Microglial exosome secretion coupled to *TREM2*: implications on neuron-like cells

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A thesis submitted for the degree of Doctor of Philosophy (Ph.D.)
I, Anna Katherina Mallach, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

- Images in Figure 2-1 were kindly provided by Dr Thomas M Piers, UCL Queen Square Institute of Neurology
- Data from the gene array (Chapter 2.2.7.1) were normalised by Dr Thomas M Piers, UCL Queen Square Institute of Neurology
- The mass spectrometry experiments, and subsequent database searches, were performed by Dr Johan Gobom in the laboratory of Professor Henrik Zetterberg, University of Gothenburg
- iPS-derived neurons were provided by Dr Charles Arber, UCL Queen Square Institute of Neurology
Abstract

Gene-wide association studies have implicated microglia, the immune cells of the brain, in the development and progression of Alzheimer’s disease (AD), the most common form of dementia. Genetic variants on the triggering receptor expressed on myeloid cells-2 (TREM2) are associated with an increased risk of developing AD. TREM2 fulfils a range of different functions in microglia and this thesis investigated the contribution of TREM2 variants on the secretion, content and effect of small extracellular vesicles, exosomes, from microglia.

To study this, patient-derived induced pluripotent stem cells (iPSC) carrying TREM2 variants were differentiated into microglia-like cells (iPS-Mg), using a newly developed protocol. These cells displayed microglia like functions and were shown to secrete exosomes. Exosome secretion was decreased from iPS-Mg carrying TREM2 variants, a deficit that could be rescued with increasing the energy availability in the iPS-Mg. Independent of the secretion rate, exosomal protein content was also influenced by the disease-relevant R47Hhet TREM2 variant. Proteomic analysis of exosomes, through mass spectrometry, revealed differences between exosomes from common variant (Cv) and R47Hhet iPS-Mg. Changes in the exosomal proteome were studied after iPS-Mg were exposed to either lipopolysaccharide (LPS), a classical treatment to activate microglia, or apoptotic neurons, a more physiological stimulus. Exosomes from Cv iPS-Mg showed a stimulus-specific shift in protein content, a response that was reduced in exosomes from R47Hhet iPS-Mg. The differences in exosomal protein translated to differential responses in neuron-like cells to these exosomes. As neuronal models SH-SY5Y and iPS-neurons were used and the effect of exosomes was analysed. The effect of exosomes on cell death and cell stress pathways appeared to be dependent on the treatment of iPS-Mg, whilst the TREM2 status of exosome-secreting iPS-Mg also played a role in inducing effects in metabolism and synaptic functioning in neuron-like cells.
Impact Statement

To study the effect of microglial exosomes, in the first part of the project I optimised a protocol to differentiate iPSC into microglia-like cells, resembling primary microglia in terms of both gene expression and function. This protocol was made available to the wider scientific community through several publications (Garcia-Reitboeck et al., 2018; Xiang et al., 2018; Piers et al., 2020), enabling other groups to benefit from this newly developed tool. This has enabled researchers to compare our protocols with others and use it to answer their own research questions.

Next to the importance of the iPSC differentiation protocol, the other results presented in this thesis can also be beneficial for the wider scientific community. Parts of the thesis, focussing on the influence of TREM2 on microglial functioning (Liu et al., 2020b; Piers et al., 2020), have been published in international peer reviewed journals. As well as contributing to other projects, the first manuscript detailing the exosome work has been accepted for publication, whilst the second one has been prepared for publication. Together these papers will give other researchers an insight into the role of microglial exosomes and their effect on neuron-like cells. These publications will disperse the knowledge and novel discoveries, which are presented in this piece of work, to the wider scientific community and will allow them to build on these findings with their own research, benefitting a range of different research avenues. In addition to publishing results obtained during my PhD, I have also presented the research at a range of different national and international conferences, interacting with experts in the field and disseminating my findings to them.

Ultimately, I hope that the work presented in this thesis will further the knowledge about the underlying working mechanisms of cellular interaction in the context of AD, which could eventually benefit patients suffering from this disease.
Acknowledgements

Firstly, I would like to thank my supervisors Professor Jennifer Pocock, Professor Selina Wray and Dr Thomas Piers for their guidance and support during my PhD. Thank you Jenny for allowing me to undertake my PhD in your lab and for guiding me throughout the project. I would also like to thank the LiDo for funding my PhD and Nadine Mogford for always having time for a quick, encouraging chat. I would like to thank the Pocock lab members, in particular Tom for teaching me any and all techniques I needed, his attention to detail when it came to formatting, and for never getting too angry at any lab mishaps that came my way. Thank you to Kat for always being available to discuss data or new experiments and for celebrating the small moments, such as a high yield in iPS-Mg. Thank you also to David for encouraging me whenever I needed it.

I would also like to thank the rest of my amazing support system I had at Wakefield Street, namely Katerina, for listen to many stories about cells and for ensuring that my pink sample box made it to Sweden, Erika, for always having time for a socially distanced chat, and my upgrade examiners, who continued to support and encourage me long after the upgrade itself, Professor Rohan De Silva and Dr Marija Sajic. I would also like to thank everyone on the 1st floor, such as Charlie, for providing both advice, and reagents, whenever I needed them. Special thanks goes out to my collaborators at the School of Pharmacy, the King’s College London Physics Department and Professor Henrik Zetterberg and Dr Johan Gobom.

Away from the lab, I would like to thank my MRes crew for reminding me of life outside of the lab and being there, even when lab life took over and thank you to Leo for countless lunches, walks and conversations, inspiring me to believe in myself, even during the difficult times. Big thanks goes out to Ben, the only one who was genuinely interested in reading this thesis, which didn’t stop him from proofreading it.

I could not have finished this thesis without the loving support of my family. Anke and Mo, you always listened to me complain, kept me sane during the long weeks of lockdown and celebrated every victory of mine even when you didn’t know why. Finally, Matt, you were always there, through the high and lows. From in-depth discussion about data analysis, providing technical support, cooked dinners after long days in the lab to making more cups of tea for me than I count, you did more for me, and my PhD, than I can put into words. Thank you.
# Table of contents

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

CHAPTER 1 INTRODUCTION

1.1 AD

1.1.1 Pathology

1.1.2 Causes and risk factors

1.1.3 Underlying disease mechanisms

1.2 MICROGLIA

1.2.1 Microglia in the healthy brain

1.2.1.1 Microglial inflammation

1.2.1.2 Migration

1.2.1.3 Support for neuronal functioning

1.2.1.4 Astrocytes and microglia

1.2.1.5 Different microglial states

1.2.2 Microglia in AD

1.2.2.1 Genetic risk factors linking microglia to AD

1.2.2.2 Microglial association with Aβ

1.2.2.3 The role of inflammation in AD

1.2.2.4 Interactions with neurons in the context of AD

1.3 TREM2

1.3.1 TREM2 variants and disease

1.3.2 TREM2 processing

1.3.3 TREM2 signalling

1.3.4 Influence of TREM2 on inflammatory processes

1.3.5 Phagocytosis

1.3.6 Survival and metabolism

1.3.7 Migration

1.3.8 Modelling TREM2 involvement in AD models

1.4 EXTRACELLULAR VESICLES (EV): EXOSOMES AND MICROVESICLES (MV)

1.4.1 Nomenclature of EV

1.4.2 Exosome formation

1.4.3 Secretion of exosomes
1.4.4 Exosomal content

1.4.4.1 Protein content

1.4.4.2 RNA content

1.4.4.3 Sorting mechanisms

1.4.5 Interaction with target cells

1.4.6 Exosomes in AD

1.5 Exosomes and TREM2

1.6 Hypothesis and aims of the project

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents

2.1.2 iPSC lines used

2.1.3 Tissue culture reagents

2.1.4 Antibodies

2.1.5 Taqman probes

2.1.6 Commercially available kits

2.1.7 Software

2.2 METHODS

2.2.1 iPSC-Mg generation

2.2.2 Cell treatment

2.2.3 Exosomal extraction protocols

2.2.4 Western Blot (WB)

2.2.5 Cell death FC

2.2.6 Calcium imaging

2.2.7 Gene expression

2.2.7.1 Gene array

2.2.8 ATP determination

2.2.9 Statistics

CHAPTER 3 CHARACTERISATION OF IPS-MG AND THEIR EXOSOMES

3.1 INTRODUCTION

3.1.1 Modelling microglia in vitro

3.1.1.1 Animal models

3.1.1.2 Immortalized cell lines

3.1.1.3 iPSC

3.1.1.4 Development of microglia
4.2.3 Database search 151
4.2.4 Analysis 156
4.2.5 Protein co-expression network analysis (ProCoNA) 156

4.3 RESULTS 162
4.3.1 Quality-controlling the data 162
4.3.2 This dataset resembles other microglial exosome datasets 166
4.3.3 R47H<sup>het</sup> exosomes contain a different proteomic profile 171
4.3.4 LPS activation of iPS-Mg change exosomal content 176
4.3.5 Changes in exosomal proteome is stimulus-specific 179
  4.3.5.1 Changes in the most abundant proteins 179
  4.3.5.2 PCA revealed cluster of samples 180
  4.3.5.3 Functional analysis of identified clusters 180
  4.3.5.4 Individual proteins 182
  4.3.5.5 Influence of TREM2 variations on specific responses to different treatments 183
4.3.6 ProCoNA 194
4.3.7 Effect on exosomal proteins on specific cellular functions 208
  4.3.7.1 Exosomal protein changes could influence synapse function in neurons 208
  4.3.7.2 R47H<sup>het</sup> exosomes contain more DAM-related proteins 210

4.4 DISCUSSION 217
4.4.1 Sample size and quality controlling the dataset 217
4.4.2 Different analysis techniques have different strengths 218
4.4.3 Differences at baseline 219
4.4.4 Effects of treatment 220
  4.4.4.1 Changes following LPS treatment 220
  4.4.4.2 Difference between LPS and apoptotic neurons as treatments 221
4.4.5 Network analysis 222
4.4.6 Implications for neighbouring cells 224
4.4.7 Future work 226
4.4.8 Conclusion 227

CHAPTER 5 EFFECT OF EXOSOMES ON NEURON-LIKE MODELS 229
5.1 INTRODUCTION 229
  5.1.1 Modelling neurons in vitro 229
    5.1.1.1 Immortalized cell lines 229
5.1.1.2 *iPS-neurons* ........................................229
5.1.2 *Neuronal stress pathways* ........................................230
  5.1.2.1 Unfolded protein response (UPR) ..........................230
  5.1.2.2 MAPK ..................................................232
5.1.3 *Synaptic functioning influenced by EV* .................232
  5.1.3.1 Neurite outgrowth ........................................232
  5.1.3.2 Synapse pruning ...........................................233
5.1.4 *Aims* ................................................................233

5.2 *METHODS* ...............................................................234
  5.2.1 *Differentiation of SH-SY5Y* ...............................234
  5.2.2 *Differentiation of iPS-neurons* ............................234
  5.2.3 *Cell treatment* ................................................234
  5.2.4 *Exosomal uptake* ..............................................238
  5.2.5 *Viability of neuron-like cells* ..............................238
  5.2.6 *MTT assay* .....................................................239
  5.2.7 *Staining* .......................................................239

5.3 *RESULTS* ...............................................................241
  5.3.1 *SH-SY5Y take exosomes up through endocytosis* ....241
  5.3.2 *Effect of exosomes on cell death* .........................244
  5.3.3 *Effect on cell stress* .........................................251
    5.3.3.1 In SH-SY5Y ...............................................251
    5.3.3.2 In other iPS-Mg ..........................................251
  5.3.4 *Exosomes have a differential effect on neuronal metabolism* 258
  5.3.5 *Cv exosomes support neuronal development* ..........266
  5.3.6 *Exosomes can influence synaptic functioning* .......271
    5.3.6.1 Changes in synaptic proteins ..........................271
    5.3.6.2 Effect on voltage gated calcium channels ...........271

5.4 *DISCUSSION* .............................................................277
  5.4.1 *Exosome treatment* ...........................................277
  5.4.2 *Cell death and stress* .......................................278
  5.4.3 *Exosomes as autocrine microglial communication pathway* 279
  5.4.4 *Metabolism* ..................................................280
  5.4.5 *Specific neuronal functions: development and synapses* 283
  5.4.6 *Future experiments* ..........................................284
  5.4.7 *Conclusion* ...................................................285

CHAPTER 6  CONCLUSION ..................................................288
REFERENCES ........................................................................................................ 292

APPENDIX ........................................................................................................ 346

7.1 CODE USED IN MATLAB FOR LC-MS ANALYSIS ........................................ 346
7.2 CODE USED FOR ProCoNA ANALYSIS IN RStudio ..................................... 349
7.3 FUNCTIONAL ANNOTATIONS ........................................................................ 357
7.4 DAM-RELATED PROTEINS ........................................................................... 361
7.5 FIJI MACROS ................................................................................................ 364
  7.5.1 Analysis of neurite length ....................................................................... 364
  7.5.2 Analysis of Tubb3 and GAP43 co-localisation .......................................... 364
7.6 LIST OF PUBLICATIONS ............................................................................... 365
List of figures

Chapter 1
Figure 1-1 Microglial functions .......................................................... 33
Figure 1-2 TREM2 variants ............................................................... 43
Figure 1-3 TREM2 signalling pathways ............................................ 48
Figure 1-4 Formation and secretion of different forms of EV ............... 58
Figure 1-5 Exosome uptake ............................................................... 64
Figure 1-6 Overview of thesis structure ............................................ 69

Chapter 2
Figure 2-1 Timeline of differentiation of iPSC into iPS-Mg using TP1 medium ___ 83
Figure 2-2 Characterisation of heat shock protocol used to generated apoptotic SH-SY5Y ........................................................................ 84

Chapter 3
Figure 3-1 Working principle of NTA .................................................. 103
Figure 3-2 Expression of classical microglial genes following different differentiation protocols to generate iPS-derived microglia ............................................................. 106
Figure 3-3 Microglial gene expression analysed using a custom gene array 108
Figure 3-4 Expression of microglial genes in different TREM2 variants .......... 109
Figure 3-5 Ability of iPS-Mg to phagocytose a range of different particles .... 111
Figure 3-6 iPS-Mg secrete both TNF-α and sTREM2 ............................... 113
Figure 3-7 Calcium response in iPS-Mg .............................................. 115
Figure 3-8 Testing two different exosome extraction protocols ................ 117
Figure 3-9 Exosomal fraction contained exosomal markers .................... 118
Figure 3-10 Cell death in iPS-Mg as a potential contaminator of exosomes ... 119
Figure 3-11 Exosomal size distribution as measured through NTA .......... 120
Figure 3-12 Number of exosomes secreted from TREM2 variants analysed with NTA ........................................................................ 125
Figure 3-13 Exosomal numbers secreted from iPS-Mg following different treatments ........................................................................ 126
Figure 3-14 Exosomal CD63 as approximation of exosomal numbers in TREM2 variants ..................................................................... 129
Figure 3-15 Controlling for contamination after apoptotic neuron treatment ... 130
Figure 3-16 Exosomal numbers following cyclocreatine treatment .......... 131
Figure 3-17 Metabolic deficits in TREM2 variants ................................ 132
Figure 3-18 Analysing p-Akt as upstream target of exosome secretion ....... 133
Figure 3-19 ASM levels in iPS-Mg at baseline and after treatment ............ 135
Figure 5-6 Cell death induced by exosomes .................................................. 246
Figure 5-7 Cv exosomes can rescue SH-SY5Y from cell death ..................... 247
Figure 5-8 Exosomes from apoptotic neuron treated iPS-Mg may not be protective
.................................................................................................................. 249
Figure 5-9 Effect of exosomes on induced cell death of iPS-neurons .......... 250
Figure 5-10 Cell stress induced by exosomes in undifferentiated SH-SY5Y ____ 253
Figure 5-11 Cell stress in differentiated SH-SY5Y ...................................... 255
Figure 5-12 Exosomes can induce inflammation in iPS-Mg ...................... 256
Figure 5-13 Effect of exosomes on metabolism ......................................... 260
Figure 5-14 Effect of exosomes on mitochondrial metabolism ............... 263
Figure 5-15 Effect of exosomes on iPS-neuron mitochondrial function ...... 264
Figure 5-16 Cv exosomes increase neurite length of iPS-neurons .............. 267
Figure 5-17 Cv exosomes induce neurite outgrowth .................................. 270
Figure 5-18 Effect of exosomes on synaptic proteins .............................. 273
Figure 5-19 VGCC changes in response to exosomes .............................. 274
Figure 5-20 Characterisation of VGCC on iPS-neurons ............................ 276
Figure 5-21 Summarising diagram of functional effect of exosomes on neighbouring cells .................................................................................. 287
List of tables

Chapter 2
Table 2-1 Composition of TP1 medium .................................................. 82
Table 2-2 Genes assessed in custom gene array .............................................. 90

Chapter 3
Table 3-1 Microglial differentiation protocols .............................................. 102

Chapter 4
Table 4-1 Exosome samples used ............................................................... 152
Table 4-2 TMT labelling .............................................................................. 155
Table 4-3 Exosomal proteins of interest ....................................................... 169

Appendix
Table 7-1 Functional annotation of modules identified in Cv network ........... 357
Table 7-2 Functional annotation of modules identified in R47H\textsuperscript{Het} network .... 359
Table 7-3 DAM-related proteins in exosomes ................................................ 361
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>ADAM10</td>
<td>A disintegrin and metalloproteinase domain-containing protein 10</td>
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<tr>
<td>ADAM17</td>
<td>A disintegrin and metalloproteinase domain-containing protein 17</td>
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<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ASM</td>
<td>Acid sphingomyelinase</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<tr>
<td>BMP-4</td>
<td>Bone morphogenetic protein 4</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>C1q</td>
<td>Complement component 1q</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
</tr>
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<td>CD200</td>
<td>Cluster of differentiation 200</td>
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<td>CD63</td>
<td>Cluster of differentiation 63</td>
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<td>Cluster of differentiation 81</td>
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<tr>
<td>CD9</td>
<td>Cluster of differentiation 9</td>
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<tr>
<td>CGC</td>
<td>Cerebellar granule cell neurons</td>
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<td>CHOP</td>
<td>C/EBP homologous protein</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>CPZ</td>
<td>Chlorpromazine</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor type-1</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement receptor type-3</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
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<td>CSF1</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>CSF1R</td>
<td>Colony stimulating factor 1 receptor</td>
</tr>
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<td>CSF2</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
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<td>Cv</td>
<td>Common variant</td>
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<tr>
<td>CX3CL1</td>
<td>C-X3-C motif chemokine ligand 1</td>
</tr>
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<td>CX3CR1</td>
<td>CX3C chemokine receptor 1</td>
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<tr>
<td>CytoD</td>
<td>Cytochalasin D</td>
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<tr>
<td>DAM</td>
<td>Disease-associated microglia</td>
</tr>
<tr>
<td>DAP12</td>
<td>DNAX-activating protein of 12 kDa</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino 2-phenylindole</td>
</tr>
<tr>
<td>Dil</td>
<td>Diotadecyl 3,3',3'-tetramethylindocarbocyanine</td>
</tr>
<tr>
<td>DiO</td>
<td>3,3' Diotadecyloxacarbocyanine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMEM-F12</td>
<td>Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium</td>
</tr>
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<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EMP</td>
<td>Erythroid-myeloid progenitors</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicles</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial Alzheimer’s disease</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal dementia</td>
</tr>
<tr>
<td>GAP43</td>
<td>Growth-associated protein 43</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>hMac</td>
<td>Human macrophages</td>
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</tr>
<tr>
<td>IRE1</td>
<td>Inositol requiring enzyme 1</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immuno-receptor tyrosine activation motif</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin transferrin-selenium</td>
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<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
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<td>KCl</td>
<td>Potassium chloride</td>
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<td>Keratin 6A</td>
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<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
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<td>LDH-A</td>
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<td>LOAD</td>
<td>Late onset Alzheimer's disease</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<td>Lipopolysaccharide</td>
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<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MAPT</td>
<td>Microtubule association protein tau</td>
</tr>
<tr>
<td>MCT-1</td>
<td>Monocarboxylate transporter 1</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
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<td>miRNA</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MTA</td>
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<td>MTG</td>
<td>Monothioglycerol</td>
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<td>Mammalian target for rapamycin</td>
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</tr>
<tr>
<td>MVB</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NHD</td>
<td>Nasu Hakola disease</td>
</tr>
<tr>
<td>NIF</td>
<td>Nifedipine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase 2</td>
</tr>
<tr>
<td>NRF1</td>
<td>Nuclear respiratory factor 1</td>
</tr>
<tr>
<td>n-SMase</td>
<td>Neutral sphingomyelinase</td>
</tr>
<tr>
<td>NT</td>
<td>Non-treated</td>
</tr>
<tr>
<td>NTA</td>
<td>Nanoparticle tracking analysis</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline solution</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline solution/1% Tween-20</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PEDF</td>
<td>Pigment epithelium-derived factor</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein kinase RNA-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator-1 alpha</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>ProCoNA</td>
<td>Protein co-expression network analysis</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density protein 95</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>Radioimmuno-precipitation assay buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROI</td>
<td>Regions of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SDC</td>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sEV</td>
<td>Small extracellular vesicles</td>
</tr>
</tbody>
</table>

22
<table>
<thead>
<tr>
<th>Protein/Abbreviation</th>
<th>Description</th>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFAIP8L2</td>
<td>Tumour necrosis factor-alpha induced protein 8 like 2 protein</td>
<td>TSN</td>
<td>Translin</td>
</tr>
<tr>
<td>TNF-R1</td>
<td>Tumour necrosis factor receptor 1</td>
<td>Unst</td>
<td>Unstained</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>TRAF1</td>
<td>Tumour necrosis factor receptor associated factor 1</td>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>TREM2</td>
<td>Triggering receptor expressed on myeloid cells-2</td>
<td>VGCC</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>TSG101</td>
<td>Tumour susceptibility gene 101 protein</td>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-CTF</td>
<td>Beta C-terminal fragment</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction

1.1 AD

AD was first defined in 1906 by Alois Alzheimer as a progressive dementia (Alzheimer, 1906). Since then, AD has been identified to be the most common cause of dementia, with nearly 50 million people living with dementia worldwide in 2015 (Prince et al., 2015). With the current increase in ageing populations in developed, but also in developing, countries, the percentage of the population suffering from dementia is expected to double every 20 years (Prince et al., 2015). The disease is characterised by progressive cognitive decline, which mainly affects short-term episodic memory (Penney et al., 2020) in a temporal gradient manner. Next to memory loss, other symptoms of AD include disorientation, but also psychiatric disorders such as depression (Donnelly, 2005; Lam et al., 2013).

1.1.1 Pathology

Symptoms are used to diagnose AD, but it is a diagnosis of exclusion, which is confirmed post mortem upon evaluation of the pathology of the brain (Murden, 1990). New imaging techniques, such as positron emission tomography (PET), have allowed researchers to visualise progression of AD pathology in patients to aid with diagnosis and to track the progression of the disease (Ennerfelt and Lukens, 2020).

The pathological hallmarks of AD are major atrophy in the medial temporal and posteromedial cortices, neuronal loss and presence of specific protein aggregates, amyloid beta (Aβ) and tau (Serrano-Pozo et al., 2011). Aβ is generated through cleavage of the amyloid precursor protein (APP) by β-secretase and then γ-secretase to secrete Aβ into the extracellular space (Zhang et al., 2011). There, Aβ monomers can aggregate into oligomers and finally plaques. The other main pathological hallmark of AD is the hyper-phosphorylation of tau, a protein associated with the cytoskeleton, which induces oligomer formation and aggregation of tau into neurofibrillary tangles (NFT) (Serrano-Pozo et al., 2011).

