
</tbody>
</table>