Aβ and NFT are not exclusive to AD nor are they the only presentations of the disease. Aβ aggregation has also been found in brains of unaffected, healthy controls of similar age (Perez-Nievas et al., 2013) and tau hyperphosphorylation is also found in other neurodegenerative diseases, such as frontotemporal dementia (FTD),
corticobasal degeneration and progressive supranuclear palsy (Spillantini and Goedert, 2013; Gao et al., 2018).

Neuronal cell death in combination with activation of microglia, the immune cells of the brain, are also common hallmarks found in AD patients (Xiang et al., 2006; Perez-Nievas et al., 2013). Preceding neuronal death is loss of synapses in the brain and widespread disturbances in brain networks (Terry et al., 1991; Bi et al., 2018).

AD-related pathology is believed to build up in the brain during a prodromal phase of up to 20 years before clinical symptoms can be observed (Braak et al., 2011; Efthymiou and Goate, 2017). This is mirrored in other neurodegenerative diseases, such as Parkinson’s disease (PD) (Kordower et al., 2013), highlighting the ability of the central nervous system (CNS) to compensate for neuronal loss to a large extent.

Characterisation of the pathological disease progression is done through the Braak staging system, first introduced in 1991 (Braak and Braak, 1991). Tracking the accumulation of hyperphosphorylated tau, through different staining approaches (Braak and Braak, 1991; Braak et al., 2006), six different stages split into three overarching units were identified. In stage I-II, staining is limited to the entorhinal regions, whilst in stage III-IV this has spread to the limbic allocortex and the neighbouring neocortex. In the final two stages, stage V-VI, pathology can also be found in the neocortex and the primary and secondary field (Braak et al., 2006).

1.1.2 Causes and risk factors

The identification of familial AD (FAD) has highlighted the importance of APP cleavage and Aβ production in the development of the disease. Genetic mutations, such as mutations in APP and APP cleavage proteins, cause FAD with symptoms appearing in the 4th and 5th decade of life and display a faster deterioration (Wattmo and Wallin, 2017; Zheng et al., 2018). These account for roughly 5-10% of the total AD cases (Zheng et al., 2018). The majority is caused by late onset AD (LOAD), which is regarded as sporadic in the population.

Genome-wide association studies (GWAS) have revealed a range of different risk factors, which could predispose individuals to developing LOAD. One major risk factor is located on apolipoprotein E (ApoE), with allele ε4 decreasing the age of onset and increasing the incidence of AD (Maestre et al., 1995).
In addition to genetic variability, other risk factors that have been linked to an increased risk of developing AD. Age is the biggest risk factor and another risk factor is gender: women are more likely than men to develop AD (Guerreiro and Bras, 2015; Podcasy and Epperson, 2016). Lifestyle factors and co-morbidities, such as cardiovascular disease, have also been shown to affect the risk of developing AD (Edwards et al., 2019; Vinicius et al., 2019).

1.1.3 Underlying disease mechanisms

The amyloid hypothesis holds that the Aβ deposits, seen in the extensive Aβ plaques, are the main causing factor of the pathology observed in AD patients (Hardy and Higgins, 1992). In short, APP can be cleaved by either α- or β-secretases, followed by γ-secretase to form a range of different cleavage products (Zhang et al., 2011). Aβ is one of these products, generated by the successive cleavage of APP by β- and then γ-secretase, through the amyloidogenic pathway. Mutations associated with FAD on APP and γ-secretase are all associated with an increase in Aβ production, namely Aβ1-42 (Kametani and Hasegawa, 2018). In addition, Down syndrome patients display AD-like pathology by the age of 40, most likely since APP is found on the triplicated chromosome 21 (Kolata, 1985). Next to aberrant production, Aβ clearance has also been hypothesized to be affected in AD (Wildsmith et al., 2013).

Aβ monomers aggregate into oligomers to fibrils and finally plaques (Heppner et al., 2015). Plaques, or intermediate Aβ aggregates, were hypothesized to injure synapses, which could in turn activate microglia (Hardy and Selkoe, 2002). This is followed by progressive neuronal injury, first through loss of synapses and then oxidative damage, and the formation of NFT. Therefore, this hypothesis stipulated that tau pathology formed downstream of Aβ abnormalities (Hardy and Selkoe, 2002), informed by the finding that genetic variants in tau cause tau pathology in FTD but not Aβ plaques (Hutton et al., 1998; Poorkaj et al., 1998), suggesting that Aβ pathology can induce NFT but not vice versa.

In support of the toxicity of Aβ, homogenates from AD patients, carrying a range of different APP cleavage products in different confirmations, were able to induce Aβ plaques in mice, supporting the idea that Aβ pathology can readily spread and amplify (Kane et al., 2000).
However, there are some problems with this hypothesis. Tau pathology is characterised by aggregates of hyperphosphorylated tau, either in NFT in the cell bodies or in threads within the neuronal processes (Braak et al., 2006). Subtle neuronal changes in tau pathology can be observed in individuals as young as 20, with Aβ lesions first occurring at age 30-40 (Braak et al., 2011). As these experiments were conducted on post mortem brain tissue, no correlation could be made between these early changes and the eventual development of AD, but it did reveal that tau pathology was observed prior to Aβ accumulation and that the effect of these changes were very subtle, as neuronal death was not observed in these pre-Braak staged brains (Braak et al., 2011).

Secondly, murine models of AD, modelling Aβ pathology, do not appear to recapitulate the whole picture, as no tau pathology can be found in addition to very little neuronal cell death (Kim et al., 2013). Clinically, Aβ load has not been shown to correlate with neuronal loss and a high Aβ plaque burden can also be observed in age-matched healthy controls (Price et al., 2009; Perez-Nievas et al., 2013; Joshi et al., 2014). Indeed, studies have suggested that Aβ deposition reaches a plateau early after onset of clinical symptoms and subsequently, displays few changes (Joshi et al., 2014). On the other hand, the spread of tau pathology was shown to be strongly correlated with clinical symptoms (Bejanin et al., 2017; Okamura and Yanai, 2017). A recent study has shown that specific post-translational modifications (PTM) of tau can influence the ability of the protein to seed tau aggregates and that these PTM are associated with worse clinical outcomes in patients with typical AD (Dujardin et al., 2020).

Soluble Aβ has been shown to cause synaptic dysfunction and spine loss, ultimately leading to neuronal death in vitro (Joshi et al., 2014). On the other hand, extracts from plaques did not have an effect of synapses, as measured through the induction of long-term potentiation (LTP) (Li et al., 2018). LTP is a long-term change to the strength of individual synapses, with a specific focus lying on hippocampal synapses in the context of AD research (Mango et al., 2019). It is hypothesized that synaptic strength, and in turn LTP, is essential for memory formation and one study has shown that altering synaptic strength, partially through LTP, was immediately responsible for memory formation in rats (Nabavi et al., 2014). Dysfunction in synaptic strength and LTP were found in AD, and other neurodegenerative diseases, and this is believed to lead to deficits in memory formation (Selkoe, 2002). More in-depth analysis of the influence of Aβ on LTP formation showed that in particular the longer
Aβ fragments, longer than 41 amino acids, inhibited LTP whilst the shorter ones did not (Li et al., 2018).

In conclusion, whilst Aβ and tau pathology are key features of AD they do not appear to describe the full picture of the disease, which could impede the development of potential treatments. Therefore, other pathological features of AD, such as neuroinflammation caused by activated microglia, need to be investigated to better understand underlying disease mechanisms and pathways.

1.2 Microglia

1.2.1 Microglia in the healthy brain

Under normal physiological conditions, the brain contains its own specialized immune cells called microglia (Brochard et al., 2009). They are the CNS substitute of lymphocytes (Ginhoux et al., 2010). They develop from the extra-embryonic yolk sac during embryonic development and migrate to the developing brain, where they proliferate and differentiate to become competent immune cells (London et al., 2013). Here the microglia support the developing neurons and continue to do so throughout life (Colonna and Butovsky, 2017) (summarized in Figure 1-1).

1.2.1.1 Microglial inflammation

When microglia detect foreign protein conformations, through pattern recognition receptors, such as toll-like receptors (TLR), a change in phenotype can be observed (Liu et al., 2012; Caldeira et al., 2014; Doorn et al., 2014). This leads to an inflammatory response, able to affect surrounding cells such as neurons and astrocytes (Minagar et al., 2002; Parkhurst et al., 2013; Caldeira et al., 2014; Jebelli et al., 2014; Miyamoto et al., 2016; Bohlen et al., 2017; Wong et al., 2017).

Human microglia have been shown to express all 10 identified TLR, which are able to detect a range of different pathogen-associated molecular pattern (PAMP) (Bsibsi et al., 2002; Jack et al., 2005; Kiellian, 2006; Fiebich et al., 2018). TLR can be activated by lipids, proteins or nucleic acids. TLR2 can sense membrane components from gram-positive bacteria as well as zymosan, whilst TLR4 senses membrane components from gram-negative bacteria, a constitute of which is LPS. TLR3, TLR7, TLR8 and TLR9 all respond to nucleic acids, including RNA (Beg, 2002; Kiellian, 2006; Fiebich et al., 2018). Next to exogenous stimuli, TLR have also been shown to be
activated by endogenous ligands. Necrotic cells were found to activate TLR2 (Li et al., 2001), whilst mRNA, released from dying cells, was found to activate TLR3 (Karikó et al., 2004; Yu et al., 2010). Most TLR couple with MyD88, leading to the activation of nuclear factor κ light chain enhancer of activated B cells (NFκB) downstream of TLR activation (Taganov et al., 2006; Tsai et al., 2014; Paschon et al., 2016).

When both TLR and the interferon gamma (IFN-γ) pathways, through the IFN-γ receptor, are activated, microglia start to mount an inflammatory response and signal the presence of noxious stimuli to their surroundings. This is achieved through production and secretion of cytokines and chemokines, such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1 beta (IL-1β) (Morgan et al., 2004; Mead et al., 2012; Jebelli et al., 2014; Jiang et al., 2016b). The production of reactive oxygen species (ROS) is also increased, impairing the supportive effect microglia have on neurons (Colonna and Butovsky, 2017).

1.2.1.2 Migration

Through their motile processes, microglia can survey the microenvironment of the CNS (Davalos et al., 2005; Nimmerjahn, 2012; Forabosco et al., 2013) and detect signals, which can encourage them to migrate to sites of injury (Davalos et al., 2005; Ohsawa et al., 2007). This migration is regulated through a range of different molecules, called chemoattractants, such as adenosine triphosphate (ATP) or macrophage colony-stimulating factor (CSF1), which has been studied both in animal models and in vitro (Davalos et al., 2005; Phillips et al., 2018). In response to neuronal injury in the mouse cortex, the processes of microglia in the immediate environment reached the site of injury and sealed it off (Davalos et al., 2005). Extracellular ATP, both released from the site of injury, but also from neighbouring astrocytes, acted through a P2RY receptor present on microglia to induce membrane ruffling and subsequently cell migration (Davalos et al., 2005). Whilst this study did not further investigate which specific microglial receptor this was signalled through, two general P2RY receptor inhibitors abolished the response. Constant presence of extracellular ATP after the initial insult was required for microglial branches to reach the site of injury (Davalos et al., 2005). Membrane ruffles are characterised as wave-like structures formed by the detachment and movement of extended cell edges, signalling possible cell migration (Imai and Kohsaka, 2002).

Microglia express both ionotropic and G-protein coupled receptor (GPCR) purinergic receptors that can be activated by extracellular nucleotides such as ATP.
(Ohsawa et al., 2007). Whilst stimulation of the ionotropic P2RX4 increases intracellular calcium concentrations in microglia, phosphoinositide 3-kinase (PI3K) generated through activation of the GPCR P2RY12 is necessary to induce migration of microglia in response to ATP (Ohsawa et al., 2007).

1.2.1.3 Support for neuronal functioning

Microglia also support neuronal tissue maintenance and homeostasis, both through secreted factors and making direct contact with neurons (Penzes et al., 2011; Parkhurst et al., 2013; Miyamoto et al., 2016).

Microglia secrete factors, such as brain-derived neurotrophic factor (BDNF) to support neuronal viability (Crotti and Ransohoff, 2016). In addition to BDNF, microglia can also upregulate the expression of insulin-like growth factor 1, another protective and growth-promoting factor (London et al., 2013). This release of neurotrophic factors by microglia has been shown to support proliferation of neuronal precursors and through this enhance survival of cerebellar granule cell neurons (CGC) (Morgan et al., 2004). This was mediated by the mitogen activated protein kinase (MAPK) and downstream PI3K signalling pathways in neurons and is directly dependent on factors secreted from microglia, but not on physical microglial contact with neurons (Morgan et al., 2004).

Through the expression of neurotransmitter receptors, microglia can sense neuronal activity and respond to it (Pocock and Kettenmann, 2007; Domercq et al., 2013). For example, glutamate can induce superoxide production in primary rat microglia through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to induce microglia to either be protective or toxic for neighbouring neurons, depending on the neurotransmitter used (Mead et al., 2012). In addition, they were able to phagocytose dendritic spines in response to decreased neuronal activity in rodent models in vivo (Schafer et al., 2012; Parkhurst et al., 2013), generating a feedback loop regulating neuronal activity.

Other than responding to neuronal activity, microglial BDNF can also modulate synaptic plasticity in neurons. Elimination of microglia, and their secreted BDNF, led to learning deficits in mice and reduction of synapse formation (Parkhurst et al., 2013). Decreases in both dendritic spine formation and elimination highlighted the importance of microglia in regulating synaptic development and plasticity (Parkhurst et al., 2013).
Besides secreted factors, microglia can also influence synaptic formation and maintenance by making direct contact with neurons. During murine neurodevelopmental periods of synapse formation, microglia contact neuronal dendrites to induce formation of filopodia in neurons (Miyamoto et al., 2016). This is mediated in the neurons through increases in the local calcium concentration and actin accumulation at the site of microglia contact (Miyamoto et al., 2016). The importance of microglia to support spine formation in neurons was shown in mice when microglia were inhibited in the developing brain. This led to decreases in spine density, functional synapses and subsequently, reductions in connectivity across the developing cortex (Miyamoto et al., 2016). This phase of synaptogenesis occurs earlier in development than the activity-dependent synaptic pruning, during which microglia are involved in the phagocytosis of excessive synapses (Miyamoto et al., 2016). This activity-dependent pruning of synapses was mediated through the complement component 3 (C3)/ complement receptor type 3 (CR3) pathway (Schafer et al., 2012). C3, and complement component 1q (C1q), localized to immature synapses in the absence of neuronal activity. C3 was recognised by the microglial CR3 to induce engulfment of presynaptic inputs. Disruption of this system led to sustained deficits of neuronal connectivity (Stevens et al., 2007; Schafer et al., 2012). Another component of this activity dependent synaptic pruning has recently been shown to be “eat me” signals on synapses, such as externalised phosphatidylserine (PS), which can directly interact with TREM2 to induce pruning of excessive synapses (Scott-Hewitt et al., 2020).

When primary rat microglia are activated with LPS, their secreted factors have been shown to reduce synaptic proteins in neurons, prior to induction of neuronal cell death (Jebelli et al., 2014). This effect was mediated through increased p53 signalling in the microglia and loss of synaptic proteins was observed in both primary rat neurons co-cultured with microglia or neurons exposed to microglial-conditioned medium, suggesting that secreted factors from microglia are capable of reducing these proteins without the need of physical contact between these two cell types (Jebelli et al., 2014).

1.2.1.4 Astrocytes and microglia

Microglia can also interact with another type of glia cell, namely astrocytes, to support neuronal functioning. Astrocytes are closely associated with neurons, enveloping up to 100,000 synapses per astrocyte (Wyss-Coray and Rogers, 2012). The effect of astrocytes in the context of neurodegeneration and in response to microglial signals is only slowly being deciphered.
In murine models of AD, inflammatory signals from microglia can recruit astrocytes to launch an inflammatory response (Heneka et al., 2005; Liddelow et al., 2017). A more in-depth analysis of this showed that secretion of microglial interleukin-1 alpha (IL-1α), TNF-α and C1q can directly induce an activation in astrocytes (Liddelow et al., 2017). This was mirrored by an increase of these reactive astrocytes in the brain of patients suffering from neurodegeneration, such as AD, PD and amyotrophic lateral sclerosis (ALS) (Liddelow et al., 2017). The damaging effect of astrocyte activation was seen in a murine PD model of neurodegeneration (Yun et al., 2018). Inhibition of microglial cytokines, with a specific agonist, prevented the activation of astrocytes and in turn protected dopaminergic neurons from degeneration (Yun et al., 2018).

In turn, activated astrocytes lost the ability to promote neuronal survival and synaptogenesis, causing them to become neurotoxic (Liddelow et al., 2017). With this, they mirror ageing astrocytes in the brain, which also display upregulated genes that eliminate synapses (Boisvert et al., 2018). Atrophy of murine astrocytes surrounding plaques was observed in a murine AD model and due to the involvement of astrocytes in modulating synaptic transmission, this could, directly or indirectly, lead to deficient neurotransmission (Olabarria et al., 2010). Expanding this further, astrocytic pathology in AD patients increased both with progression of disease, characterised through increases in Braak stages, and correlated well with the increased burden of Aβ plaques (Simpson et al., 2010). In the PD model, inhibition of astrocyte activation prevented behavioural deficits associated with this animal model from manifesting, highlighting the damaging effect of microglial-induced astrocytic activation (Yun et al., 2018).

Overall, microglia can signal a host of different messages to neighbouring cells, including neurons and astrocytes, which can be altered by processes occurring during neurodegeneration and the development of AD.
Microglia fulfil different functions in the CNS. They secrete cytokines and neurotrophic factors. In addition, they can sense ATP and adenosine diphosphate released from injured tissue, leading to migration and sealing off the site of injury. Through phagocytosis, microglia can clear cellular debris and proteins from the environment. In addition, expressing a range of different receptors, such as TREM2, microglia can sense signals from the extracellular space. Both through secretion of factors and direct contact, microglia can support neuronal development, spine formation and synaptic activity of neurons. Based on Pocock and Piers, 2018.

Figure 1-1 Microglial functions
1.2.1.5 Different microglial states

Depending on the stimulus, peripheral macrophages can either adopt an M1 or M2 state, either pro-inflammatory or regenerative respectively (Mills et al., 2000; Cherry et al., 2014; Zhou et al., 2017). Based on this paradigm and the observation that microglia readily change their morphology in disease, some work has been conducted to apply a similar paradigm to microglial states. Thus, microglia were categorised into three different states: resting microglia, pro-inflammatory M1 microglia and anti-inflammatory M2 microglia (Minagar et al., 2002). M1 microglia were characterised in primary murine microglia to be pro-inflammatory in response to stimuli, such as LPS or IFN-γ leading to an increased expression of nitric oxide synthase 2 (NOS2) (Michelucci et al., 2009), whilst treatment of interleukin-4 (IL-4) was hypothesized to induce an M2 microglial state, characterised through the increased expression of arginase 1 and chitinase-3-like protein (Heppner et al., 2015; Krasemann et al., 2017). This paradigm, classifying microglia into either pro- or anti-inflammatory and therefore toxic or supportive respectively, has recently been called into question for its usefulness, through a range of different studies (Ransohoff, 2016).

In animal models of traumatic brain injury (TBI), a broad spectrum of inflammatory responses were observed in microglia and macrophages of the brain parenchyma, which appeared to express both classical M1 and M2 genes simultaneously (Morganti et al., 2016). Another study into brain macrophages following a mouse model of TBI using single cell ribonucleic acid (RNA) sequencing showed that even on a single cell level there were a range of different polarization states and that individual cells could not neatly be categorised as either M1 or M2 (Kim et al., 2016).

These studies were also expanded to look specifically at microglia, and not just generally at immune cells in the brain. When gene expression was measured in microglia from both aged mice and mice modelling AD, activated microglia did not resemble clear M1 or M2 types and did not even represent intermediate phenotypes (Holtman et al., 2015). More in-depth experiments lead to the classification of microglia based on region, age and disease-association and the interplay between all of these factors, leaving the M1/M2 paradigm behind. During ageing, murine microglia lose some of their regional differences, with hippocampal microglia becoming less discrete from microglia of other regions (Grabert et al., 2016). Region-specific differences were mainly due to differences in the bioenergetics and immune pathways, represented in differences in genes such as Clec4e and Stat1 and genes...
involved in the glycolytic pathway and the electron transport chain (Grabert et al., 2016). Analysis of human microglial genetic signature revealed a distinct age-related profile, with hippocampal microglia showing an increased fold-change in core microglia genes, such as AIF1 and ABI3, with ageing, whilst the same was not observed in microglia from the entorhinal cortex (Patir et al., 2019).

The number of microglia also changed in an age-related manner and was correlated with regional atrophy and inflammatory processes. In addition, murine microglia ageing was also shown to be region-dependent, with microglia of different regions, displaying different kinetics (Grabert et al., 2016). Through ageing, microglia have been shown to decrease the expression of genes involved with young homeostatic microglia, such as Tmem119, but this was not mirrored by increases in a macrophage-like signature, such as with increased expression of Alox5, showing that even aged microglia form a distinct population from macrophages (Grabert et al., 2016).

Distinct changes were also observed in microglia in the brains of AD patients, such as genes involved in microglia activation and immune response, including PYCARD and CX3CR1 changing most in AD and TSPO being upregulated, independently of age-related changes (Forabosco et al., 2013; Patir et al., 2019). Singe cell analysis of microglia in a range of diseases has revealed that rather than adopting a simple binary state, both murine and human microglia can display a range of different stages, based on changes in a range of different proteins (Keren-Shaul et al., 2017; Zhou et al., 2020). In neurodegenerative diseases, individual microglia were found to be on a spectrum ranging from homeostatic microglia to disease-associated microglia (DAM). In human DAM both M1 and M2 markers were found (Forabosco et al., 2013) and other studies also struggled to identify these clear cut microglial states in vivo (Sousa et al., 2017). A study in a murine model of AD found that Aβ deposition significantly enhanced the activated microglial states, which were also found in aged microglia albeit at lower levels, indicating an overlap between ageing and dementia (Frigerio et al., 2019).

The changes of the microglia in separate brain regions mirrored the vulnerability of the region to AD pathology (Patir et al., 2019). This was supported by another study, showing independent clusters of age and disease-related microglia in AD patients (Zhang et al., 2013). Another study in mice has suggested the important role of ApoE in mediating the switch of homeostatic microglia to microglia associated
with neurodegeneration, both in suppressing homeostatic factors and inducing an inflammatory programme associated with neurodegenerative context (Krasemann et al., 2017). Overall, this suggests that microglia can adapt a range of different signatures, depending on the received stimuli such as regional specificities, age and disease.

1.2.2 Microglia in AD

Microglia are believed to play an important role in AD with research continuing to identify whether microglia activity is a cause or effect of neurodegeneration.

1.2.2.1 Genetic risk factors linking microglia to AD

A number of GWAS, studying risk factors that could contribute to the development of AD in the general population, have revealed a range of risk factors that are associated with microglia and the function of the immune system (Lambert et al., 2009, 2013; Guerreiro et al., 2013a; Reitz et al., 2013; Gjoneska et al., 2015).

One protein associated with risk factors increasing the risk of developing AD is complement receptor type-1 (CR1). CR1 is the main receptor for C3, binding of which can induce the complement cascade and the activation of microglia in response to it (Efthymiou and Goate, 2017). Through association with C1q, another agonist for CR1, Aβ can bind to CR1, initiate the activation of the complement system and induce the phagocytosis of Aβ (Jiang et al., 1994; Velazquez et al., 1997).

Variants of TREM2 have been linked to LOAD, but also Nasu Hakola disease (NHD) (Paloneva et al., 2002; Dardiotis et al., 2017). TREM2 is a receptor, solely expressed on microglia in the CNS, which senses lipids, such as anionic carbohydrates and phospholipids, in the extracellular environment (Daws et al., 2003; Filipello et al., 2018; Shirotani et al., 2019). Through downstream signalling pathways, including extracellular signal-regulated protein kinase (ERK) and Akt, TREM2 can influence phagocytosis, survival and migration of microglia (Wang et al., 2015; Efthimiou and Goate, 2017; Zhong et al., 2017b; Garcia-Reitboeck et al., 2018; Konishi and Kiyama, 2018; Phillips et al., 2018; Piers et al., 2020).

Another protein, whose variants are associated with LOAD, is CD33 (Bradshaw et al., 2013). Through downstream signalling partners, CD33 and other proteins from the same protein family, can induce immunosuppression (Estus et al.,
and one specific variant causing the ligand binding site of CD33 to be non-functional has been shown to be protective against AD (Hollingworth et al., 2011; Naj et al., 2011), potentially as it reduced the levels of Aβ in murine models of AD (Griciuc et al., 2013, 2019). CD33 could interact with TREM2, with TREM2 knock out (KO) in a murine model of AD reversing the benefits of a CD33 KO (Griciuc et al., 2019).

1.2.2.2 Microglial association with Aβ

Microglia were found in close association with extracellular Aβ plaques, one of the pathologies associated with the disease (Minagar et al., 2002). There are several hypotheses regarding this close association and the impact on disease progression.

The interaction of plaques with microglia could be damaging, as Aβ can interact with microglia through a range of different receptors, such as scavenger receptor class A, receptor for advanced glycation end (RAGE) products and CD36. Binding of fibrillary Aβ to the CD36 receptor has been shown to induce the activation of primary murine microglia, characterised by increases in microglial ROS and secretion of IL-1β (Bamberger et al., 2003). Following association of murine microglia with fibrillary Aβ plaques, they adopt an inflammatory phenotype, characterised by the expression of major histocompatibility complex (MHC) class 1 and class 2 peptides in addition to secretion of inflammatory cytokines (Bamberger et al., 2003).

Microglia could also reduce Aβ deposition by phagocytosing these particles. Murine microglia have been shown to internalize small aggregates of Aβ through scavenger receptor (SR) and clear it from the extracellular space (Paresce et al., 1996). However, binding of Aβ to SR has also been shown to activate inflammatory responses in the microglia (Paresce et al., 1996). Besides Aβ phagocytosis leading to changes in inflammatory cytokines in microglia, ROS were also generated in microglia exposed to Aβ (Minagar et al., 2002). The released cytokines and ROS can have a detrimental effect on neighbouring neurons but also astrocytes (Minagar et al., 2002). Through binding of Aβ to one of the expressed receptors, microglia subsequently phagocytose Aβ (Paresce et al., 1996; Bamberger et al., 2003; Liu et al., 2005). This process could be impaired in AD, as inefficient clearance of Aβ is regarded as a contributor to the pathogenesis of AD (Griciuc et al., 2013). Patients with LOAD do not carry FAD-linked mutations affecting Aβ production, so in this large patient group, increased accumulation of Aβ could be influenced by inefficient Aβ clearance mechanisms, as described in AD patients (Mawuenyega et al., 2010). GWAS also suggested that phagocytosis could be impaired, as microglial genes
identified through these studies, such as TREM2, encode proteins implicated in phagocytosis (Garcia-Reitboeck et al., 2018; Piers et al., 2020).

Microglia appear to fulfil another role in close association with plaques, as elimination of microglia did not reduce Aβ burden in a mouse model of AD but still produced a beneficial effect, reducing neuronal loss and improving memory (Spangenberg et al., 2016). Increased proliferation of microglia in AD patients, mediated through the colony stimulating factor 1 receptor (CSF1R), was shown to positively correlate with disease severity (Olmos-Alonso et al., 2016). Impairing CSF1R decreased microglial proliferation and rescued behavioural deficits, however the number of Aβ plaques was unaffected, indicating that microglia perform additional functions in the AD brain than simply Aβ phagocytosis (Jankowsky et al., 2004; Olmos-Alonso et al., 2016; Spangenberg et al., 2016). In a murine model of AD, areas of Aβ deposition that were not surrounded by microglia showed higher neuronal degeneration, highlighting the protective role microglia play around plaques (Condello et al., 2015). This protective barrier was shown to decrease with increased ageing, increasing the number of plaques lacking microglial processes and being more toxic (Condello et al., 2015). This indicated that microglia may be able to contain toxic Aβ into plaques and masking the toxicity from neighbouring tissue through forming a protective physical barrier in a murine model of AD (Yuan et al., 2016).

A recent theory suggested that as well as forming a barrier between Aβ and surrounding tissue, microglia may also phagocytose damaged synapses within the plaques to prevent neuronal degeneration caused by damaged synapses (Edwards, 2019). This was supported by work showing that microglia mediate synapse loss in murine models of AD (Hong et al., 2016; Spangenberg et al., 2016) and the presence of increased dystrophic neurons surrounding plaques when microglial function was impaired (Yuan et al., 2016).

Microglial activation was found early in disease progression with brain tissue from LOAD patients suggesting that in early stages of the disease, when few plaques are found, microglia already display an altered phenotype (Xiang et al., 2006). In addition, increased microglia activation was also accelerated by plaque deposition, indicating a vicious cycle developing. But in later stages of AD, a functional impairment of microglia was also observed (Heneka et al., 2005).
1.2.2.3 The role of inflammation in AD

Studies on animal models of this disease and on patients suggest that pro-inflammatory cytokines are increased in the disease (Heneka et al., 2013; Zhang et al., 2013). The chronic secretion of cytokines has been shown to contribute to neurodegenerative changes in the surrounding tissues by producing a chronic toxic reaction (Frank et al., 2008).

Phagocytosis of Aβ can result in activation of microglia and increased inflammatory cytokine production, signalling threats to the neighbouring cells. Prolonged secretion of these cytokines, such as TNF-α, IL-6 and IL-1β, can be toxic to both neurons and could lead to dysfunctions in the microglia themselves (Liu et al., 2001; Jung et al., 2005). In addition to being damaging to neurons, inflammatory stimuli have also been shown to induce tau phosphorylation, further propagating pathological processes (Lee et al., 2010a).

Immortalized neuronal cell lines exposed to inflammatory cytokines secreted from microglia display an increased production of Aβ through the upregulation of the APP-cleaving β-secretase (Sastre et al., 2003; Heneka et al., 2005). This can be inhibited when the activation of microglia was prevented with anti-inflammatory drugs (Sastre et al., 2003). This highlights the importance of inflammatory cytokines to maintain and drive AD pathology. In the tissue of AD patients, increased microglial activation was also positively correlated with pathology (Xiang et al., 2006), indicating a link between microglial activation and Aβ accumulation.

Even in murine tauopathy models, modelling the hyperphosphorylated tau tangles found in AD, neuroinflammation in the form of IL-1β and cyclooxygenase was observed (Bellucci et al., 2004). In turn, murine microglial inflammation, induced by LPS, was shown to promote the phosphorylation of tau in an animal model of early disease processes (Bhaskar et al., 2010). This was further enhanced in mice without the CX3C chemokine receptor 1 (CX3CR1), which showed less ramified microglia and increases in Nos2 and Ccl2 (Bhaskar et al., 2010).

In another murine model of ageing, activation of microglia with LPS to induce secretion of inflammatory IL-1β, specifically reduced synaptic proteins, such as presynaptic synaptophysin (Sheppard et al., 2019). This was found to be a direct consequence of microglial activation, as synaptic proteins recovered once microglial activation was stopped.
1.2.2.4 Interactions with neurons in the context of AD

The C-X3-C motif chemokine ligand 1 (CXC3CL1)/CX3CR1 system between neurons and microglia can also mediate neuronal survival (Fuhrmann et al., 2010; Lee et al., 2010b). AD mice with a CX3CR1 KO showed a reduction of Aβ deposition (Lee et al., 2010b), which was linked to reduced levels of inflammatory cytokines, including TNF-α. In addition, another study found that CX3CR1 KO mice crossed with AD mice showed that neuronal loss associated with AD was prevented, as the signalling system, which is normally involved in recruiting microglia to injured neurons, was disrupted (Fuhrmann et al., 2010). Specifically, microglial migration to neurons was impaired in microglia missing CX3CR1, as microglial phagocytosis of Aβ was shown not to be affected in this KO model (Fuhrmann et al., 2010). In addition, microglia were found to internalize neurites in a mouse model of AD before plaques were starting to form (von Saucken et al., 2020), a change which may be regulated through the expression of CCL2 and CCL3 on the neurons (Wellikovitch et al., 2020).

1.3 TREM2

TREM2, expressed almost exclusively in microglia in the CNS, has a range of functions. Its gene, TREM2, is located on chromosome 6, clustered with other TREM genes, including TREM1 (Benitez et al., 2014; Replogle et al., 2015). The protein is a transmembrane receptor and associates with DNAX-activating protein of 12 kDa (DAP12) to mediate downstream signalling within microglia (Peng et al., 2010). Further downstream signalling cascades include the PI3K-Akt signalling pathway, MAPK and Rac signalling (N'Diaye et al., 2009; Peng et al., 2010; Zhong et al., 2017b). Activation of TREM2 results in a range of different immune-cell responses such as phagocytosis, cell migration, survival and production of cytokines (Forabosco et al., 2013; Gawish et al., 2015). DAP12 has been shown to negatively regulate the response of TLR receptors. Both DAP12-deficient and TREM2-deficient cells show a hyper-responsiveness to activation of TLR4, TLR9 and the TLR2/6 heterodimer (Ito and Hamerman, 2012).

1.3.1 TREM2 variants and disease

Homozygous null TREM2 variants, namely p.T66M, were initially identified to be a cause of NHD, a rare recessive syndrome with symptoms including dementia and bone cysts (Paloneva et al., 2002; Klunemann et al., 2005; Guerreiro et al., 2013b). Onset of clinical symptoms in NHD can be as early as the second decade of
life, with the earliest symptoms being bone fractures (Klunemann et al., 2005). A decline in cognitive function, such as dementia and frontal lobe syndrome leading to behavioural changes can be observed from the 4th decade of life onwards (Klunemann et al., 2005). Recently, another homozygous TREM2 mutation, p.W50C, has been associated with NHD (Dardiotis et al., 2017). Heterozygous mutations of TREM2, such as the arginine 47 to histidine substitution, R47H, have been associated with an increased risk of developing AD (Guerreiro et al., 2013a; Jonsson et al., 2013). The effect size of TREM2 variants reached a similar magnitude to the ApoE ε4 allele (Guerreiro et al., 2013a; Ruiz et al., 2014). Whilst patients carrying the R47H variant also displayed an earlier disease onset, many developing symptoms before the age of 65, the disease progression was typical of AD (Slattery et al., 2014). Another study found that despite a typical progression of AD, patients carrying TREM2 variants showed a faster progression through the different stages of AD (Del-Aguila et al., 2018). Next to these studies, whole-exome sequencing also implicated TREM2 variants in increasing the risk of early-onset AD (Bellenguez et al., 2017).

The link between TREM2 variants and other neurodegenerative disorders, such as FTD, is less well established. Whilst a recent analysis showed that the R47H TREM2 variant did not contribute to the increased risk of developing FTD (Ruiz et al., 2014), other studies found that this variant was more common in patients with ALS (Cady et al., 2014), showing an association with both FTD and PD (Rayaprolu et al., 2013; Cady et al., 2014). Another TREM2 variant, W198X, was also linked to a family with autosomal FTD (Giraldo et al., 2013). Other TREM2 variants that are associated with an increased risk of developing AD are R62H, D87N, T96K, E151K, L211P (Guerreiro et al., 2013a; Jin et al., 2014; Song et al., 2017) (see Figure 1-2).

Subsequent network analysis of GWAS studies have highlighted the importance of TREM2 as a risk factor for AD. Weighted gene co-expression network analysis, used to group genes into co-expressed modules, showed that TREM2 is a central gene within the innate/adaptive immune system (Forabosco et al., 2013). This module was also enriched for other genes implicated in AD and the study suggested that under normal conditions these risk genes can be involved in promoting microglial functions (Forabosco et al., 2013).

Another study, ranking modules based on the risk the associated genes infer on developing AD, showed that the microglial module was the most important (Zhang et al., 2013). This suggested that the microglial module carried genes implicated with
the highest risk of developing AD, highlighting the importance of microglia and immune functions in AD development. One key driver of this microglial module was DAP12, the binding partner of TREM2 (Zhang et al., 2013). This was supported in animal models, in which Dap12 KO induced expression changes that overlapped with the human DAP12 network (Zhang et al., 2013). Regional differences were detected in this analysis as well, highlighting the prefrontal cortex as the area with the biggest changes (Zhang et al., 2013).
TREM2 variants associated with AD, NHD and FTD are located on both the extracellular immunoglobulin (Ig)-like V-type domain and the intracellular part of the TREM2 protein, separated out by the transmembrane (TM) domain. Adapted from Yeh et al., 2016.
1.3.2 TREM2 processing

TREM2 is expressed on both macrophages and microglia (Takahashi et al., 2005; Turnbull et al., 2006), with TREM2 expression increasing during ageing and AD progression (Zheng et al., 2018).

TREM2 undergoes a range of different maturation steps, which were studied both in primary rodent cells and in immortalized human cell lines. Following translation, TREM2 is core glycosylated in the endoplasmic reticulum (ER). Afterwards, this immature TREM2 is shuttled to the Golgi apparatus, where further glycosylation occurs to mature TREM2 (Gawish et al., 2015; Park et al., 2015). This leads to increases in molecular weight in the protein (Kleinberger et al., 2014).

The mature TREM2 then migrates to the membrane, whilst misfolded protein or immature TREM2 is retained within the cell (Kleinberger et al., 2014). Studies in an immortalized human cell line have implicated presenilin 1 in playing a role in transporting TREM2 to the cell surface (Zhao et al., 2017). Particularly at the membrane, TREM2 experiences a short half-life of less than 1hr (Thornton et al., 2017), as it is sensitive to degradation, by both disintegrin and metalloproteinase domain-containing protein sheddases and γ secretases (Wunderlich et al., 2013; Gawish et al., 2015; Feuerbach et al., 2017; Schlepckow et al., 2017; Thornton et al., 2017; Ewers et al., 2019). TREM2 can be cleaved by a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) or a disintegrin and metalloproteinase domain-containing protein 17 (ADAM17) (Feuerbach et al., 2017; Schlepckow et al., 2017; Thornton et al., 2017) to secrete the C-terminal fragment of TREM2 into the extracellular space, called soluble TREM2 (sTREM2). Following the shedding of the ectodomain, the remaining part of TREM2 at the cell membrane is processed by γ secretase (Wunderlich et al., 2013). This frees up DAP12 to find a new binding partner, as a study using immortalized human and monkey cell lines showed that inhibition of γ secretase activity impairs downstream DAP12 signalling (Wunderlich et al., 2013).

Research into the production of sTREM2 has suggested that both ADAM10 and ADAM17 can generate sTREM2. Inhibition of ADAM17 in an immortalized human monocyte cell line was effective at blocking constitutive shedding of sTREM2 (Feuerbach et al., 2017), whilst treatment of cells with phorbol myristate acetate induced additional sTREM2 shedding, using additional mechanisms next to ADAM17,
such as ADAM10 (Feuerbach et al., 2017). Other studies have shown that ADAM10 KO, in mouse primary microglia and in an immortalized murine cell line, prevented sTREM2 secretion (Kleinberger et al., 2014; Thornton et al., 2017). Independently of the sheddase used for sTREM2 generation, the cleavage site has been reported between the H157 and S158 sites (Feuerbach et al., 2017; Schlepckow et al., 2017; Thornton et al., 2017). One TREM2 variant that is associated with an increased risk of developing AD, H157Y (Jiang et al., 2016a), is located at this cleavage site. Both murine primary microglia and human immortalized cell lines show enhanced sTREM2 shedding in cells carrying the H157Y variant (Schlepckow et al., 2017; Thornton et al., 2017), in turn leading to reduced surface expression of TREM2 and decreases in TREM2-dependent phagocytosis (Schlepckow et al., 2017). Reducing sTREM2 shedding through antibodies that compete for the ADAM-mediated shedding led to stabilization of TREM2 at the surface and increased downstream activity, such as spleen tyrosine kinase (SYK) activation (Schlepckow et al., 2020).

sTREM2 still contains the TREM2 binding domain, suggesting that it could still bind to ligands, potentially competing for ligands with the full-length membrane bound TREM2 (Colonna and Wang, 2016; Zhong et al., 2017a). This was supported by a study showing that sTREM2 was found to bind to Aβ in mice expressing human TREM2 (Song et al., 2018).

Whilst the functional role of sTREM2 is not fully understood yet, studies have shown a positive correlation between levels of sTREM2 in the cerebrospinal fluid (CSF) of AD patients with levels of phosphorylated tau (Colonna and Wang, 2016). In AD patients carrying the R47H TREM2 variant, higher levels of sTREM2 were found (Suárez-Calvet et al., 2019). In line with this, a decrease in sTREM2 was found when TREM2 was specifically activated with an antibody in a murine model of AD, which was interpreted as being reflective of TREM2 activation (Wang et al., 2020). Independent of TREM2 status, sTREM2 levels was shown to increase in all AD patients in the early stages of the disease (Gispert et al., 2016; Suárez-Calvet et al., 2016; Suárez-Calvet et al., 2019) and is associated predominantly with changes in tau pathology and grey matter volume changes (Henjum et al., 2018). A recent longitudinal study has shown that higher CSF sTREM2 levels were associated with attenuated Aβ deposition over two years and lower tau levels as detected through PET (Ewers et al., 2020). Elevated levels of sTREM2 in the CSF was also found in patients with other inflammatory neurological disorders, such as multiple sclerosis (Piccio et al., 2008). On the other hand, a recent study found plasma sTREM2 levels
did not change with AD disease progression or TREM2 risk variants (Ashton et al., 2019), indicating that the changes of CSF sTREM2 may not be translatable to plasma sTREM2.

Whilst these studies have mainly used CSF sTREM2 as a readout for TREM2 activation in the CNS, the precise nature of this correlation is not known. Recently, there have been studies investigating the role sTREM2 can play independently of TREM2. sTREM2 has been shown to promote the survival of primary murine microglia and stimulate the production of inflammatory cytokines (Zhong et al., 2017a), an effect which was reduced in sTREM2 carrying the R47H TREM2 variant. A similar effect was seen when sTREM2 was administered in vivo (Zhong et al., 2017a).

The T66M mutation leads to a functional loss of TREM2, induced by increased accumulation of TREM2 in the ER (Kleinberger et al., 2014; Zhao et al., 2017). This has been shown to lead to impaired trafficking of TREM2 to the cell surface, leading to subsequent decreases in sTREM2 (Kleinberger et al., 2014; Garcia-Reitboeck et al., 2018). Supporting the idea that the T66M mutation is indeed a functional loss of TREM2, KO of TREM2 in iPSC differentiated into microglia-like cells also showed reductions in TREM2 levels and significant decreases in sTREM2 (McQuade et al., 2020). Whilst one study suggested changes in the glycosylation status of TREM2 carrying the R47H mutation (Park et al., 2015), others have found that this mutation does not affect glycosylation status nor trafficking to the cell membrane (Ma et al., 2016; Zhao et al., 2017; Cosker et al., 2020). It is still unknown whether the R47H TREM2 variant affects sTREM2 levels, as both decreased (Kleinberger et al., 2014), unchanged (Cosker et al., 2020) and increased (Hall-Roberts et al., 2020) sTREM2 levels have been reported in cells carrying the R47H TREM2 variant.

Structural analysis of TREM2 carrying the R47H mutation has shown that this mutation in particular affects the binding site of TREM2 (Kober et al., 2016; Sudom et al., 2018). This was tested on a range of different substrates with the capacity of TREM2 to bind to lipids such as PS being found to be decreased in R47H TREM2 (Wang et al., 2015) in addition to decreased binding ability to a TREM2 ligand (Kober et al., 2016). Whilst binding of TREM2 to low µM of Aβ1-42 oligomers was not impaired in TREM2 carrying the R47H substitution, deficits were detectable in the activation of TREM2 as measured by a downstream reporter (Lessard et al., 2018). However, this could also be due to the high affinity binding of Aβ to TREM2, which could mask any small differences between AD-linked and Cv TREM2. Increased lysosomal
degradation of the protein could lead to this decreased function (Borroni et al., 2014; Park et al., 2016; Kleinberger et al., 2017).

1.3.3 TREM2 signalling

Once shuttled to the membrane, TREM2 can bind to a range of molecules, such as anionic lipids (Wang et al., 2015), PS (Wang et al., 2015), bacterial products (N'Diaye et al., 2009), phospholipids such as ApoE (Daws et al., 2003; Atagi et al., 2015; Yeh et al., 2016), high and low density lipoproteins (Yeh et al., 2016; Song et al., 2017) and apoptotic cells (Hsieh et al., 2009; Shirotani et al., 2019). Through its binding partner DAP12, TREM2 can elicit a range of different signalling pathways (Bouchon et al., 2001) (see Figure 1-3). Upon binding of a ligand, the immunoreceptor tyrosine activation motif (ITAM) on DAP12 is phosphorylated, which in turn recruits SYK (Glebov et al., 2016; Konishi and Kiyama, 2018). Following SYK kinase phosphorylation, downstream pathways such as ERK and PI3K are recruited (Konishi and Kiyama, 2018). DAP12 is also an adaptor and signalling partner of CD33 (Haure-Mirande et al., 2017).

It has also been reported that binding of a low affinity ligand to TREM2, induced partial ITAM phosphorylation and subsequent dephosphorylation of SYK, indicating that TREM2/DAP12 could also induce inhibitory signals (Hamerman et al., 2006; Turnbull et al., 2006; Turnbull and Colonna, 2007; Peng et al., 2010).
Figure 1-3 TREM2 signalling pathways

Proposed TREM2 signalling pathways upon activation of the receptor. Adapted from Xing et al., 2015; Colonna and Wang, 2016.
1.3.4 Influence of TREM2 on inflammatory processes

Inhibition of the TREM2 signalling cascade in *TREM2* KO mice leads to decreases in inflammatory cytokines release from microglia (Shirotani et al., 2019). On the other hand, other studies have suggested that *TREM2* KO mice show an increase in cytokine production when challenged with LPS (Gawish et al., 2015), as TREM2 can negatively regulate TLR induced responses. This was supported by another study, showing an increased production of inflammatory cytokines in *TREM2* KO murine macrophages (Glebov et al., 2016). In addition to an increase in cytokines, *TREM2* KO mice also show a more rapidly resolving inflammatory response (Gawish et al., 2015). *TREM2* KO murine macrophages showed increased TNF-α production in response to activation of TLR2, 4 and 6 (Turnbull et al., 2006). This was replicated in another study using mice, in which knock down (KD) of *TREM2* led to increases in TNF-α secretion (Takahashi et al., 2005). Furthermore, rats carrying the AD-related R47H *TREM2* variant display a gene dosage dependent increase in TNF-α secretion (Ren et al., 2020).

On the other hand, decreases in TNF-α secretion were observed in human iPS-Mg carrying a range of different TREM2 disease variants, such as R47H (Piers et al., 2020). In human iPSC-derived microglia carrying the T66M variant, no changes in TNF-α secretion following LPS stimulation was observed (Garcia-Reitboeck et al., 2018).

1.3.5 Phagocytosis

Another mechanism through which microglia interact with the environment is through phagocytosis of particles, both foreign and endogenous. Immortalized microglia cell lines and human iPSC-derived microglia carrying a *TREM2* KO, or the *TREM2* variants T66M and W50C, which prevent the maturation of the protein, both showed deficits in phagocytosis (Garcia-Reitboeck et al., 2018; Phillips et al., 2018). Interestingly, in the study with the disease-relevant *TREM2* variants, only phagocytosis of apoptotic cells and Aβ and not bacterial substrates, such as *E. coli*, was impaired (Garcia-Reitboeck et al., 2018; Piers et al., 2020). In brains of AD patients, carriers of the R47H^het^ and R62H^het^ *TREM2* variant also displayed reduced synaptic elements in microglia, indicating disrupted phagocytosis (Gratuze et al., 2020). This was replicated in animal models of *TREM2* KO showing decreases in CD68, a molecular indicator for microglial phagocytosis (Filipello et al., 2018). Due to this phagocytosis deficit, an increase in synaptic numbers was also observed,
highlighting the importance of synapse pruning by microglia (Filippello et al., 2018). The phagocytosis deficits also extended to the uptake of myelin debris in TREM2 KO mice (Poliani et al., 2015).

TREM2 KD cells also displayed reductions in the phagocytosis of apoptotic neurons, whilst overexpression increased phagocytosis (Takahashi et al., 2005), which was mirrored by phagocytosis deficits of human iPS-derived microglia carrying the T66M, W50C and R47H variant (Garcia-Reitboeck et al., 2018; Piers et al., 2020). Exposed PS on the apoptotic neurons is believed to drive the binding to TREM2 and subsequent phagocytosis (Wang et al., 2015; Garcia-Reitboeck et al., 2018; Cosker et al., 2020). In line with this, PS-containing liposomes were phagocytosed by microglia in a PS concentration dependent manner and phagocytosis was decreased in TREM2 KO primary murine microglia (Scott-Hewitt et al., 2020). Reductions of TREM2 can also cause a deficit in the binding and uptake of bacteria into a hamster cell line (N'Diaye et al., 2009). Following phagocytosis of apoptotic neurons, TREM2-induced signalling involving ApoE was shown to mediate a switch in the microglial phenotype to a phenotype associated with neurodegeneration in a mouse model of AD (Krasemann et al., 2017).

Another system through which microglia could recognise degenerating cells or other structures, which require phagocytosis, is the complement system. This system is capable of recognising molecular patterns, for example through association with C1q to promote phagocytosis into microglia, as microglia express the corresponding CR3 (Wyss-Coray and Rogers, 2012).

### 1.3.6 Survival and metabolism

TREM2 has been found to interact with CSF1R signalling through SYK kinase, which has implications for microglial survival (Wang et al., 2015). In low CSF1 or granulocyte-macrophage colony-stimulating factor (CSF2) conditions, murine microglia with a TREM2 KO showed decreased viability (Wang et al., 2015; Zhong et al., 2017a) and aged mice with a TREM2 KO also have fewer microglia (Poliani et al., 2015).

In addition to this, addition of sTREM2 was shown to promote primary murine microglial survival through the Akt-glycogen synthase kinase 3 beta (GSK-3β) signalling pathway when CSF2 was removed from the cells, independent of the full-
length membrane-bound TREM2 (Zhong et al., 2017a). This effect was attenuated by the PI3K/Akt inhibitor, LY294002. The beneficial effect of sTREM2 was reduced in sTREM2 carrying the R47H variant (Zhong et al., 2017a). The T66M variant of TREM2, causing a lack of membrane-associated TREM2 similar to TREM2 KO, has been shown to reduce glucose metabolism in the brain (Kleinberger et al., 2014).

The link between TREM2 and metabolic processes has also been investigated, as mammalian target for rapamycin (mTOR) was found to be a downstream target of TREM2/DAP12 signalling (Ulland et al., 2017). T66M<sup>hem</sup> knock-in mice have been found to display reductions in glucose metabolism in the brain, indicating a metabolic deficit, in addition to abundant autophagic vesicles (Ulland et al., 2017). This deficit was also found in TREM2 KO mice, caused by decreased levels of mTOR. Subsequently, the macrophages display a defective energetic state, which is further exacerbated by stress (Ulland et al., 2017). Increased autophagy was detected, a mechanism through which it is thought the cells try to increase the available energy. Using patient-derived iPS-Mg, it was shown that even microglia carrying the R47H<sup>het</sup> variant display a metabolic deficit in addition to the inability to switch their energy production to glycolysis when activated (Piers et al., 2020). This metabolic deficit could explain the reduced ability of R47H<sup>het</sup> microglia to phagocytose Aβ (Piers et al., 2020).

Next to energy metabolism, TREM2 deficient microglia also have an impaired cholesterol metabolism (Nugent et al., 2020). TREM2 can bind to ApoE, the main cholesterol transporter in the brain (Wolfe et al., 2019), and a KO of TREM2 or APOE can cause impairment in the cholesterol efflux from microglia with a damaging effect on neuronal health (Nugent et al., 2020).

### 1.3.7 Migration

Activation of TREM2 increases cell migration (Glebov et al., 2016), likely as stimulation of TREM2 induced the reorganization of the cytoskeleton (Takahashi et al., 2005). In a microglia cell line lacking TREM2, microglia failed to form membrane ruffles and migrate in response to attractants such as CSF1 or ATP (Phillips et al., 2018). This was replicated in another study using a human model of microglia, where human iPSC-derived microglia carrying the NHD-related mutations variants, T66M and W50C, also showed deficits in cell migration (Garcia-Reitboeck et al., 2018).
1.3.8 Modelling TREM2 involvement in AD models

To better understand the role of TREM2 in the context of AD, a range of studies were conducted in AD murine models, looking at both models that mimic Aβ accumulation and tau phosphorylation to investigate different TREM2-related functions.

TREM2 KO mice crossed with animal models of AD showed increases in Aβ accumulation (Wang et al., 2015). This was matched by two other studies which showed that TREM2 upregulation of activation showed decreases in Aβ deposition and subsequent burden (Lee et al., 2018; Price et al., 2020). As previous studies have shown, the interaction of Aβ with lipoproteins, such as ApoE, was required for TREM2-mediated uptake of Aβ into microglia (Yeh et al., 2016). In line with the increases in Aβ accumulation in TREM2 KO models, in the absence of functional TREM2, modelled with the T66M TREM2 variant, plaque associated ApoE was reduced in this animal model (Parhizkar et al., 2019).

Potentially through the lack of ApoE interaction, TREM2 deficient mice show reduced expression of genes that were associated with the microglial response to Aβ (Colonna and Wang, 2016). In a recent analysis of the influence of TREM2 KO on a murine model of AD, microglia showed a reduced response to Aβ accumulation, reflected in a reduction in DAM (Zhou et al., 2020). Interestingly, one study found that in cells that only carried one copy of TREM2, modelling a partial TREM2 KD, the Aβ burden was not affected (Ulrich et al., 2014).

As microglia can also surround Aβ plaques (Minagar et al., 2002; Bamberger et al., 2003), a range of studies have investigated the migration and association of microglia with Aβ plaques. They found that as microglia migrate to surround Aβ plaques, an increase in plaque-associated TREM2 can also be observed (Frank et al., 2008), in particular on the edges that directly contact the plaques themselves (Yuan et al., 2016). In TREM2 KO mice, crossed with a mouse model of AD, a reduction in plaque-associated microglia was observed (Jay et al., 2015). This was replicated in a T66M TREM2 model showing reduced clustering of microglia around plaques (Parhizkar et al., 2019) and in a KD model of TREM2 (Ulrich et al., 2014).

This lack of microglia surrounding the plaques was found to have an immediate effect on the behaviour of the plaques themselves. The plaques in a TREM2 KO AD
model were more diffuse, which resulted in increases in neuronal death (Wang et al., 2016a). This was also replicated in mice carrying the R47H TREM2 variant, which showed a spread of dying neurons around Aβ plaques (Leyns et al., 2019), and a KD of TREM2, which also showed a higher degree of neuronal dystrophy was found around the plaques, particularly around small plaques (Yuan et al., 2016). The effect of TREM2 KO was reversed in two recent studies which showed that activation of TREM2 through specific antibodies led to an increased number of microglia being recruited to Aβ plaques (Price et al., 2020) and an overall decreased plaque load (Wang et al., 2020).

The interaction between TREM2 and tau accumulation has also been studied in both AD patients and tau models. Whilst AD patients carrying the TREM2 R47H variant displayed higher levels of tau in their CSF (Cruchaga et al., 2013), TREM2 expression was also positively correlated with phosphorylated tau in the post mortem brain samples of AD patients (Jiang et al., 2016b).

In both DAP12 and TREM2 KO models, increased tau phosphorylation was observed in a tau mouse model (Audrain et al., 2019). This was supported by another study, showing that TREM2 KO in a model of tau pathology increased tau pathology, which could be rescued with TREM2 expression on microglia (Jiang et al., 2016b) and another murine model, which showed increased tau aggregation in both mice carrying a TREM2 KO or the R47H TREM2 variant (Leyns et al., 2019). In an interesting study, TREM2 deficiency was shown to increase tau pathology in an animal model, but the same was not observed in a total TREM2 KO (Sayed et al., 2018).

Looking beyond Aβ and tau pathology, in TREM2 KO models, a decrease in inflammatory markers released from microglia was observed (Jay et al., 2015; Leyns et al., 2017). Interestingly, another study found that TREM2 activation were linked to suppressed inflammation (Jiang et al., 2016b).

TREM2 KO has been linked with increased neuronal death, even away from Aβ plaques (Wang et al., 2016a), which was shown to be reversed with specific TREM2 activation (Wang et al., 2020). In a model of tau pathology, increases in TREM2 also ameliorated neuronal loss (Jiang et al., 2016b). However, another study suggested that due to decreases in phagocytic ability of microglia with the R47H TREM2 variant, both synapse loss and overall atrophy were decreased in a tau model (Gratuze et al., 2020). This matched a previous study which showed that a TREM2
KO may be protective in a tau environment, whilst a mere KD was not (Sayed et al., 2018). This study would suggest that TREM2 KO and KD led to vastly different phenotypes and disease progression, even in a controlled environment, highlighting the need to model patient specific TREM2 variants. The majority of pathological changes that were influenced by TREM2 preferentially occurred in the hippocampus, which may suggest a region-specific effect of TREM2, however this could either a direct or indirect effect of TREM2 (Colonna and Wang, 2016).

Ultimately, any differences between studies could be explained with differences between the use of various animal models of AD, in terms of both which pathology is modelled and the timeframe during which said pathology develops. Overall, there appears to be a consensus though that TREM2 deficiency prevents migration of microglia to Aβ plaques, which could have a detrimental effect on development of further pathology. Furthermore, there appear to be differences in the effect of TREM2 KO and KD. As the AD-linked R47H variant is likely to induce even more subtle effects than a TREM2 KD, these studies highlight the importance of studying disease-relevant variants, as findings in KD or KO models may not be easily transferrable.

1.4 Extracellular vesicles (EV): exosomes and microvesicles (MV)

EV were characterised for the first time in the 1940s (Chargaff and West, 1946) and were subsequently defined as ‘pro-coagulant platelet derived particles’ (Wolf, 1967). Later they were also found in serum and plasma (Hawari et al., 2004; Graner et al., 2009; Baranyai et al., 2015). Whilst initially they were thought to contain cellular debris, it has now been shown that they contain a distinct subset of proteins and RNA. EV can be divided into exosomes and MV. Exosomes are formed by the endocytosis of multivesicular bodies (MVB) and are quite uniform in shape and size, ranging from 30-150nm (Mathieu et al., 2019). MV on the other hand are bigger and more variable in size, with sizes ranging from 0.1-100μm, and are formed by direct shedding of the membrane (see Figure 1-4) (Muralidharan-Chari et al., 2009). Distinguishing between these two different types of EV can be difficult for a number of reasons.

1.4.1 Nomenclature of EV

Exosomes and MV are distinguished from one another based on their formation and secretion mechanisms. Exosomes, formed within cells, are believed to
be smaller than MV, which are formed upon membrane shedding. However, as reported in the literature, the sizes of these two groups of EV can overlap (Mathieu et al., 2019). Therefore, generating pure exosomal factions is difficult, which is further confounded by the presence of classical exosome markers in MV and vice versa (Bianco et al., 2005; Glebov et al., 2015; Kowal et al., 2016; Hessvik and Llorente, 2018; Chen et al., 2019). MV were initially believed to expose PS on their surface membrane, but exosomes were then identified to also contain PS and so protocols separating out vesicles with Annexin-V may not be as exclusive as previously believed (Bianco et al., 2005).

Both exosomes and MV have been shown to transport cargo to neighbouring cells and confer an effect on these cells. With technical difficulties separating out these two EV groups, the question is whether a distinction between these groups is even necessary. Recently, the suggestion has been made to differentiate between small EV (sEV) and large EV (Kowal et al., 2016).

1.4.2 Exosome formation

Within cells, invagination of the membrane in early endosomes leads to the formation of MVB. When MVB fuse with the cellular membrane, the intraluminal vesicles (ILV) contained within the MVB are secreted as exosomes (see Figure 1-4). The formation of ILV therefore influences both exosome formation and content. This process is associated with four endosomal sorting complexes required for transport (ESCRT) complexes: ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III. Some components of the ESCRT complexes have been used as classical exosomal markers, including tumour susceptibility gene 101 protein (TSG101), which is an ESCRT-0 complex associated protein and ALIX, which is associated with ESCRT-III (Hessvik and Llorente, 2018). The involvement of these proteins with the ESCRT machinery and the role they play in exosome secretion has been studied in KD models. TSG101 KD reduced the total amount of exosomes secreted whilst ALIX KD altered the composition of the exosomes instead (Hessvik and Llorente, 2018), suggesting that formation and packaging of ILV go hand-in-hand and the different ESCRT complexes play a different part in this pathway.

After formation of the MVB containing ILV, transport of the MVB to the membrane is dependent on the rearrangement of the cellular cytoskeleton. Increases
in the actin binding protein, cortactin, is linked with an increase in exosome secretion (Sinha et al., 2016).

### 1.4.3 Secretion of exosomes

Fusion of the MVB with the cell membrane leads to the secretion of exosomes into the extracellular space. The fusion process itself is regulated by a range of different proteins, some of which are also involved in the secretion of synaptic vesicles. Rab GTPases have been implicated in the secretion of exosomes from cells. KO of Rab35 prevents secretion of exosomes and Rab27a and Rab27b prevents docking of MVB to the plasma membrane (Ostrowski et al., 2010; Bobrie et al., 2012; Hoshino et al., 2013). SNAP receptor (SNARE) proteins and SNARE associated proteins, such as VAMP7, are also necessary for the secretion of exosomes (Fader et al., 2009).

Several intracellular mechanisms can also regulate release of exosomes. Increased intracellular calcium leads to increases in exosome secretion and KD of the calcium sensor synaptotagmin-7 has been shown to lead to a decrease in exosome release from cells (Hoshino et al., 2013).

However, next to secretion, MVB can also be degraded in the lysosome to recycle the contents. Whilst cell stress, such as ER stress, has been shown to increase the number of MVB, the number of MVB undergoing autophagy also increased (Fader et al., 2009; Hessvik et al., 2016). ISGlyation of the late endosome is believed to be controlling this process (Villarroya-Beltri et al., 2016; Hessvik and Llorente, 2018). In addition to ISGlyation, ubiquitination of the ESCRT machinery itself could determine the fate of MVB (Moreno-Gonzalo et al., 2018). The pH of the MVB has also been implicated in directing the MVB down either secretory or degradation pathways (Edgar et al., 2016). In turn, lysosomal dysfunctions have been shown to increase secretion of exosomes (Alvarez-Erviti et al., 2011).

Interestingly, another study has found the opposite effect of autophagy and exosome secretion. In fibroblasts exosome release was shown to be negatively regulated by mTOR activity, in particular the mTOR complex 1 (mTORC1) (Zou et al., 2019). mTORC1 coordinates a range of different metabolic activities in cells and inhibition of its activity, for example in response to nutrient deprivation, was shown to induce exosome secretion from the cells (Zou et al., 2019). In addition to nutrient and
growth factor deprivation inducing exosome secretion, autophagy is concomitantly upregulated in the cells as well. Despite changes in exosome secretion though, the protein profile of the vesicles appeared to be unchanged (Zou et al., 2019).
A cell can secrete a range of different EV. Loading of proteins into ILV in the early endosome is regulated through ceramide and the ESCRT pathway. The formed MVB, containing vesicles, can either be targeted for lysosomal degradation or migrate down the cytoskeleton and be released through SNARE activity, to release exosomes into the extracellular space. MV, bigger than exosomes, are formed directly through budding for the cytoplasmic membrane. Apoptotic bodies are generated through blebbing for the membrane, for example, when the cell is stressed or undergoing apoptosis. Based on Basso and Bonetto, 2016; Gustafson et al., 2017; Gurunathan et al., 2019; Mathieu et al., 2019.
Ceramide has been shown to induce exosome secretion from a range of different cell lines (Yuyama et al., 2012). The importance of ceramide was underscored after it was found that ceramides play a role in exosome formation, in particular in the early endosome and that ceramide is found in the membrane of exosomes (Properzi et al., 2015; Mathieu et al., 2019). Inhibition of ceramide formation, through inhibiting its producing enzyme sphingomyelinase, has become common practice to suppress the secretion of exosomes (Bianco et al., 2009; Antonucci et al., 2012; Yuyama et al., 2012; Dinkins et al., 2014; Asai et al., 2015).

In macrophages and microglia, several stimuli have been studied that lead to an increase secretion of EV. Extracellular ATP has been shown to lead to increases in MV secretion, through the activity of acid sphingomyelinase (ASM) causing an increase in ceramide leading to spontaneous membrane blebbing (Bianco et al., 2005, 2009). This process is mainly mediated through P2RX7 on microglia. ATP could mediate the secretion of exosomes through changes in intracellular calcium levels (Properzi et al., 2015), as ATP application induces changes in intracellular calcium.

A range of different calcium sensing components are involved in the release of exosomes, indicating that changes in intracellular calcium can influence the secretion rate of exosomes (Savina et al., 2005; Krämer-Albers et al., 2007). Independent of this pathway, cytokines, such as IFN-γ and IL-4, can induce MV production and secretion in microglia and monocytes (Colombo et al., 2018), a process that was dependent on transcription in the cells but independent of P2RX7 activation.

Secretion of exosomes appears to be under strict control, with neurons displaying polarised exosome secretion, secreting different kinds of exosomes from dendrites and the cell body (Properzi et al., 2015). Such a polarised release was also shown in antigen presenting cells and T-cells. However, whether the contents of these secreted exosomes are intended to signal to neighbouring cells or are an alternative route of waste disposal is still being studied. Overall EV, and in particular exosome, secretion is tightly regulated by a range of different cellular processes, such as intracellular calcium, stress, extracellular stimuli and metabolic processes.
1.4.4 Exosomal content

1.4.4.1 Protein content

Protein cargo of exosomes, and EV in general, have been shown to change depending on both the cell of origin and stimuli these cells receive. In the next section, I will mainly focus on EV released from myeloid cells.

Macrophages infected with mycobacterium release exosomes containing disease-initiating glycopeptidolipids, which can be transferred to unaffected macrophages. In addition, these exosomes can induce an inflammatory response through activation of both TLR2 and TLR4 (Bhatnagar and Schorey, 2007). Intercellular pathogens can induce PAMP on exosomes, which can activate pattern recognition receptors in recipient cells. Interestingly, macrophages can incorporate foreign molecules, processed in phagosomes, into exosomes (Bhatnagar et al., 2012). Exosomes can also contain functional enzymes and protein analysis of exosomes secreted from a murine microglia cell line revealed that the exosomes contain proteins, such as CD13 and monocarboxylate transporter 1 (MCT-1), associated with a whole metabolic pathway for anaerobic glycolysis (Potolicchio et al., 2005; Mukherjee et al., 2020).

In addition to content such as proteins, exosomes also contain receptors, such as the TNF-receptor 1 (TNF-R1) (Hawari et al., 2004; Zhang et al., 2006), which can bind to extracellular TNF-α (Hawari et al., 2004). Bound forms of TNF-α have been identified on exosomes from fibroblasts from patients with arthritis, which can be cytotoxic to target cells (Zhang et al., 2006).

EV, including exosomes, often lose their membrane asymmetry leaving PS exposed and blocking PS with Annexin V can mediate adhesion of EV to target cells, as blocking of this protein prevented astrocytes to react to microglial EV (Drago et al., 2017).

Cytokines have been implicated as a long-range communication signal between cells, but many cytokines have been found to be secreted in association with EV (Fitzgerald et al., 2018). A systematic review of cytokines secreted from different cell types in response to different stimuli revealed that association of cytokines with EV is dependent on the cell and stimulus type and not an inherent cytokine property (Fitzgerald et al., 2018). Cytokines associated with EV can either be encapsulated by
the lipid bilayer or bound to the surface of EV, but independent of this, even encapsulated cytokines can elicit a biological effect on target cells (Fitzgerald et al., 2018).

1.4.4.2 RNA content

Exosomes have been shown to contain a range of different RNA species. In addition to messenger RNA (mRNA), microRNA (miRNA) was also found in exosomes (Bellingham et al., 2012). miRNA is short, non-coding RNA that target mRNA transcription by binding to its 3’ untranslated region. miRNA cargo has been shown to change in exosomes following different stimuli with one study showing that glioblastomas can be diagnosed based on the miRNA signature found in serum MV (Skog et al., 2008). In addition prion-infected neurons have been shown to secrete exosomes carrying a distinct miRNA signature (Bellingham et al., 2012).

Exosomes from motor neurons carrying mutant SOD1 contain more inflammatory miRNA which can subsequently reduce microglial phagocytosis and increase inflammation (Pinto et al., 2017). In particular, miRN-124 was associated with the activation of microglia.

1.4.4.3 Sorting mechanisms

For exosomes to contain specific enriched proteins and RNA, sorting of these molecules into ILV has to be specifically regulated. As protein composition within exosomes does not match the composition of the parental cell, active protein sorting is involved in exosome formation (Moreno-Gonzalo et al., 2018). This can be mediated either through an ESCRT-dependent or and ESCRT-independent mechanism (Moreno-Gonzalo et al., 2018).

As many proteins found inside exosomes are ubiquitinated, ubiquitination is a proposed mechanism through which proteins could be directed towards being loaded into ILV (Hessvik and Llorente, 2018; Moreno-Gonzalo et al., 2018). The ESCRT machinery can control the cooperation of ubiquitinated proteins into ILV and subsequently into exosomes. For example, ESCRT-0 can bind ubiquitin, which could function as a recognition molecule (Moreno-Gonzalo et al., 2018), in addition to TSG101 also binding ubiquitinated proteins. Investigation of exosome cargo has also revealed a distinct glycosylation signature in EV, indicating that glycans could be a sorting motif of proteins into EV (Liang et al., 2014). Cluster of differentiation (CD63), a glycoprotein, appears to play a role in determining protein trafficking into EV.
Specific loading of RNA, and in particular miRNA, into exosomes relies on a specific recognition sequence targeting miRNA for exosomes in a human system (Hobor et al., 2018). This exosome sequence on miRNA allows RNA binding proteins to specifically target these miRNA into ILV and subsequently into exosomes. Loading of RNA cargo into the ILV is slowly being understood, with one elegant study showing that hnRNPA2B1 regulates the loading of miRNA into ILV (Villarroya-Beltri et al., 2016), as sumoylation of hnRNAPA2B1 is required for miRNA binding and subsequent loading. In addition to hnRPA2B1, the RNA binding protein SYNCRIPI has also been implicated in sorting specific miRNA into exosomes. With its ability to bind to the exosome motif on miRNA, it guides miRNA for inclusion into exosomes (Santangelo et al., 2016). Whether each ILV generated contains the same cargo or whether heterogeneous cargo is found in the MVB is not yet understood.

1.4.5 Interaction with target cells

There are different uptake mechanisms for any cells to incorporate extracellular material including EV and exosomes. Phagocytosis and macropinocytosis are mainly used by cells highly capable of uptake, such as macrophages and microglia, and involved engulfment of the particles through actin polymerization and reorganisation (Kuhn et al., 2014). Whilst this process is compatible with sEV/exosome, there is an upper size limit of particles that can be taken up along this route (Kanada et al., 2015). Other uptake pathways are endocytosis, namely clathrin-mediated endocytosis and caveolae-dependent endocytosis (Kuhn et al., 2014), with endocytosis appearing to be the major entry mechanism for EV into cells (Trotta et al., 2018) (see Figure 1-5).

Using specific inhibitors of each uptake mechanism and exosomes from a range of cancerous cell lines, it was shown that the uptake mechanism was dependent on the target cell and not on the cell from which the exosomes originated (Horibe et al., 2018). Uptake of exosomes into colon cell lines, COLO205 and HCT116, was blocked by inhibitors of clathrin-mediated uptake, with uptake into COLO205 also inhibited by caveolin-mediated endocytosis (Horibe et al., 2018). On the other hand, uptake of exosomes into the A549 lung cell lines was not inhibited by either, possibly suggesting uptake mechanisms involving phagocytosis or macropinocytosis. Interestingly, another study found that nanoparticle uptake into the A549 line was prevented with caveolin and clathrin inhibitors (Kuhn et al., 2014). The difference in results could be explained with the different substrate used with the 40nm
nanoparticles potentially being smaller than the exosomes used in the later study (Kuhn et al., 2014; Horibe et al., 2018).

Uptake of exosomes into a mouse macrophage cell line was decreased when actin polymerization and clathrin-mediated endocytosis were blocked (Kuhn et al., 2014). Whilst phagocytosis may not appear to be the preferred route of uptake of exosomes into epithelial cells, they could still take it up through this pathway given longer incubation times (Kuhn et al., 2014).

Differences in the ability of various cell types to take up exosomes could also be influenced by the ability of exosomes to bind to recipient cells, in addition to the phagocytic potential of the recipient cells. Whilst exosomes from neuroblastoma cell lines were found to bind to both neurons and glia cells, they were preferentially taken up by glia (Chivet et al., 2014). On the other hand, exosomes from glutamate-treated neurons only bound to other neurons but not glia, ensuring they were only taken up by other neurons (Chivet et al., 2014). One study showed that sEV from oligodendrocytes were preferentially internalised by microglia rather than neurons (Fitzner et al., 2011), however whether this was due to the overall higher ability of microglia to take up extracellular particles was not shown, so this could be only a correlation rather than a direct effect.

Uptake of EV into epithelial cells has been shown to be time and dose dependent with the uptake also depending on the microenvironment. Whilst some studies suggested that uptake of EV is not energy dependent (Schneider et al., 2017), this was not found in every study (Gupta and Pulliam, 2014), indicating that energy availability could play a positive role in uptake, which is most likely dependent on the specific uptake pathway.

Independent of uptake, EV can also exert effects on cells through receptor-mediated signalling via surface bound ligands, through transferring surface receptors to target cells and finally through delivering functional proteins to target cells (Choi et al., 2013). For example, EV containing MHC-peptide complexes have been shown to activate T cell receptors (Tkach et al., 2017). After internalization, EV cargo are either degraded, re-secreted or affect the cells.
There is a range of different ways that exosomes can influence target cells. The vesicles can be taken up through endocytosis, such as clathrin-mediated endocytosis, caveolin-mediated endocytosis or lipid-raft mediated endocytosis. Phagocytic cells are also capable of phagocytosis and macropinocytosis. In addition, exosomes can also fuse with the cell membrane directly releasing their content into the cytoplasm or dock with the membrane, for example binding to receptors, to induce downstream signalling cascades. Once taken up, exosomes are transported to the endosome, where their content can either be released into the cytoplasm of the cell to induce signalling cascades or transported to the lysosome for degradation. Based on Urbanelli et al., 2013; Mulcahy et al., 2014; Mathieu et al., 2019.
1.4.6 Exosomes in AD

The first indication that exosomes could play a role in AD was the increased accumulation of classical exosomal markers, such as ALIX and flotillin-1 around Aβ plaques (Yuyama et al., 2012; Gupta and Pulliam, 2014).

Exosomes have been shown to be able to increase Aβ degradation in the extracellular space through the secretion of the Aβ degrading enzyme, insulin degrading enzyme (IDE) (Tamboli et al., 2010). The increased secretion of IDE in association with exosomes was stimulated by statins. In turn, levels of soluble Aβ were increased in mice depleted of microglia (Tamboli et al., 2010). This suggested that IDE, located at the surface of exosomes, was to be able to degrade Aβ and highlighted the role metabolism plays in regulating exosomal packaging and secretion.

Exosomes have also been shown to influence Aβ aggregation (Yuyama et al., 2012). In the presence of exosomes, soluble Aβ aggregated into fibrils at a higher rate and without the intermediate step of Aβ oligomers. As Aβ oligomers in particular are associated with neurodegeneration and neuronal cell death, Aβ incubated with exosomes was shown to be less toxic than Aβ on its own (Yuyama et al., 2012). Aβ incubated with exosomes, forming fibrils, was also shown to be preferentially taken up by microglia, suggesting that exosomes could play an important role in both Aβ formation and clearance (Yuyama et al., 2012).

Another study found that exosomes from astrocytes can also stimulate the aggregation of Aβ but that this could interfere with Aβ uptake into microglia (Dinkins et al., 2014), exacerbating disease processes. Inhibition of exosome secretion through inhibitors of sphingomyelinase was shown to reduce exosome secretion in the CNS in vivo and in turn reduce the Aβ load. There also appeared to be an effect of gender on this effect, with male mice showing a larger reduction of the Aβ load (Dinkins et al., 2014).

In contrast, another study has shown that microglial MV exposed to Aβ can be highly toxic to neurons through converting aggregated Aβ into soluble Aβ species (Joshi et al., 2014). Furthermore, microglia exposed to Aβ were shown to phagocytose Aβ and subsequently release soluble Aβ oligomers in association with MV (Joshi et al., 2014).
Exosomes secreted from a neuroblastoma cell line express glycosphingolipids at their surface, which Aβ can bind to (Yuyama et al., 2015). Subsequently, exosome bound Aβ can be internalized by microglia and subsequently degraded (Yuyama et al., 2015). Aβ-binding was found to be a specific function of neuronal, but not glial exosomes, and infusion of neuronal exosomes into murine AD models decreased both Aβ levels and deposition in the brain (Yuyama et al., 2015).

A range of different proteins of the APP processing machinery were reported to be contained within exosomes (Sharples et al., 2008; Perez-Gonzalez et al., 2012). Following cleavage of APP by β-secretase, the resulting β-C-terminal fragment (β-CTF) fragment can accumulate in the MVB, leading to the secretion of β-CTF in exosomes (Perez-Gonzalez et al., 2012). In addition, full-length APP and β-secretase were also reported in exosomes, next to cleaved Aβ (Sharples et al., 2008). The presence of both APP and secretases in the exosomes could suggest that at least part of the APP cleavage can occur within the exosomes. Proteins packaged into exosomes contain a range of different PTM, such as phosphorylation, and Aβ proteins could highjack this process by being increasingly packaged into exosomes (Saman et al., 2012).

In addition to interacting with Aβ, exosomes also appear to play a role in the propagation of the other pathological marker of AD, tau. Microglia have been shown to phagocytose tau aggregates (Asai et al., 2015), but when exposed to LPS, microglial tau has been shown to get ubiquitinated, priming it to be packaged into exosomes. After subsequent stimulation of microglia with ATP, to induce exosome secretion, this study showed that the secreted exosomes do indeed contain tau. This is a pathway through which tau can be transported from microglia to neurons, where it has been shown to be phosphorylated, propagating tau pathology (Asai et al., 2015). In line with this research, another study showed that exosome-associated tau could also be detected in CSF of AD patients and that it was hyper-phosphorylated, in line with tau from AD patients (Saman et al., 2012). In addition, this study also found other proteins in exosomes involved in tau processing, suggesting that tau could be processed within the exosomes (Saman et al., 2012).

1.5 Exosomes and TREM2

In this project, the interplay between TREM2 and microglial exosomes will be studied.
TREM2 has been shown to modulate how microglia respond to extracellular stimuli, through migration, phagocytosis and secretion of a range of different factors. Exosomal secretion and content can both be influenced by extracellular stimuli, inducing differential effects in target cells. Therefore, it seems to be conceivable that deficits of microglia to sense the environment, through disease-relevant *TREM2* variants, could have an influence on exosome secretion and content. Several studies support this hypothesis.

On a cellular level, *TREM2* activation has been shown to induce PI3K signalling (Peng *et al.*, 2010). As the formation of MVB is influenced by the PI3K-Akt pathway (Gangoda *et al.*, 2015), abnormalities in the PI3K pathway through *TREM2* loss of function could negatively influence both exosome content and secretion. Microglia carrying *TREM2* variants also display cytoskeletal deficits, characterised by the inability to migrate (Phillips *et al.*, 2018), whilst the movement of MVB to the membrane for fusion and secretion of exosomes, requires cytoskeletal rearrangement and mobilization (Mathieu *et al.*, 2019).

*TREM2* variants, displaying deficits in the PI3K-Akt signalling pathway and cytoskeletal changes (Takahashi *et al.*, 2005; Peng *et al.*, 2010; Phillips *et al.*, 2018), are hypothesized to have impaired MVB trafficking to the membrane, which could in turn lead to changes in exosome secretion from these variants. Since *TREM2* variants also display an inability to respond appropriately to extracellular stimuli and such stimuli can be instrumental in altering exosome content, this project will also test whether content is altered in exosomes secreted from *TREM2* variants.

### 1.6 Hypothesis and aims of the project

I hypothesize that *TREM2* variations, reducing or fully preventing *TREM2* functioning and its downstream signalling pathways, can lead to changes in exosome secretion and content. This mechanism may have an influence on the increased risk of AD in patients with these variants.

To this end, this project aims to:

- Investigate the effects of *TREM2* variants on exosome secretion rate in iPS-Mg with defined *TREM2* mutations associated with AD and NHD
• Compare the changes in exosome content caused by \textit{TREM2} variants in iPS-Mg

• Investigate the effect of exosomes from \textit{TREM2} variant iPS-Mg on the functioning and viability of neuron-like cells
This project was split into three parts to elucidate the effect of TREM2 variants on exosomal secretion and the downstream effect this has on neuron-like cells. In the first part of the project, the iPS-Mg model will be characterised including their exosome secretion rate (blue). Subsequently, the proteome of the secreted exosomes from TREM2 variant iPS-Mg treated with different stimuli will be analysed in detail (red). Based on the proteomic analysis, the effect of these iPS-Mg exosomes on a range of different neuron-like cells will be tested (yellow).
Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Reagents

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<th>Source</th>
<th>Catalogue number</th>
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<tr>
<td>16% paraformaldehyde (PFA)</td>
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2.1.5 Taqman probes

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<td>System Biosciences</td>
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2.1.7 Software

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2.2 Methods

2.2.1 iPS-Mg generation

Dr Garcia-Reitboeck and Dr Piers induced fibroblasts from patients with the R47H<sup>het</sup> mutation to become iPSC using 3.4μg Oct3/4, 3.2μg hsk and 3.4μg hUL.
(Garcia-Reitboeck et al., 2018). The R47H<sup>het</sup> lines (ADRC 8, ADRC 26 and ADRC27) were karyotyped and developing cells stained for markers of different germ layers, confirming their ability to differentiate into embryoid body (EB) (Brownjohn et al., 2018). The T66M<sup>hom</sup> iPSC clones (IST84) were generated in collaboration with the Livesey group, University of Cambridge, and their pluripotency was assessed (Brownjohn et al., 2018). For the R47H<sup>het</sup> lines, three clones per patient cell line were used for the experiments. In addition to three R47H<sup>het</sup> patients, two clones from a patient-derived T66M<sup>hom</sup> line was used.

Next to patient-derived cell lines, a pair of isogenic cell lines (BIONi010-C and BIONi010-C7) were used, where the BIONi010-C line was genetically engineered to harbour the R47H<sup>hom</sup> TREM2 variant.

iPSC were clustered into EB for 5 days using E8 medium with SCF, BMP-4, VEGF and ROCK inhibitor, using previously established protocols (van Wilgenburg et al., 2013). After collection, 150 EB were transferred into a T175 flask containing X-VIVO 15 with mercaptoethanol, IL-3 and CSF1 and maintained for 2-3 months (see Figure 2-1). Progenitor cells were collected once a week and plated for final differentiation. Different media compositions (Chapter 3.3.1) were tested for the differentiation of the myeloid progenitors and assessed before a final maturation protocol, TP1, was chosen (Xiang et al., 2018). Briefly, the base medium contained IL-34, CSF1 and TGF-β1 for 2 weeks before CX3CL1 and CD200 was additionally added for another 3 days (Table 2-1 and Figure 2-1). This medium composition was used throughout the thesis unless otherwise specified.

2.2.2 Cell treatment

After iPSC-Mg were fully differentiated, medium was changed on the cells 24hrs before the experiments to remove any exosomes secreted during the differentiation process. The cells were then treated with 100ng/ml LPS for 24hrs, apoptotic neurons:iPSC-Mg at a ratio 2:1 for 24hrs, 100μM glutamate for 1hr, 50μM ATP for 1hr or 4hrs, 10mM cyclocreatine for 24hrs and 20μM LY294002 for 24hrs.

Apoptotic neurons were generated by subjecting SH-SY5Y cells (a kind gift from Professor Rohan de Silva, UCL Queen Square Institute of Neurology) to a heat-shock for 2hrs at 45°C (Garcia-Reitboeck et al., 2018; Cosker et al., 2020). Cell death,
and exposure of PS, was confirmed through Annexin/PI staining assessed through flow cytometry (FC) (Figure 2-2).

After the treatment, the iPS-Mg supernatant (SN) was spun down at 300g for 15mins to remove cell debris, before it was processed for exosome extraction. The iPS-Mg were then lysed with radioimmunoprecipitation assay buffer (RIPA buffer) for future experiments and to normalise exosomal number to total cell protein amount.
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<td>N2 supplement</td>
<td>1% v/v</td>
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<tr>
<td>MTG</td>
<td>200μm</td>
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<td>GlutaMax</td>
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<td>NEAA</td>
<td>1x</td>
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<tr>
<td>Insulin</td>
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<td>CD200</td>
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Figure 2-1 Timeline of differentiation of iPSC into iPS-Mg using TP1 medium

Undifferentiated iPSC (far left, picture provided by Dr Piers) form EB (left, picture provided by Dr Piers) in the primitive haematopoiesis step of the protocol. Throughout the differentiation, the EB contain a CD34 positive cell population (middle graphs). The EB were dissociated, stained with CD34-FITC and analysed via FC. Progenitor cells released from the EB are collected once a week and further matured for 2 weeks using serum-free medium containing IL34, CSF1 and TGFβ1. Final maturation is achieved with the addition of CD200 and CX3CL1 for 3 days to generate iPS-Mg (right, picture provided by Dr Piers).
Figure 2-2 Characterisation of heat shock protocol used to generated apoptotic SH-SY5Y

SH-SY5Y were subjected to heat-shock for 2hrs at 45°C. The cells were stained with 10µl Annexin-V FITC for 30mins and 1µl PI 1min in the dark (A) and 10µl Annexin-V FITC, 1µl PI and 1µl Hoechst for 30mins in the dark (B). Heat-shock induced cell death in SH-SY5Y, as characterised by an increase in both PI and Annexin-V staining was shown (Ai). Exposure of PS, measured with Annexin-V staining, was significantly increased in heat-shocked SH-Sy5Y compared to untreated, alive SH-SY5Y (Aii). This was further verified through staining, which showed co-localisation of Annexin-V and PI staining in heat-shocked SH-SY5Y (B). Scale bar represents 10µm. n = 3, t-test between alive and heat-shocked SH-SY5Y in Aii with * p < 0.05.
2.2.3 Exosomal extraction protocols

Initially, two different protocols were tested to extract exosomes from the SN of iPS-Mg. The exoEasy kit followed a filter-approach, allowing exosomes to bind to the filter and washing them following manufacturer's instructions. Subsequently, the exosomes were further concentrated by filtering them through 3kDa NMWL columns, whilst keeping the eluent as a negative control. The other protocol used was the ExoQuick-TC kit that cleared the SN of cell debris by spinning them for 30mins at 3000g before adding the ExoQuick-TC solution overnight. Subsequently, the exosomes were pelleted and suspended in either PBS or lysis buffer depending on the downstream analysis. After analysis of both protocols (Figure 3-8), ExoQuick-TC was used for all subsequent experiments.

2.2.4 Western Blot (WB)

Cell lysates or exosomal pellets, resuspended in RIPA buffer, were boiled in sample buffer with NuPage Reducing agent for 5mins and then loaded onto a 4-12% SDS gradient gel. For CD63 analysis, proteins were deglycosylated prior to denaturing them. All Blue Prestained Protein Standards were used as a molecular weight marker and 10µg standard exosomes were used to assess specificity of some antibodies.

The proteins were transferred onto nitrocellulose membranes in 1x Tris-Glycine Buffer (25mM Tris, 192mM glycine, pH 8.3) for 90mins at 90V and blocked in 5% non-fat dried milk in PBS-0.1% Tween20 (PBST) for 1hr. Membranes were washed in PBST before they were incubated in primary antibody (see Chapter 2.1.4) in 5% milk or 1% BSA in PBST overnight at 4°C. Subsequently, the membranes were washed in PBST and incubated with the corresponding fluorescence-conjugated secondary antibody for 1hr diluted 1:5000 in a 50:50 mix of PBST and Superblock. Using an Odyssey FC (Li-Cor Biosciences, US), the membranes were imaged and band density was quantified in ImageJ (https://imagej.nih.gov/ij/). For exosomal CD63, bands were normalised to protein concentration of the corresponding cell lysates. For cell lysates, the bands were normalised either to the housekeeping protein β-actin or, in case of phospho-antibodies, the bands of the total corresponding protein.
2.2.5 Cell death FC

Following heat-shock, apoptotic neurons were stained with Annexin V-FITC following manufacturer’s instructions (Miltenyi Biotech). Briefly, the cells were washed with 1x Binding buffer, diluted in water, and incubated with 1:10 Annexin V conjugate in the dark for 15mins. The cells were washed with Binding buffer and pelleted with a 10mins spin at 300g. The cells were then resuspended in 300μl and 2μl PI was added right before the sample was analysed using a FACSCalibur flow cytometer. As a negative control, unstained cells (unst) were used. Additionally, cells boiled at 95°C for 1hr were used as a positive control. For cell death analysis of iPS-Mg or SH-SY5Y, the cells were detached using PBS without Ca²⁺ and Mg²⁺ and 1μl PI was added before FC using a FACSCalibur. Again, unstained cells and boiled cells were used as negative and positive controls respectively. Analysis was completed with the Flowing Software 2 program. Mean fluorescent activity (MFI) was plotted, apart from where indicated.

2.2.6 Calcium imaging

Changes in intracellular calcium were measured using Fura-2 AM, a probe made up from a calcium chelator and a fluorescence reporter. Without calcium bound to it, electron transfer from the chelator quenches the fluorescence and binding of calcium induces a shift in the excitation spectrum from 380nm to 340nm (Paredes et al., 2009; Lock et al., 2015). Fura-2 is therefore a ratiometric dye, as the levels of intracellular calcium are expressed as a ratio of 340nm to 380nm, controlling for uneven loading, bleaching and dye leakage (Paredes et al., 2009), thereby offering clear advantages over other, single wavelength indicators such as Fluo-4. In addition, by tracking both wavelengths, artefacts can be detected (Kopp et al., 2014).

Cells were seeded on 1mm thick coverslips and differentiated. iPS-Mg, SH-SY5Y and iPS-neurons were loaded with 2μg/ml Fura-2 AM for 10mins, 30mins and 20mins respectively. To specifically inhibit the function of P/Q type calcium channels, iPS-neurons were pre-incubated with 1μM of the previously extracted and validated spider toxin, ωAgatoxin IV (Pocock and Nicholls, 1992; Pocock et al., 1993; Sidach and Mintz, 2000; Pringos et al., 2011; Nimmrich and Gross, 2012), for 30mins before imaging.

The cells were then mounted in the imaging chamber in imaging solution (137mM NaCl, 27mM KCl, 1mM MgCl₂, 1.8mM CaCl₂, 0.2mM NaH₂PO₄, 12mM
NaHCO₃, 5.5mM D-Glucose, pH: 7.4) warmed to 37°C. The images were acquired at 20x on an Olympus IX70 inverted fluorescence microscope with the Crain high intensity Arc lamp and a 20x objective using the OptoMorph software. The cells were imaged at both the 340mm and 380mm channel, acquiring information for both channels every second.

At the indicated times, drugs were added at the following concentrations: 10μM or 100μM ATP, 50mM KCl to SH-SY5Y, 30mM KCl to iPS-neurons, 5μM NIF, 10mM glutamate or 125μM 4-aminopyridine (4-AP). These concentrations are in line with previously published studies (Pocock and Nicholls, 1992; Evans and Pocock, 1999; Hooper et al., 2005; Sun et al., 2010; Sousa et al., 2013; Wu et al., 2013).

After recording using the OptoMorph software, up to 20 regions of interest (ROI) were manually selected and the 340/380 ratio calculated for each time point. In the case of the iPS-neurons, 20 ROI along five different processes and at least five independent cell bodies were selected. The average trace for each recording was plotted in GraphPad prism and further analysed.

2.2.7 Gene expression

Cells were washed with PBS before being lysed with TRIzol. RNA was extracted using the DirectZol RNA MiniPrep Plus kit, including the DNAse digestion step, and RNA was eluted in 50μl nuclease-free water. RNA concentration and purity was assessed and for subsequent experiments, RNA concentration was normalised between the different samples. Reverse transcription was carried out according to the high-capacity cDNA Reverse Transcription kit using an Eppendorf Mastercycler. Quantitative polymerase chain reaction (qPCR) was conducted using specific TaqMan primers (see Chapter 2.1.5) and TaqMan Universal Master Mix on a Stratagene Mx3000P. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene to calculate the fold change relative to the control samples, following the Livak-Schmittgen method (Livak and Schmittgen, 2001). For this, the $2^{-\Delta\Delta CT}$ was calculated using:

$$2^{-(Ct_{GOI} - Ct_{REFA}) - (Ct_{GOIB} - Ct_{REFB})}$$

With GOI referring to the gene of interest and REF to the reference gene, GAPDH in this case. A and B refer to different samples, with B representing the
averages of the control sample, allowing for the $2^{\Delta \Delta C(T)}$ of each sample to be normalised to the average of the control sample.

2.2.7.1 Gene array

To test a range of different microglial genes within the cells, a custom qPCR plate was designed with 28 different genes and 4 reference genes (Table 2-2). The plates were run according to manufacturer’s instructions using a Stratagene Mx3000P machine and the results compared to the gene expression of a primary human microglia sample. The ΔCt values were calculated with GAPDH being used as reference gene. The heatmaps and principal component analysis (PCA) were generated using the Bioinformatics Toolbox in MATLAB R2019b using the following code:

```matlab
% Import data with "Genes" as string and "Samples" containing the sample names
M = table2array(AllLines); % convert imported table into array

% To generate PCA analysis
STrans = M'; % Transpose the data
sz = 50;
[pc, zscores, pcvars] = pca (STrans);
hold on
scatter(zscores (1,1), zscores (1,2), sz, [0.15 0.15 0.15], 'filled')
scatter(zscores (2:4,1), zscores (2:4,2), sz, [0.41 0.84 0.41], 'filled')
scatter(zscores (5:7,1), zscores (5:7,2), sz, [0.6 0.8 1], 'filled')
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scatter(zscores (12,1), zscores (12,2), sz, [0 0 1], 'filled')
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scatter(zscores (19:21,1), zscores (19:21,2), sz, [1 0 0], 'filled')
scatter(zscores (22:23,1), zscores (22:23,2), sz, [0.53 0.05 0.14], 'filled')
hold off
legend ('iPSC', 'X-VIVO', 'NB', 'NB5', 'hMg', 'hMac', 'NB 2 weeks', 'NB5 2 weeks', 'TP1', 'TP2')
xlabel ('First Principle Component, 41.4%')
ylabel ('Second Principle Component, 24.3%')
title ('PCA analysis on Gene Array')

% To generate heatmap

88
cg_s = clustergram(M, 'RowLabels', Genes, 'ColumnLabels', Samples, 'Colormap', hot, 'ColumnLabelsRotate', 30, 'DisplayRange', 19);
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2.2.8 ATP determination

ATP levels were determined in iPS-Mg and SH-SY5Y using a commercially available ATP determination kit (Thermo Fisher Scientific). After maturation of the iPS-Mg and differentiation of SH-SY5Y, the cells were treated for 24hrs before they were lysed in cell lysis reagent and spun down at 1,000g for 1min. 10µl of sample and ATP standards were added to 100µl of the ATP reaction mix, after the background luminescence was read. Luminescence was read on a Tecan 10M. Data were normalised to the protein concentration, as determined through BCA assay.

2.2.9 Statistics

The results were presented as mean ± standard error of the mean (SEM) with p < 0.05 being considered statistically significant. Before the statistical tests were performed, outliers, as determined through the Grubb’s test, were excluded, as indicated, and normal distribution of the data was tested. With the GraphPad Prism 5 software, independent t-tests and either one-way or two-way analysis of variance (ANOVA) with a Tukey’s post hoc test were carried out. Experiments were repeated at least 3 times in biologically independent samples with as many different cell lines of each mutation being used as were available at the time.
Chapter 3 Characterisation of iPS-Mg and their exosomes

3.1 Introduction

3.1.1 Modelling microglia in vitro

GWAS and clinical studies suggest that microglia play a role in the development and progression of neurodegenerative diseases, however, so far, studying these processes in experimental studies has been limited by the availability of appropriate microglial models.

3.1.1.1 Animal models

Primary microglia extracted from either mice or rats have historically been the most widely used and available model (Kingham et al., 1999; Hooper and Pocock, 2007; Jebelli et al., 2014; Jiwrajka et al., 2016). However, previous studies have suggested that microglia can vary in their function depending on their species (Pocock and Piers, 2018; Zhou et al., 2020). In particular, regulators of the complement system, a system believed to play a role in synapse pruning (Hong et al., 2016), is differentially regulated between mouse and human microglia (Gosselin et al., 2017). Next to this, limited overlap between microglial genes associated with ageing was found between mice and humans, indicating that these two species age differently (Galatro et al., 2017). In addition to this, single nucleus RNA sequencing revealed that the microglial signature in an AD mouse model was distinct from the signature to AD patients (Zhou et al., 2020).

In addition to synapse pruning, inflammatory processes themselves appear to be differentially regulated in rodents and humans. Production of inducible nitric oxide synthase (iNOS) by microglia occurs in inflammatory conditions, such as application of LPS. iNOS is produced by NOS2 and transcription of this gene can be regulated by miRNA-939 in humans, whilst this seems not to be the case in mice cells (Guo et al., 2012; Hoos et al., 2014).

Next to differentially regulated components of the inflammatory system, rodent models of neurodegeneration also have the additional disadvantage of missing orthologues of human genes associated with AD (Mancuso et al., 2019), making it difficult to study the contribution of these risk genes in rodent models of neurodegeneration.
When studying genetic polymorphisms of disease-associated genes, human genes have to be genetically knocked-in in these animals to make viable models. This is associated with its own set of risks and off-target effects, such as upregulation of TREML1 downstream of TREM2 and impaired splicing of TREM2 causing degradation (Carbajosa et al., 2018; Kang et al., 2018; Xiang et al., 2018). This limits the uses of non-human derived microglia cells.

3.1.1.2 Immortalized cell lines

In addition to using primary cells extracted from animal models, much research has been carried out using immortalized microglial cell lines, such as N9, BV2 and RAW264.7, a rodent model for macrophages (Liu et al., 2012; Butovsky et al., 2014; Song et al., 2018). Whilst these cell lines are easy to maintain and experiment on in the lab, they come with limitations of their own. Compared with primary cells, they show a differential expression of important microglial genes such as TREM2 and P2RY12 (Butovsky et al., 2014). In addition, immortalized cell lines have been shown to be more prone to an inflammatory status, in addition to being sensitive for genetic drifts, which has to be carefully controlled for (Sousa et al., 2017).

Particularly when studying genetic polymorphisms leading to an increased risk of developing disease, immortalized cell lines reach their limits as they themselves do not express the disease-associated polymorphisms (Pocock and Piers, 2018). Genetically modifying the immortalized cell lines has the possibility of introducing off-target effects, as previously discussed.

3.1.1.3 iPSC

With the development of human iPSC, it has become possible to differentiate human microglia cells from iPSC, which carry the disease-associated polymorphisms. In recent years, more and more protocols have been developed for this, trying to recapitulate the developmental origins of microglia cells (Pocock and Piers, 2018; Hasselmann and Blurton-Jones, 2020).

3.1.1.4 Development of microglia

Yolk sac stem cells give rise to erythroid-myeloid progenitors (EMP) and some of these EMP migrate to the developing brain early in development where they further develop into microglia (Ginhoux et al., 2010). Other tissue resident macrophages, such as Kupffer cells in the liver and Langerhans cells in the skin, are also EMP-
derived, whereas macrophages from peripheral blood monocytes arise later in development from definitive, and not primitive, haematopoiesis (Buchrieser et al., 2017). In addition, macrophages are dependent on Myb as a transcription factor (TF), whereas microglia and tissue resident macrophages rely on the PU.1 and RUNX1 (Buchrieser et al., 2017).

3.1.1.5 Assessing iPS-microglial models

With increasingly more papers being published detailing different iPS microglia differentiation protocols, assessing the protocols for their reliability and specificity in generating microglia is imperative.

Modelling embryonic development is a good approach to ensure the resulting cells are reliable, which means for microglia generation differentiation from iPSC is preferable than generation from monocytes, which have already travelled down different developmental trajectory. In addition to this, understanding the environment the cells are exposed to is important. It has been shown that primary cells rapidly change their expression profile once cultured in vitro (Gosselin et al., 2017) due to changes in their environment. Therefore, iPS microglia protocols have taken to co-culturing microglia with neurons, to model the environment of the CNS (Haenseler et al., 2017). However, this can introduce variability in the resulting cells depending on the proportion of cell types and the specific neuronal cell type used. Providing the developing microglia specifically with brain-specific cues (Abud et al., 2017) could avoid this problem.

The most common way of assessing the reliability of microglial differentiation protocols is to compare the expression profile of the cells with primary microglia and other cell types in the CNS (Abud et al., 2017; Gosselin et al., 2017; Haenseler et al., 2017; Xiang et al., 2018). This is necessary, as there is not one specific microglial marker that can be used to confidently and reliably identify microglia relative to macrophages or other brain cells.

In addition to gene expression analysis, generated cells can also be assessed in their ability to function like primary microglia cells. Through studies in rodents, a range of different microglial functions was identified. As immune cells, microglia can readily respond to signals in their microenvironment by migrating to them. ATP, released from ruptured cells, is one well-studied chemoattractant to microglia (Davalos et al., 2005). Upon activation of the ATP receptor P2RX4 and P2RY12 on
microglia with ATP, intracellular microglial calcium increases (Bianco et al., 2005; Ohsawa et al., 2007).

In response to threat signals and activation of receptors, such as toll like receptors with pathogen-associated molecular patterns, microglia have been shown to change their baseline secretion of cytokines. The classically identified cytokines released from microglia in response to inflammatory stimuli such as LPS are IL-1β, TNF-α and IL-6, but anti-inflammatory cytokines such as interleukin-18 have also been identified (Yoon and Kim, 2015).

Demonstrating at least some of these microglia functions in addition to similarities in gene expression between the cells and primary human microglia is a commonly used strategy to verify the identity of the iPS- microglia.

3.1.2 Exosome characterisation

3.1.2.1 Extracting exosomes

Exosome extraction has to take several challenges into account. As these vesicle types are categorized and differentiated from other vesicle types by their secretion mechanism, there is not one unique marker present in exosomes that can distinguish them from other EV (Hessvik and Llorente, 2018; Mathieu et al., 2019). Their formation means that they are smaller than other types of EV, such as MV, but protocols are still being developed to confidently separate vesicles ranging from 20-150nm from MV with a size range of 100nm-1μm.

One commonly used technique is ultracentrifugation, separating out vesicles using a glucose gradient to distinguish between different densities (Hooper et al., 2012; Van Deun et al., 2014). The advantage of this is that the presence of specific surface proteins is not required, as it is in some pulldown kits, but other lipoproteins present in the medium, introduced for example through the addition of FBS in in vitro experiments, have been shown to co-purify with exosomal fractions (Skotland et al., 2017).

Size exclusion chromatography separates out the vesicles by size, most likely producing pure fractions as well and this can be used at the end step of any other extraction protocol, increasing the purity of presumed exosomal fractions. This on the
other hand dilutes the final exosomal fraction, reducing the applications this protocol can be used for (Hessvik and Llorente, 2018).

Whilst the extraction protocols so far listed can produce pure population of exosomes, one common downside of them is the loss of material during the protocol. Many exosomes are lost in the extraction process and therefore, large quantities of starting material and exosomes are needed. This makes these protocols inappropriate to use in experiments with precious, less readily available samples.

Other techniques have been developed to extract exosomes based on either pull-down techniques or columns that are commercially available. These products produce less pure exosomal fractions but can be used with less material (Hessvik and Llorente, 2018).

To assess the quality and purity of extracted exosomes, several approaches can be taken. As exosomes are mainly characterised through their size, size analysis either through electron microscopy (EM) or NTA are appropriate techniques (Witwer et al., 2013). The advantage of NTA is exosome concentration can also be analysed, but the variability between different specific machines remains a limiting factor.

Classical exosomal markers have been identified with which exosomal fractions can be probed. CD63, CD9 and CD81 are all tetraspanins, molecules involved in membrane organization and therefore essential for exosome formation itself (Andreu and Yáñez-Mó, 2014). Proteins involved in the ESCRT machinery are also used to identify exosomes, such as ALIX and TSG101 (Colombo et al., 2013). Whilst these markers can be used to determine the presence of exosomes in any fraction, negative markers can be used to confirm the purity of exosomes and the absence of any other particles or impurities (Witwer et al., 2013). It is widely believed that organelle-specific proteins, such as calnexin for the ER, ATP synthase proteins in the mitochondria and histone-associated proteins in the nucleus, are not found in exosomes themselves and presence of these markers indicates impurities in the extraction protocol.

3.1.2.2 Exosome secretion

Exosome secretion can be modified depending on the stimuli received by both changing the rate of exosome secretion and their size.
Treatment with LPS, a commonly used activator of macrophages and microglia, has been shown to lead to an increase in exosome secretion (Yang et al., 2018) and ATP via the P2RX7 receptor on microglia has also been shown to increase the amount of EV secreted from microglia (Bianco et al., 2005). Microglia also secrete more exosomes in response to neurotransmitters, such as serotonin (Glebov et al., 2015). Interestingly, oligodendrocytes also secrete more exosomes in response to neurotransmitters, this time the specific transmitter used was glutamate (Frühbeis et al., 2013). Finally, Wnt3a has been shown to increase the number of exosomes secreted from primary rat microglia (Hooper et al., 2012).

To analyse the secretion of exosomes, a range of different techniques has been used. Measuring the direct particle concentration in a sample using NTA is often used (Frühbeis et al., 2013; Villarroya-Beltri et al., 2016; Yang et al., 2018), however due to the lack of availability of these machines, other techniques have also been applied. Quantification of particle number is rarely done through EM, but size quantification using this technique can be more accurate than NTA (Yuyama et al., 2012; Chen et al., 2019). Often, exosomal markers are used to quantify exosome concentration with different treatments. CD63 (Villarroya-Beltri et al., 2016; Yang et al., 2018; Chen et al., 2019), ALIX (Hooper et al., 2012; Frühbeis et al., 2013; Chen et al., 2019) and Flotillin-1 (Glebov et al., 2015; Chen et al., 2019) are more commonly used markers for quantifying exosomal concentration but CD9 (Chen et al., 2019), CD81 (Villarroya-Beltri et al., 2016), TSG101 (Villarroya-Beltri et al., 2016) and HSP70 (Frühbeis et al., 2013) have also been used.

3.1.3 Aims

The aim of this chapter is to establish the experimental methods for the project, in addition to characterising microglial exosomes. In order to do so, the following aims are:

- Optimise the protocol to generate iPS-Mg
- Test and optimise exosome extraction
- Quantify microglial exosome secretion and the effect TREM2 variants have on this
3.2 Methods

3.2.1 Assessing iPS-Mg differentiation protocols

iPSC were differentiated out into myeloid progenitors as described above (Chapter 2.2.1) and different media compositions were tested (Table 3-1, Figure 3-3). TP1 (Table 2-1) was chosen as the medium composition capable of inducing the most microglia-like state and therefore has been used throughout the thesis unless stated otherwise.

3.2.2 Phagocytosis FC

Aβ1-42 HiLyte-488 or 555 was prepared by diluting it in 100% hexafluoro-2-propanol and incubating it at room temperature (RT) for 1hr, as previously described (Whitcomb et al., 2015). The resulting solution was sonicated and then dried using nitrogen gas. Aβ1-42 was stored at -80°C at a concentration of 1mM in dimethyl sulfoxide. Before use, the peptides were diluted to 25µM and rotated at 37°C for 2hrs to generate oligomers. The presence of oligomers has been validated through EM by Claudio Villegas-Llerena, a former PhD student in the Pocock lab. Cells were incubated with the oligomers for 2hrs at a final concentration of 500nM (Piers et al., 2020).

pHrodo Green Zymosan or E. coli Bioparticles have been used in the past as standard particles to assess the phagocytic potential of microglia (Bohlen et al., 2017; Brownjohn et al., 2018; Garcia-Reitboeck et al., 2018; Hasselmann et al., 2019). Zymosan is thought to be a TLR2 agonist, whilst E. coli, as gram-negative bacteria, can bind to TLR4 (Ribes et al., 2009; Taghavi et al., 2018; Lax et al., 2020). Additionally, E. coli have been shown to be opsonized to allow for uptake via the LRP1 receptor on microglia (Cockram et al., 2019). Overall, these bioparticles can be used to assess the ability of microglia to phagocytose foreign pathogens (Garcia-Reitboeck et al., 2018). 2mg of pHrodo Green Zymosan or E.coli Bioparticles was resuspended in Live Cell Imaging buffer at a final concentration of 1mg/ml and sonicated for 5mins. The solution was added neat to iPS-Mg for 30mins before the cells were washed and detached with PBS to analyse their fluorescence using FC (Garcia-Reitboeck et al., 2018).

To image the ability of iPS-Mg to phagocytose exosomes, exosomes were incubated with vybrant DiI following manufacturers’ instructions (1:200) at 37°C for
30mins. The excess unbound dye was removed using 3kDa NMWL columns by spinning the columns at 14000g for 20mins to elute particles larger than 3kDa. The concentrated exosomes were added to the iPS-Mg for 2hrs. As a negative control to account for Dil-carryover, a PBS sample was treated just like the exosomes. After incubation, the cells were incubated for 10mins at 37°C with PBS and subsequently detached by washing the cells off.

10µM CytoD pre-treatment for 30mins was used as a negative control for phagocytosis (Magae et al., 1994; Garcia-Reitboeck et al., 2018). FC was performed using a FACs Calibur and analysis was completed with the Flowing Software 2 program, plotting MFI as a readout.

3.2.3 TNF-α ELISA

TNF-α secreted from iPS-Mg was measured with the commercially available Quantikine TNF-α ELISA kit. SN was collected from iPS-Mg and centrifuged for 15mins at 300g to remove cell debris. The SN were then analysed with a kit following manufacturer’s instructions and read on the Tecan 10M.

3.2.4 sTREM2 ELISA

SN was collected from the iPS-Mg and spun down at 300g for 15mins to remove cell debris. sTREM2 was then measured through an in house-generated ELISA system (Garcia-Reitboeck et al., 2018). For this, MaxiSORP 96 well plates were coated with 1µg/ml of rat anti-mouse/human (R&D systems) overnight at 4°C. The plates were washed with PBST and then blocked with 1% BSA in PBST for 45mins at RT, followed by 3 washes with PBST. 15µl of samples and standards (recombinant human TREM2-His, 0-20ng/ml in BSA-PBST) were then incubated for 2hrs at RT, followed by 3 washes with PBST. For detection, plates were incubated for 1.5hrs at RT with 0.1µg/ml biotinylated sheep anti-human TREM2 antibody diluted in BSA-PBST. Plates were subsequently washed four times with PBST, followed by an incubation with 0.2µg/ml streptavidin-HRP diluted in PBST for 45mins at RT. Plates were washed 3 times with PBST followed by the addition of TMB in the dark. The reaction was terminated with the addition of stop solution (0.16M H₂SO₄) and the absorbance was read at 450nm on a Tecan 10M. sTREM2 levels were normalised to total cell number of the samples the SN was collected from.
3.2.5 NTA

Exosomes were diluted in 100µl PBS and recorded twice for 30secs on the NanoSight LM10 (Malvern Panalytical) at the Physics Department, King’s College London and the School of Pharmacy, UCL. 5.5µg of the human CSF exosome standard was also recorded for comparative purposes. Analysis was completed with the NTA software version 3.2. For size analysis, both the mean measurement and the D90, representing the size of 90% of all particles, were plotted. To control for varying cell densities between the lines, the results for exosome secretion rate were normalised to cell lysates.
<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
<th>Duration</th>
<th>Reasoning</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-Vivo</td>
<td>100ng/ml CSF1</td>
<td>1 week</td>
<td>Based on previous publications, this protocol was initially developed in the lab (Garcia-Reitboeck et al., 2018) and used to compare new differentiation protocols to.</td>
</tr>
<tr>
<td>NB</td>
<td>100ng/ml IL-34, 25ng/ml CSF1</td>
<td>1 week/ 3 weeks</td>
<td>Based on previous publications (Abud et al., 2017; Haenseler et al., 2017), different concentrations of TGF-β1 were tested in addition to two different time points.</td>
</tr>
<tr>
<td>NB5</td>
<td>100ng/ml IL-34, 25ng/ml CSF1, 5ng/ml TGF-β1</td>
<td>1 week/ 3 weeks</td>
<td></td>
</tr>
<tr>
<td>NB10</td>
<td>100ng/ml IL-34, 25ng/ml CSF1, 10ng/ml TGF-β1</td>
<td>1 week/ 3 weeks</td>
<td></td>
</tr>
<tr>
<td>NB50</td>
<td>100ng/ml IL-34, 25ng/ml CSF1, 50ng/ml TGF-β1</td>
<td>1 week/ 3 weeks</td>
<td></td>
</tr>
<tr>
<td>TP1</td>
<td>100ng/ml IL-34, 25ng/ml CSF1, 5ng/ml TGF-β1, 100ng/ml CX3CL1, 100ng/ml CD200</td>
<td>2.5 weeks with only the final 3 days including CX3CL1 and CD200</td>
<td>Based on previous publications (Abud et al., 2017).</td>
</tr>
<tr>
<td>TP2</td>
<td>100ng/ml IL-34, 10ng/ml CSF2, 5ng/ml TGF-β1, 100ng/ml CX3CL1, 100ng/ml CD200</td>
<td>2.5 weeks with only the final 3 days including CX3CL1 and CD200</td>
<td>Based on previous publications (Douvaras et al., 2017).</td>
</tr>
</tbody>
</table>
For NTA, particles were suspended in PBS. A laser beam was shown through the suspension and light scattering from the particles imaged with a microscope. From the scattering, movement and size of the particles using Brownian motion will be extrapolated.
3.3 Results

3.3.1 Gene expression with different media

The protocol used for differentiation of iPSC into microglia-like cells was initially optimised using qPCR, looking at specific genes associated with a microglia phenotype. The relative gene expression was compared with the cells used originally in the lab (Garcia-Reitboeck et al., 2018). Only the final differentiation stages, after the haematopoiesis step (Figure 2-1) were optimised.

Based on published literature, the medium composition was altered to include TGF-β1 in addition to an additional time point of differentiation of three weeks, rather than the previously used one week. The other two media compositions used for analysis by qPCR were based on a previous publication (Abud et al., 2017), which used high concentrations of IL-34 instead of CSF1. Whilst both activate CSF1R, IL-34 has been shown to be the main ligand for this receptor in the brain (Nakamichi et al., 2013; Lin et al., 2019). Using high IL-34 concentrations, MERTK expression markedly increased with prolonged incubation of low TGF-β1 concentrations over 3 weeks and a similar trend was seen with AIF1 (Figure 3-2). P2RY12 expression was reduced in all tested media compositions except TP1. No significant difference was seen in TMEM119 expression and only cells grown in high concentrations of TGF-β1 over 1 week had an increase in TREM2.

The next logical step following the results from these experiments was to compare the different levels of gene expression with mature human primary microglia. Additionally, a more comprehensive overview of genes expressed in microglia rather than the five mentioned was used to confidently decide which medium composition yields iPS-derived microglia most similar to human ones. With this in mind, a custom-gene array was designed, which contained 28 microglial associated genes (Table 2-2) to test the different media composition. In addition, the choice was made to only differentiate the cells for 2 weeks rather than 3 due to decreases in cell viability in the 3rd week. The relative expression of these genes from cells grown in different maturation medium was subsequently compared to undifferentiated iPSC and human microglia (hMG) and macrophage (hMac) samples. Clustering analysis revealed that the original differentiation protocol (Garcia-Reitboeck et al., 2018) led to cells more similar to hMac than hMG in their gene expression (Figure 3-3A). Conversely, the TP1
protocol, led to cells that clustered most closely to the hMG sample (Figure 3-3B). This protocol was subsequently used for the differentiation of iPS-Mg from iPSC.

Using the same custom-designed gene array, the microglial phenotype of the varying TREM2 variants was assessed. TREM2 variations did not appear to have an impact on the ability of the cells to differentiate into iPS-Mg (Figure 3-4).
Figure 3-2 Expression of classical microglial genes following different differentiation protocols to generate iPS-derived microglia

Different media composition used to mature progenitors were tested for their ability to influence expression of microglial genes in the cells. MERTK increased in cells grown in neurobasal medium (NB/ NB5/ NB10/ NB50) with varying TGFβ (A) for 3 weeks. P2RY12 decreased with cells matured for 3 weeks in NB (B), whilst AIF1 was significantly increased in the NB10 medium, grown for 3 weeks. TMEM119 did not change significantly in response to any of the treatments (D), whilst TREM2 was unaffected in any of the conditions apart from NB50 for 1 week (E). In all graphs, n = 3, one-way ANOVA with Tukey’s post hoc test. * p < 0.05, ** p ≤ 0.01.
Figure 3-3 Microglial gene expression analysed using a custom gene array

Using a custom-gene array, the influence of different media compositions on microglial maturation were assessed, in comparison to undifferentiated iPSC, hMG and hMac. ΔCt normalised to GAPDH was plotted using PCA (A) and clustered dendrograms (B). PCA showed the stark differences of iPSC (black) to differentiated iPSC. Three clusters were identified, with hMac clustering with the original X-vivo protocol (blue cluster) whilst another cluster containing the TP2 and NB 2 weeks media becomes apparent (green cluster). Several media compositions, TP1, NB5 2 weeks and NB, cluster closely with the human microglia sample (red cluster). To further distinguish between these different media, the different samples and genes were clustered in dendrograms based on the Euclidian distance between the different samples (B). Here, it appears that TP2, NB5 and NB 2 weeks form their own cluster (indicated in red) and it becomes obvious that TP1 samples clustered the closest with the hMG sample (indicated in pink). Based on this, TP1 was chosen as the ideal medium to generate cells closely associated with human microglia. n = 3 for the different media composition plotted, apart from TP2 n = 2 and iPSC n = 1.
Figure 3-4 Expression of microglial genes in different TREM2 variants

To assess whether TREM2 variants influenced the differentiation of iPSC into iPS-Mg using TP1, the gene expression of 28 microglial genes was analysed in the 4 control lines (BIONi010-C, AD03, SFC840, CTRL1) and three clones of each R47Hhet patient line. No big difference in Euclidian distance was observed between the Cv and TREM2 variants, showing that the R47Hhet TREM2 variant did not affect the ability of iPSC to be differentiated into iPS-Mg. ΔCt normalised to GAPDH was plotted. n = 1 for each cell line.
In addition to assessing microglial gene expression, the ability of these iPS-Mg to perform microglial functions was also assessed.

Phagocytosis of a range of different particles was assessed in the different TREM2 variants, with the phagocytosis inhibitor CytoD being used as a negative control. No deficit was observed in the uptake of pHrodo E.coli and zymosan in the TREM2 variants (Figure 3-5A, B), as reported previously (Garcia-Reitboeck et al., 2018). On the other hand, T66M
het iPS-Mg showed a deficit in uptake of Aβ1-42, a trend that was seen independent of the tag-size (Figure 3-5C, D). iPS-Mg carrying R47H
het and R47H
hom displayed an intermediated phagocytosis potential.

The uptake of iPS-Mg exosomes was also assessed. Again, the TREM2 variants displayed a deficit in exosome uptake (Figure 3-5E, F). This uptake deficit was not specific to exosomes extracted from Cv iPS-Mg, but also to exosomes from R47H
het iPS-Mg (Figure 3-5F), indicating that exosome phagocytosis was dependent on TREM2 status of the target cell, but not the exosome-secreting cell.

Another function of microglia is to respond to inflammatory stimuli, such as LPS, with an increase in cytokine release. The ability of the iPS-Mg to secrete TNF-α in response to LPS was not affected by the R47H
hom TREM2 variant (Figure 3-6Ai). The increase in TNF-α secretion was mirrored in an increase expression of TNF in Cv iPS-Mg exposed to LPS for 24hrs, however R47H
het iPS-Mg displayed lower levels of TNF (Figure 3-6Aii), in line with previous results (Piers et al., 2020). The iPS-Mg also displayed an ability to secrete sTREM2, with no sTREM2 being found in SN from a TREM2 KO line and T66M
hom line, however sTREM2 were not affected by the R47H TREM2 variant (Figure 3-6Bi). sTREM2 secretion was found to be decreased after the cells were stimulated with LPS (Figure 3-6Bii).

The ability of the iPS-Mg to respond to extracellular ATP with intracellular calcium increases was also tested and the cells were able to respond to both 10µM and 100µM ATP (Figure 3-7). This assay however was highly variable, so the difference in calcium flux between different TREM2 variants was not quantified.
Figure 3-5 Ability of iPS-Mg to phagocytose a range of different particles
Phagocytosis of various particles into the iPS-Mg was assessed. No difference in MFI was seen when Cv, R47Hhet and R47Hhom phagocytosed pHrodo E.coli and zymosan after 30mins (A, B), with 30mins CytoD pre-treatment reducing the uptake of the particles. The TREM2 variants on the other hand showed deficits in the uptake of labelled Aβ after 2hrs, both HiLyte 488 and 555 labelled Aβ showing that this deficit is not due to the label itself. Particularly the T66Mhom phagocytosed less Aβ oligomers (C, D). Uptake of iPS-Mg exosomes into the iPS-Mg was also analysed. After 2hrs Cv iPS-Mg phagocytosed exosomes more readily than TREM2 variants (E), with this deficit being true for both exosomes originating from Cv and R47Hhet iPS-Mg (F). n = 4 for A, B and n = 3 for remaining experiments. One-way ANOVA, apart from F: 2-way ANOVA with Tukey’s post hoc test. * p < 0.05 and n.s. not significant.
TNF-α secretion from microglia in response to TLR4 activation with LPS has been well reported. The ability of the iPS-Mg to respond similarly was assessed with a TNF-α ELISA.
showing that both Cv and R47H<sup>het</sup> iPS-Mg were able to significantly and massively increase the secretion of TNF-α in response to LPS in comparison to non-treated (NT) iPS-Mg (Ai). This was mirrored by an increase of TNF secretion, shown for both Cv and R47H<sup>het</sup> iPS-Mg (Aii).

sTREM2 secretion was measured with an in-house ELISA kit to assess the cells ability to secrete sTREM2. iPS-Mg carrying the T66M<sup>hom</sup> TREM2 variant and iPS-Mg with a TREM2 KO secreted no measureable sTREM2 (Bi). The other cell lines showed a significant decrease in sTREM2 in response to LPS treatment. Ai: n = 2 for Cv, n = 6 for R47H<sup>hom</sup>, Aii: n = 3. B: n = 4 with n =2 for T66M<sup>hom</sup> and TREM2 KO. One-way ANOVA for Bi and two-way ANOVA for Aii and Bii, with Tukey's post hoc test * p < 0.05 and n.s. not significant.
Figure 3-7 Calcium response in iPS-Mg

The response of iPS-Mg to ATP was measured with the calcium dye Fura-2, showing an increase in intracellular calcium as measured through the 340/380 ratio with curves shown for Cv iPS-Mg (A) and R47H\textsuperscript{het} iPS-Mg (B). 20 ROI per coverslip were analysed with the mean plotted and the SEM represented as the dotted line. Both Cv and R47H\textsuperscript{het} showed the potential to respond to 10µM and 100µM ATP.
3.3.3 iPS-Mg secrete exosomes

Both exosomal extraction protocols, the filter and the pull-down kit (Chapter 2.2.3), were tested for their ability to extract exosomes with a consistently high purity and quality. Using NTA, the size distribution of samples from both extraction methods was tested and no big difference was found between the two different approaches (Figure 3-8A, B). Both methods produced EV within the expected range of close to 100nm, with a few larger particles being detected. Whilst they also displayed exosomal markers, such as CD81, they did not have ER associated markers, such as calnexin (Figure 3-8D). This suggested a relatively high purity of the extracted fractions (Witwer et al., 2013). Both kits preformed similarly well, with the pull-down ExoQuick-TC kit potentially outperforming the exoEasy kit in extracting a more homogenous population of small particles (Figure 3-8B) and so for this, and for ease of use, the pull-down kit was used for all future experiments. The exosomes extracted using the ExoQuick-TC pull-down kit displayed different classical expected exosomal markers, such as CD63, CD9, HSP70 and ALIX (Figure 3-9).

Another potential source of impurities for exosomal fractions could be apoptotic bodies generated by cell death (Hristov et al., 2004; Turola et al., 2012; Witwer et al., 2013). Apoptotic bodies form during late stages of apoptosis when membrane fraction occurs (Cummings and Schnellmann, 2004; Beyer and Pisetsky, 2010; Xu et al., 2019), indicating that they are formed right before or whilst cells could be stained with PI. Due to the long nature of the incubations of 24hrs, absence of high levels of PI staining in the iPS-Mg without and with various treatments therefore indicated that apoptotic bodies are most likely not present in the cell medium and therefore are not likely to be co-purified using the pull-down kit (Figure 3-10).

Exosomes from each cell line were assessed using NTA to investigate their size distribution. A similar distribution in size with an average size of 146nm was found, with no significant difference in exosome size between the different TREM2 variants (Figure 3-11).
The efficiency of different exosome protocols was assessed. Representative size distribution between the filter method (A) and pull-down kit (B) showed that the pull-down kit performed better at extracting small particles. As comparison, no exosomes were found in the TP1 medium, representing a negative control (C). The exosomal marker CD81 was found in the concentrate of all kits and calnexin was found only in the cell lysate (D), highlighting the purity of the extractions.

Figure 3-8 Testing two different exosome extraction protocols
The exosomal fraction of the iPS-Mg expressed the expected exosomal markers such as CD63, CD9, HSP70 and ALIX. As positive controls, standard exosomes from a Jurkat clone and a mouse macrophage line were used.
A possible confounding factor for any exosome extraction method is the presence of apoptotic bodies in the SN. Cell death in the iPS-Mg was analysed before and after treatment by PI FC to assess cell death rate (A). Different TREM2 variants did not show significant differences in baseline cell death (B) and treatment did not appear to change levels PI staining (C), indicating that any changes in particles seen after treatment are not due to increases in the secretion of apoptotic bodies. At least n = 3. One-way ANOVA with Tukey’s post hoc test (B) and two-way ANOVA with n.s. not significant.

Figure 3-10 Cell death in iPS-Mg as a potential contaminator of exosomes
Figure 3-11 Exosomal size distribution as measured through NTA

Size distribution was analysed for exosomes extracted from each cell line of iPS-Mg grown in TP1 (A). The distribution curves generated through NTA suggested that TREM2 mutations do
not appear to have an effect on size. This was verified through plotting the mean size across the different variants (B) and the mean size distribution curves (C) with the dotted line representing the SEM. \(n = 4\) for Cv, \(n = 8\) for R47H\textsuperscript{het}, \(n = 2\) for CSF standard and \(n = 1\) for T66M\textsuperscript{hom} and R47H\textsuperscript{hom}. T-test between Cv and R47H\textsuperscript{het} in B with n.s. not significant.
3.3.4 *TREM2* variants secrete less exosomes

The quantity of secreted exosomes from *TREM2* variants was assessed through a range of different methodologies, investigating whether *TREM2* variant iPS-Mg had a reduced ability to secrete exosomes.

Interestingly, looking at total particles/ml acquired through NTA, the samples from R47H\textsuperscript{het} iPS-Mg were shown to secrete less exosomes than were secreted from Cv iPS-Mg in 24hrs (Figure 3-12). Following up from this data, NTA was used for a more in-depth analysis of exosomal number. Repeating the experiments, again a decrease in exosome number and concentration was seen in R47H\textsuperscript{hom} iPS-Mg at baseline (Figure 3-13A). For treatments, SN was collected before and after treatment was applied. This meant that varying cell numbers were not a confounding variable, as every treatment was normalised back to the number of exosomes secreted from the same cells over 24hrs. Whilst LPS did not appear to have an influence on exosome secretion (Figure 3-13B), a significant increase was observed in both cell types following treatment with glutamate (Figure 3-13C), showing that glutamate is capable of increasing exosome production similarly to serotonin (Glebov et al., 2015). ATP, activating P2RX7 on microglia, appeared to have a time-dependent effect on exosome secretion. Addition of ATP for 1hr significantly decreased exosome secretion in both cell lines, but longer treatments for 4hrs brought the levels back to the untreated control (Figure 3-13D).

Following up from the NTA results suggesting decreases in exosomal secretion rate in the R47H\textsuperscript{het} and R47H\textsuperscript{hom} iPS-Mg, the classical exosomal marker CD63 was used to assess the rate of exosome secretion. This marker has been used in the past by others to ascertain the amount of exosomes secreted (Liang et al., 2014; Villarroya-Beltri et al., 2016; Yang et al., 2018; Chen et al., 2019). Again, a decrease in exosomal numbers secreted from R47H\textsuperscript{het} and T66M\textsuperscript{hom} cells, normalised to the number of cells was found (Figure 3-14B). Following treatment with LPS, exosome amount, as measured through CD63, did not change significantly in *TREM2* variants (Figure 3-14D). Glutamate treatment appeared to increase exosomal CD63 in some *TREM2* variants, however this trend was not significant (Figure 3-14D).

However, treatment with apoptotic neurons increased CD63 in R47H\textsuperscript{het} and R47H\textsuperscript{hom} to levels similar to NT Cv (Figure 3-14D). This could be due to specific activation of TREM2 by PS exposed on the apoptotic neurons (Figure 2-2) or by the
energy supplied by the lipids of the apoptotic neurons (Cosker et al., 2020). Therefore, the increase in exosomal numbers, as approximated with CD63, in response to apoptotic neuron treatment was further investigated.

To control for any potential contamination from the apoptotic neurons that could interfere with reliable CD63 measurements following treatment of iPS-Mg with apoptotic neurons, the size distribution of the extracted particles before and after addition to apoptotic neurons was tested (Figure 3-15A, B). No differences in size distribution were observed, excluding the possibility that apoptotic bodies secreted from the neurons were co-precipitated in the exosome extraction. In addition to testing particle size following apoptotic neuron treatment, the ability of these cells to contaminate the exosomal fraction with standard exosomal markers, such as ALIX and CD63 was tested (Figure 3-15C). Performing the exosomal extraction protocol on a serial dilution of apoptotic neurons revealed that with increasing material, both ALIX and CD63 signals can be detected in exosomal fractions, but that at low levels used for the experiments, this contamination was minimal.

To test whether increases in energy availability following the apoptotic neuron treatment was responsible for the observed increase in exosome secretion, iPS-Mg were supplied with another form of energy, cyclocreatin, and exosome secretion was measured again (Figure 3-16). Cyclocreatin has been used to provide TREM2 variants with more energy (Ulland et al., 2017), decreasing their metabolic deficit and indeed, exosome secretion increased in R47Hhet following treatment with cyclocreatin (Figure 3-16B). On the other hand, cyclocreatin did not increase exosome secretion from T66Mhom iPS-Mg, in line with previous findings that apoptotic neurons also did not significantly increase exosomal numbers, measured through CD63, secreted from T66Mhom iPS-Mg (Figure 3-14D).

ATP levels were measured in iPS-Mg to confirm that indeed both apoptotic neurons and cyclocreatin are able to supply the cells with energy. At baseline, the iPS-Mg carrying the R47Hhet and R47Hhom TREM2 variant, displayed a metabolic deficit in terms of lower ATP levels in a gene-dosage dependent manner in comparison to Cv iPS-Mg (Figure 3-17A). This matched previous publication, which also suggested a decrease in metabolic activity in TREM2 variants (Ulland et al., 2017; Piers et al., 2020). Furthermore, apoptotic neurons and cyclocreatin both increased energy availability in R47Hhet and R47Hhom iPS-Mg (Figure 3-17B).
In addition to directly assessing exosomal numbers, through NTA and exosomal protein levels, pathways upstream of exosome secretion in iPS-Mg were also analysed. The PI3K-Akt pathway was shown to play a role in exosome secretion, through the regulation of MVB formation (Gangoda et al., 2015) and so the phosphorylation status of Akt at baseline was assessed. Whilst T66M\text{hom} cells had a significantly reduced phosphorylation of Akt, the decrease in Akt phosphorylation was not significant in the R47H\text{het} and R47H\text{hom} TREM2 variants (Figure 3-18B). To directly link Akt to exosome secretion, Akt phosphorylation was reduced with the LY294402 inhibitor. LY294402 is an inhibitor of PI3K signalling, which has been shown to reduce overall Akt phosphorylation (Shinohara et al., 2006; Tanaka et al., 2018). The effectiveness of LY294402 to reduce phosphorylation of Akt at the Thr308 site was verified (Figure 3-18C) and exosome secretion analysed in Cv iPS-Mg. Interestingly, exosome secretion was not reduced as expected (Figure 3-18E), showing that Akt phosphorylation was not sufficient in inducing exosome secretion from iPS-Mg.

Ceramide is involved in the trafficking of exosomes to the cell membrane (Verderio et al., 2018) and is produced by sphingomyelinases. ASM was analysed in iPS-Mg and appeared to decrease in T66M\text{hom} iPS-Mg, whilst it was significantly increased in R47H\text{hom} iPS-Mg (Figure 3-19B). Following LPS treatment, ASM significantly increased in Cv, R47H\text{het} and T66M\text{hom} iPS-Mg, whilst showing no change after exposure of iPS-Mg to glutamate (Figure 3-19C).
Exosomal particles/ml were analysed by NTA, giving an indication of exosomes secreted from each cell line after 24hrs. R47H\text{het} variants were shown to secrete significantly less exosomes over 24hrs in comparison to Cv iPS-Mg. $n = 6$ for Cv and R47H\text{het}, $n = 1$ for T66M\text{hom} and R47H\text{hom}. T-test to determine difference between Cv and R47H\text{het} condition with * $p < 0.05$. 

Figure 3-12 Number of exosomes secreted from TREM2 variants analysed with NTA
Figure 3-13 Exosomal numbers secreted from iPS-Mg following different treatments

iPS-Mg were grown in NB5 for 2 weeks (see Table 3-1) and the amount of secreted exosomes over 24hrs analysed by NTA. Baseline secretion rate was normalised to total cell number (A) in which a drop in exosomal number for R47H<sup>hom</sup> was observed, which is in line with previous results. To compare exosome secretion following treatment, the number of particles was normalised to the number of particles before treatment was added, normalising the exosomal numbers to the cell amount directly. LPS treatment did not seem to influence exosome
numbers significantly (B), but a significant increase in exosomal numbers was observed after glutamate was added for 1hr (C). Short ATP treatment decreased exosomal numbers in both Cv and R47H<sup>hom</sup>, whilst longer incubations returned exosomal levels back to normal. A: n = 11, B and C: n = 4 and D: n = 3. T-test for A and two-way ANOVA for B-D with Tukey’s post hoc test and * p < 0.05 and n.s. not significant.
Exosomal number was approximated through the amount of exosomal CD63 secreted from iPS-Mg after 24hrs. CD63 significantly decreased in all disease-associated TREM2 variants tested in comparison to Cv (B). Whilst LPS treatment did not change exosomal numbers, measured through CD63, treatment of R47H\text{het} and R47H\text{hom} iPS-Mg with apoptotic neurons induced an increase in exosomal CD63 (D). Glutamate treatment also showed a trend of increasing exosomal numbers secreted from R47H\text{het} and T66M\text{hom} iPS-Mg, albeit this trend not being significant. One-way ANOVA (B) and two-way ANOVA (D) with Tukey’s post hoc test and * p < 0.05, ** p ≤ 0.01.
Figure 3-15 Controlling for contamination after apoptotic neuron treatment

Treatment with apoptotic neurons could lead to some potential cross-contamination from vesicles, including apoptotic bodies, secreted from apoptotic neurons into exosome fraction collected from the iPS-Mg. To test that this contamination did not skew any results, exosomal size was analysed before and after apoptotic neurons were added. The mean size (A) and D90 of exosome particles (B) analysed through NTA were not significantly different before and after apoptotic neuron treatment, suggesting no carry-over of the bigger apoptotic bodies. In addition to size analysis, the presence of exosomal markers in apoptotic bodies was also tested. To do this, apoptotic neurons of varying amounts were added to TP1 and the exosome extraction protocol was performed. Whilst ALIX was only detected in the positive control, increasingly high concentration of apoptotic neurons (2x10⁶ to 4x10⁶ apoptotic neurons/ml) did produce a CD63 signal, indicating that only high concentrations of apoptotic neurons could potentially skew WB results. Based on this, a concentration of 1x10⁶ apoptotic neurons/ml used for the experiments did not appear to cause any noticeable contamination of the exosomal fraction which could interfere with the experiments. A, B n = 4 with t-test and n.s. not significant.
Figure 3-16 Exosomal numbers following cyclocreatinne treatment

To test whether the increases in exosomal CD63 following treatment with apoptotic neurons could be explained by an increase in energy availability in the iPS-Mg, iPS-Mg were treated with the ATP supplier, cyclocreatinne, and exosomal CD63 analysed subsequently, showing that cyclocreatinne treatment significantly increased exosomal CD63 from R47H\textsuperscript{het} but not T66M\textsuperscript{hom}. n = 3, two-way ANOVA with Tukey's post hoc test and * p < 0.05 and n.s. not significant.
Figure 3-17 Metabolic deficits in TREM2 variants

The metabolic ability of iPS-Mg carrying TREM2 variants was assessed with an ATP assay. At baseline, R47H\text{het} and R47H\text{hom} iPS-Mg showed a reduction in ATP levels in comparison to Cv iPS-Mg in a gene-dosage dependent manner (A). Apoptotic neurons and cyclocreatine were both able to increase ATP availability in the R47H\text{het} and R47H\text{hom} iPS-Mg to levels comparable to Cv ATP levels, whilst these treatments had no effect on ATP levels in Cv iPS-Mg (B). n = 5, one-way (A) and two-way ANOVA (B) with Tukey’s post hoc test and * p < 0.05, ** p ≤ 0.01.
Figure 3-18 Analysing p-Akt as upstream target of exosome secretion

Through phosphorylation of Akt, TREM2 signalling could directly affect exosome secretion. Phosphorylation of Akt at the Thr308 was measured in baseline iPS-Mg and was found to be decreased only in the T66M\textsuperscript{hom} variant (B). To directly link phosphorylation of Akt to exosome secretion, Akt phosphorylation was inhibited through LY294402 (effect shown in C). No decrease in exosome secretion was seen as analysed through exosomal CD63 (E). n = 3. One-way ANOVA with Tukey’s post hoc test and t-test for B and E respectively with ** p ≤ 0.01 and n.s. not significant.
ASM levels were assessed in iPS-Mg. At baseline, both R47H\textsuperscript{het} and T66M\textsuperscript{hom} TREM2 variants showing a trend of decreased ASM levels, whilst ASM were significantly increased in R47H\textsuperscript{hom} iPS-Mg (B). When looking at ASM levels after treatment of cells with LPS or glutamate, LPS treatment significantly increased ASM levels in Cv, R47H\textsuperscript{het} and T66M\textsuperscript{hom} iPS-Mg (C), with glutamate having no effect on ASM levels. One-way ANOVA (B) and two-way ANOVA. $n = 5$ apart from $n = 3$ for R47H\textsuperscript{hom} for NT condition and $n = 3$ for LPS. One-way ANOVA (B) and two-way ANOVA (C) with Tukey’s post hoc test and * $p < 0.05$ and ** $p \leq 0.01$. 
3.3.5 Exosome size does not change following treatment

Previous publications have studied the size of EV, with one study showing an increased size of EV after BV2 were activated with LPS (Yang et al., 2018). On the other hand, another study saw no difference in size distribution and average particle size after treatment of oligodendrocytes with glutamate (Frühbeis et al., 2013). In light of this and the fact that ASM is particularly linked to the increased secretion of MV (Bianco et al., 2009; Antonucci et al., 2012), particles larger than exosomes, the size of the secreted particles was also assessed. Particle size was analysed through NTA analysis in iPS-Mg differentiated out with NB5 medium for 2 weeks (Table 3-1), as ample data had been collected from these conditions.

Both the mean size and D90 size were plotted, with the D90 previously having been shown to be more sensitive to changes in exosomal sizes (Yang et al., 2018). No significant differences in both of these measurements were found following treatment of Cv and R47H\textsuperscript{hom} treatment with LPS (Figure 3-20A), glutamate (Figure 3-20B) or ATP (Figure 3-20C). In part, this corroborated previous findings that exosomal size distribution was not changed after exosome secretion (Frühbeis et al., 2012).
Changes in size distribution was analysed in exosomes extracted from iPS-Mg grown in NB5 medium for 2 weeks (see Table 3-1) before and after treatments. The mean size did not change in either the Cv or R47H\text{hom} (Ai) and neither did the D90 (Aii) following LPS treatment. Similarly, following glutamate treatment, the mean size (Bi) and D90 did not change neither
Cv nor R47H<sup>hom</sup> (Bii). There also was no significant difference between exosome size following treatment with ATP for 1hr or 4hrs (Ci, Cii). A: n = 4 and B,C: n = 3. Two-way ANOVA with n.s. not significant.
3.4 Discussion

3.4.1 Generation of iPS-Mg

The original protocol used to generate iPS-derived microglia in the lab (Garcia-Reitboeck et al., 2018) was optimised in this part of the project. The optimised protocol further took the developmental origin of microglia into account and supplied the developing cells with signals, such as CX3CL1 and CD200, which are secreted from other cells of the brain. The advantage of using a gene array to compare gene expression of the cells with a human microglial sample was that it represented a more holistic picture of microglial gene expression rather than simply focusing on five key microglia signature genes, which were previously identified and are still prominently used to characterise microglia (Butovsky et al., 2014; Garcia-Reitboeck et al., 2018). For the future, using bulk RNA sequencing would give an even better insight into the identity of the cells and could inform future optimisation steps. Future optimisation steps could include oxygen deprivation to model low oxygen availability during development, which has been used by other methods and has been shown to improve maintenance and renewal of myeloid progenitors (Hawkins et al., 2013; Tiwari et al., 2016; Abud et al., 2017). Another approach to surround developing iPS-Mg with the required brain-related factors to induce maturation can be transplantation of the microglia into mice brains as recently published (Hasselmann et al., 2019; Mancuso et al., 2019; Svoboda et al., 2019). However, the advantage of the current protocol is the scalability, allowing the generation of a few million microglial progenitors once a week required for large scale exosome experiments.

Another advantage of this protocol is the absence of FBS in the medium. As exosomes can be found in serum, FBS-supplemented media can contain contaminating exosomes, making it difficult to distinguish between serum exosomes and exosomes secreted from the cells themselves. The absence of serum in this differentiation protocol therefore not only makes the generation of iPS-Mg more reliable with no dependence on specific serum lots, it also excludes serum-exosomes as a possible confounding variable for future exosome experiments.

The generated iPS-Mg displayed functions expected of microglia cells in vivo (Pocock and Piers, 2018) and in line with previous publications. Loss of function of TREM2, as seen in the T66M<sup>hom</sup> variant, led to significantly decreased phagocytosis (Figure 3-5) (Filipello et al., 2018; Garcia-Reitboeck et al., 2018; Phillips et al., 2018).
Another study found a decrease in Aβ uptake in microglia differentiated from human embryonic stem cell lines carrying the R47H<sub>hom</sub> TREM2 variant or TREM2 KO (Liu et al., 2020a), in part corroborating the results obtained here (Figure 3-5C). In line with other publications showing phagocytic deficits in R47H TREM2 variants, specifically in response to endogenous stimuli (Garcia-Reitboeck et al., 2018; Cosker et al., 2020), a deficit in microglial exosome uptake in TREM2 variants was also found (Figure 3-5E, F). This deficit was seen independent of the TREM2 status of the exosome secreting iPS-Mg (Figure 3-5F) and only seemed dependent on the TREM2 status of the recipient cell. This was supported by a previous study highlighting the importance of the recipient cell type over the secreting cell type in terms of exosome uptake (Horibe et al., 2018).

 Whilst T66M<sub>hom</sub> mutation also led to an abolishment of sTREM2 secretion (Figure 3-6Bi), as previously described, the R47H<sub>het</sub> TREM2 variant did not impair sTREM2 secretion (Cosker et al., 2020), despite another study suggesting this (Kleinberger et al., 2014). The iPS-Mg also displayed other expected microglial functions, such as the ability to secrete TNF-α in response to LPS (Figure 3-6A) and respond to ATP with changes in intracellular calcium (Figure 3-7).

### 3.4.2 Exosomes can be extracted reliably

Different exosome extraction protocols carry different advantages and disadvantages. Choosing the correct one is based on the starting material and the downstream applications of the exosomes. Due to the relatively small amount of SN available per experiment, preserving exosomes during extraction was imperative. The absence of serum in the medium meant that extraction protocols used for this project did not have to be able to clear away lipoproteins or albumin, as they were not present.

With these constraints, two different extraction protocols were tested at the beginning of the project, one based on filter-size exclusion and the other on pull-down of particles based on size. Both protocols performed as expected with the pull-down kit extracting particles with a smaller size distribution (Figure 3-8). Thorough testing of the extraction protocol was performed to ensure that no contaminants co-purified with the exosomal fraction both at baseline and after treatments (Figure 3-9, Figure 3-10 and Figure 3-15) and testing that TREM2 variants all had the ability to secrete exosomes with similar size distribution curves and average sizes (Figure 3-11).
3.4.3 Exosome secretion is influenced by metabolic deficits caused by TREM2 variants

At baseline, it appeared that disease-associated TREM2 variants secreted less exosomes over the same period of time in comparison to Cv iPS-Mg. This was verified through direct measures of exosomal number such as NTA (Figure 3-12 and Figure 3-13A) and exosomal protein CD63 (Figure 3-14). Whilst previous studies have suggested a link between PI3K signalling and exosome secretion, inhibition of Akt phosphorylation did not decrease exosome secretion from the cells (Figure 3-18). The inhibitor may not be effective in inhibiting Akt phosphorylation, however it has been used successfully in similar studies (Motti et al., 2005; Jo et al., 2011) and WB showed it to be effective in inhibiting Akt phosphorylation in the iPS-Mg (Figure 3-18C), indicating that there were Akt independent mechanisms of exosome secretion, which may be capable of compensating for inhibition of Akt phosphorylation. Several different pathways have been linked to exosome secretion and some of them have also been shown to be independent of each other. For example, both tetraspanins and ceramide could induce ESCRT-independent secretion (Escola et al., 1998; Turola et al., 2012), whilst Wnt3a independent of GSK-3β and ceramide independent of ASM were also shown to induce exosome secretion (Hooper et al., 2012; Colombo et al., 2018).

Baseline secretion of exosomes from iPS-Mg, including those with TREM2 variants, was detected through both NTA and WB. Whilst some literature has detected no baseline secretion of exosomes from microglia (Hooper et al., 2012), others have also found that microglia can secrete exosomes basally (Potolicchio et al., 2005; Drago et al., 2017). This may be a question of the sensitivity of the analysis and the amount of material used.

One pathway influencing exosome secretion is energy availability and metabolic deficits have been reported in a range of TREM2 variants (Ulland et al., 2017; Piers et al., 2020). This energy deficit was shown in both the R47H\textsuperscript{het} and R47H\textsuperscript{hom} iPS-Mg (Figure 3-17A). Apoptotic neurons have been shown to supply iPS-Mg with energy (Cosker et al., 2020), which was also verified (Figure 3-17B) and in line with this, R47H TREM2 variant iPS-Mg were shown to display increases in exosomal secretion, as measured through CD63 (Figure 3-14). This increase was shown to be due to increases in energy supply to the iPS-Mg by using cyclocreatine as an alternative energy source. In line with previous studies (Ulland et al., 2017),
cyclocreatine increased ATP availability in R47H TREM2 variants (Figure 3-17) and lead in R47H<sup>het</sup> to an increase in exosomal numbers as well (Figure 3-16). On the other hand, iPS-Mg carrying the T66M<sup>hom</sup> TREM2 variant did not show changes in their exosomal numbers, after either treatment with apoptotic neurons or cyclocreatine (Figure 3-14 and Figure 3-16 respectively). It could be that the treatments did not rectify the metabolic deficit of these iPS-Mg, as they have been shown to be more severe than in the R47H<sup>het</sup> iPS-Mg (Piers et al., 2020), however without testing the ATP levels in these cells following apoptotic neuron or cyclocreatine treatment, this remains a hypothesis.

Following treatment, changes in exosome secretion were also analysed. NTA is the most direct measure of exosomal particle analysis, as exosomes are directly imaged and quantified, both in their concentration, but also size. However, NTA experiments are time-consuming, requiring both a lot of troubleshooting and a lot of time to collect the necessary data points. Therefore, the experiments were only conducted with the R47H<sup>hom</sup> cell line and its isogenic control (Figure 3-13). This limited the variability and reduced the number of replicates needed to achieve the necessary power. NTA analysis on these cells indicated that LPS did not affect exosomal secretion rate (Figure 3-13B). Whilst one study found an increase in exosome number following LPS treatment (Yang et al., 2018), the findings presented here are in line with another study which suggested that LPS and IFN-γ treatment did not affect the secretion rate of MV from BV2 (Grimaldi et al., 2019). This was also backed up by further analysis of exosome numbers using CD63 as an approximation, which again showed no effect of LPS treatment (Figure 3-14D).

As a positive control, glutamate was used to increase exosome secretion. Neurotransmitters have been shown to induce exosome release from microglia (Glebov et al., 2015) and other glia cells, such as oligodendrocytes (Frühbeis et al., 2013). Microglia have been shown to express functional receptors for neurotransmitters (Pocock and Kettenmann, 2007; Mead et al., 2012), and therefore, due to the implications of glutamate-induced excitotoxicity in AD, the effect of glutamate on exosome secretion was probed.

Whilst an increase in exosomal particles was observed in both Cv and R47H<sup>hom</sup> following glutamate treatment (Figure 3-13C), this was not replicated in the analysis of exosomal CD63 (Figure 3-14D). This could be due to the different sensitivities of
the assays used or the small changes in the microglial differentiation protocol used to
generate iPS-Mg for these experiments.

The fact that inhibition of Akt phosphorylation does not reduce exosome
secretion from iPS-Mg suggests that phosphorylation is not essential for exosome
secretion but could rather support secretion as an upstream factor (Gangoda et al.,
2015).

Sphingomyelinases regulate the production of ceramide, a sphingolipid
important for the spontaneous curvature formation of the membrane (Verderio et al.,
2018). Both ASM and neutral sphingomyelinase-2 (n-SMase) have been implicated
in the formation and secretion of EV (Bianco et al., 2009; Yuyama et al., 2012;
Verderio et al., 2018). Whilst inhibition of n-SMase has been used to reduce exosome
secretion in previous publications (Yuyama et al., 2012), ASM is also implicated in the
production of EV, namely in a series of publications implicating it in the secretion of
MV (Bianco et al., 2009; Antonucci et al., 2012). To control for potential secretion of
MV alongside the exosomes collected and extracted from the iPS-Mg, ASM was
analysed. Whilst R47H hom iPS-Mg showed higher levels of ASM (Figure 3-19B), LPS
treatment led to a significant increase of ASM levels in the TREM2 variants, whilst
glutamate treatment did not affect ASM levels (Figure 3-19C). This could suggest that
more MV are secreted from iPS-Mg following LPS treatment but that MV secretion is
not affected by glutamate treatment. This would be in line with previous studies which
have indicated that EV from microglia activated with LPS display an increase in
particle size, indicating an increased secretion of MV (Reales-Calderón et al., 2017;
Colombo et al., 2018; Yang et al., 2018). To investigate whether MV are preferentially
secreted from the iPS-Mg following LPS treatment, particle size following LPS and
glutamate treatment were analysed (Figure 3-20). No difference in particle size was
seen in either condition.

Whilst the ASM analysis suggested an increase in MV secretion from iPS-Mg
following LPS treatment, the secreted particles did not increase in size. This indicated
that if MV were increasingly secreted from iPS-Mg, they would be similar in size to
exosomes. One study suggests that MV secretion from microglia in response to long
term cytokine exposure is independent from ATP-induced ASM changes (Colombo et
al., 2018). Previous studies have not shown an increase in exosomes secreted from
cells following glutamate treatment, so these results are in line with them (Frühbeis et
al., 2013).
Exosomal markers and NTA results did not always follow the same trend in the same study (Villarroya-Beltri et al., 2016), therefore it is important to use several methods to probe exosomal numbers in tandem. Through combining different techniques, the one trend that was persistently shown was a decreased exosome secretion in disease-associated TREM2 variants, particularly R47H<sup>het</sup> iPS-Mg. This could be due to decreased energy availability in these disease-associated TREM2 variants. Supplying the iPS-Mg with energy rescued exosome secretion. LPS treatment appeared not to have an effect on exosomal secretion, whilst the effect of glutamate and ATP could not be conclusively determined. In the next chapters, the effect of these exosomes will be probed in more detail to study their content and the downstream effect they have on neurons.

3.4.4 Conclusion

In summary, in this chapter I have presented a reliable protocol to differentiate iPSC into iPS-Mg, which resembling human primary microglia both phenotypically and functionally. From these iPS-Mg, exosomes could be extracted reliably, with deficits in baseline exosome secretion observed in iPS-Mg carrying disease-associated TREM2 variants. Through a range of experiments, I developed the hypothesis that TREM2 dependent energy deficits could cause the reduction in exosome secretion and that this can be efficiently reversed when iPS-Mg are exposed to additional energy sources, such as apoptotic neurons (summarised in Figure 3-21).
A summary diagram showing exosome secretion is impaired in disease-associated $TREM2$ variants, in particular in the $R47H^{het}$ variant, iPS-Mg. This deficit, most likely caused by an energy deficit, can be reversed through the addition of apoptotic neurons, or other molecules reversing the metabolic deficit.
Chapter 4 Exosomal content

4.1 Introduction

4.1.1 Measuring exosomal content

4.1.1.1 Specific proteins

Exosomal fractions can be investigated for specific proteins using methods such as WB. In addition to this, ELISA can be used, which has the added benefit that more samples can be processed at the same time (Yang et al., 2018). In a recently developed technique, exosomes can be captured using specific antibodies and surface exosomal makers can subsequently be resolved, which can provide information on a single exosome basis (Wu et al., 2019). However, any of these techniques can only be used when specific antibodies are available. In addition, it is a top-down analytical approach, assuming the presence of specific markers and probing for them.

4.1.1.2 Proteomics

As a bottom-up strategy to analyse the protein content of exosomes in an unbiased manner, a more holistic approach can be taken to analyse the protein content. Separation of the proteins on an SDS-PAGE gel can enable the analysis of specific bands of proteins, in addition to comparing the overall exosome composition between different conditions (Hooper et al., 2012). This allowed for the identification of the most abundant exosomal proteins. In addition to a SDS-PAGE gel, which separates the proteins by molecular weight, they can additionally be separated out in a second dimension based on their charge (Mears et al., 2004; Graner et al., 2009). This can provide a more in-depth analysis of exosomal proteins. Both approaches however are only able to detect a few dozen proteins within exosomes, mainly the most abundant proteins.

High throughput proteomics can shine a light on the more subtle changes and the less concentrated proteins in exosomes. Mass spectrometry enables the separation of peptides based on their specific mass to charge ratio. This can be preceded by chromatography, to separate out the peptides based on their retention time. Analysis of the mass to charge ratios and the intensities of the peaks can be matched with proteins in subsequent analysis to give the relative abundances of hundreds of different proteins in a sample (Karpievitch et al., 2010; Xie et al., 2011).
4.1.2 Microglial EV protein content

The content of exosomes has been shown to be influenced by the origin of the secreting cell and specific state of the cell (Hooper et al., 2012; Choi et al., 2013; Yang et al., 2018).

4.1.2.1 At baseline

Across a range of different studies, EV from microglia have been shown to contain cell-specific makers. These include proteins that are found specifically in microglia in comparison to other CNS or myeloid cells. In exosomes from primary murine microglia, these markers include Olfml3, Hexb, Fabp5, Cspg4, Fam3 and Lgals1 (Murgoci et al., 2020). Other microglia specific markers found in exosomes include galectin-3, HSP90, enolase and CD11b (Prada et al., 2013; Drago et al., 2017). Microglial exosomes have also been shown to contain CD13, which is involved in the degradation of enkephalins (Potolicchio et al., 2005; Prada et al., 2013). This enzyme is not found in exosomes from other hematopoietic cell lines, indicating it could be used to distinguish microglial EV from EV of other cell types (Prada et al., 2013).

In addition to these specific microglial proteins, proteomic analysis also revealed components of the endosomal compartment, from which EV arise, to be found in exosomes, such as Lamp1 and Lamp2, as well as established exosomal markers, such as ALIX and CD63 (Potolicchio et al., 2005; Hooper et al., 2012). EV can also contain enzymes, such as GAPDH (Trotta et al., 2018). In addition to GAPDH, other metabolic enzymes found in exosomes include MCT-1, which functions as a lactate transporter in neurons (Potolicchio et al., 2005). Other proteins involved in basic microglial functioning such as C1q, which is part of the complement cascade that microglia are involved in to prune aberrant synapses, are also found in exosomes from microglial cells (Stevens et al., 2007; Drago et al., 2017).

The overall proteome of microglial exosomes appeared to fulfill functions towards protein and RNA/nucleotide binding. In addition, proteins involved in actin reorganization, such as Wipf1, were also found in exosomes (Drago et al., 2017). Proteins involved in the cytoskeletal structure, such as actin, and associated proteins, such as keratin, were found in exosomes from microglia (Potolicchio et al., 2005; Prada et al., 2013; Yang et al., 2018).
sEV from homeostatic microglia have revealed that there are differences in exosomal proteomics depending on the location-specific phenotype of microglia, with sEV from primary microglia from the cortex displaying a different profile than sEV from microglia originating from the spinal cord (Murgoci et al., 2020). The differences in protein content meant that microglial sEV from the spinal cord were more inflammatory than those from cortical microglia, but functionally this translated in sEV from spinal cord microglia enhancing neuronal growth cone more than cortical microglia sEV (Murgoci et al., 2020).

4.1.2.2 Exosomal proteome changes following LPS activation

Traditionally, inflammatory activation of microglia is induced using LPS. Stimulation of microglia with LPS can induce changes in EV content, as demonstrated in a range of different experimental paradigms.

Commonly reported were increases in inflammatory cytokines in EV in response to LPS stimulation. These cytokines include TNF-α and IL-6, as shown in exosomes from BV2 exposed to LPS (Yang et al., 2018). Following LPS stimulation, IL-1β was also found to be increasingly packaged in MV from microglia (Paolicelli et al., 2018). Anti-inflammatory markers, such as Annexin A1 and A2, were also found in primary rat exosomes (Drago et al., 2017). These two proteins were also found in the secretome of anti-inflammatory M2 macrophages, indicating that these could be important pro-regenerative markers that a range of myeloid cells are capable of secreting (De Torre-Minguela et al., 2016). Following LPS and IFN-γ treatment of both primary mouse microglia and the mouse microglial cell line, BV2, a fall in the anti-inflammatory marker IL-4 was reported in MV (Grimaldi et al., 2019). Treatment of microglia with IFN-γ led to increases in the MHC class 2 proteins (Prada et al., 2013).

Besides classical inflammatory markers, the proteomic content of microglial exosomes also shifted to contain more proteins involved in RNA binding and the extracellular matrix (Yang et al., 2018).

Comparing sEV from different microglial populations in response to LPS lead to more variable sEV profiles (Murgoci et al., 2020). The response of microglial exosomal packaging also appeared to depend on the specific pathway used to induce activation, as treatment with LPS, activating TLR4, induced the packaging of GAPDH, but this was not recapitulated with TLR5 stimulation (Paolicelli et al., 2018).
4.1.2.3 Following other treatments

Another treatment used to induce EV secretion from microglia is ATP, which has been shown to induce EV secretion through the activation of the P2RX7 receptor and downstream signalling pathways (Bianco et al., 2005; Drago et al., 2017). In addition to changes in exosome secretion rate, ATP released from damaged cells and astrocytes also induced changes in EV proteins. MV collected from microglia treated with ATP contained pro-IL1β and the enzyme required to mature this protein into functional IL-1β, caspase 1 (Trotta et al., 2018).

Proteomic analysis also showed that exosomes from rat primary microglia treated with ATP contained more proteins involved in energy metabolism (Drago et al., 2017). The presence of important enzymes such as lactate dehydrogenase A (LDH-A) and malate dehydrogenase-2 suggested that EV can contain proteins necessary to induce changes in metabolism in target cells (Drago et al., 2017). Another function that also seems to be altered in EV from ATP-treated microglia appears to be cell adhesion. This suggests that EV from ATP-treated microglia may be able to adhere more strongly to target cells, which was shown using optical tweezers (Drago et al., 2017).

Wnt3a has also been used to induce exosome release from primary microglia and a shift in exosomal proteomics was also reported in this study (Hooper et al., 2012). In response to Wnt3a treatment, the exosomes were reported to contain Wnt3a but also compartments of the cytoskeleton, such as β-actin (Hooper et al., 2012).

4.1.3 Aims

The aims of this chapter are to:

- Test the influence of the R47H\textsuperscript{het} TREM2 variant on the proteomic content of exosomes secreted from iPS-Mg
- Determine whether exosomal content is altered by different treatments
- Study the contribution of the R47H\textsuperscript{het} TREM2 variant to these changes
4.2 Methods

4.2.1 Exosomal preparation

Exosomes were collected from iPS-Mg, carrying the Cv and R47H<sup>het</sup> TREM2 variant, before and after treatment with 100ng/ml LPS or 2:1 apoptotic neurons:iPS-Mg as previously described (Chapter 2.2.2). The extracted exosomes were lysed in RIPA buffer and protein concentration determined through BCA protein assay. Exosomes from different repeats were pooled into three independent samples (Table 4-1) containing 50µg of exosomal protein in 240µl of RIPA buffer each.

4.2.2 Liquid chromatography mass spectrometry (LC-MS)

LC-MS was performed by collaborators Dr Johan Gobom in the laboratory of Professor Henrik Zetterberg at the University of Gothenburg, with the workflow described in Figure 4-1. Samples were reduced with 100nM dithiothreitol for 30mins at 60°C. For subsequent filter aided sample preparation, 8M urea was added to the samples before the samples were added to 30kDa MWCO filters (Nanosep, Pall). Ultrafiltration was performed by centrifugation at 10,000g for 10mins at RT, and subsequently discarding the flow-through. The filters were washed twice by adding 200µl 8M urea followed by centrifugation as above. The filters were then washed with 200µl 0.5% SDC (sodium deoxycholate, 50mM TEAB triethylammonium bicarbonate (digestion buffer)). Alkylation of cysteine disulfides was performed by adding 100µl 18mM iodoacetamide in digestion buffer to the filters, and incubating for 20mins at RT in darkness. The liquid was removed by centrifugation and the filters were washed twice with 100µl digestion buffer. The filters were incubated with 0.1µg/ml trypsin in digestion buffer at 37°C overnight. Digested peptides were collected by centrifugation. Tandem Mass Tag (TMT) 10-plex reagents (8µg dissolved in 42µl acetonitrile) were added to the samples followed by incubation for 1hr at RT on a shaker. Hydroxylamine (8µl, 5%) were added and the samples were incubated for 15mins to inactivate excess TMT reagent, after which the individually TMT-labelled samples were pooled into a multiplex sample (Table 4-2). The samples were acidified by addition of 10% trifluoroacetic acid until pH<2 to precipitate the SDC. The sample was centrifuged at 18000g for 10mins and the SN was recovered. The TMT labelled peptides were fractionated by reversed-phase chromatography at basic pH, using a previously established protocol (Batth et al., 2014). Briefly, the multiplex sample was re-dissolved in 16µl 2 % AcN, 5 mM ammonium hydroxide and loaded on an Ultimate 3000 HPLC system (Thermo Fisher). Offline peptide separation was performed over
an XBridge BEH130 C18 3.5 µm, 2.1 mm x 250 mm separation column at a flow of 100µl/min, using a linear gradient and collecting 1 fraction/min for 72mins. Collected fractions were then concatenated to 12 fractions, which were dried by vacuum centrifugation. For LC-MS, the fractions were dissolved in 7µl 3% AcN and 0.1% formic acid.

4.2.3 Database search

The LC-MS data were processed using the software Proteome Discoverer 2.2 (Thermo Fisher Scientific) by Dr Johan Gobom in the laboratory of Professor Henrik Zetterberg at the University of Gothenburg, according to the following: protein identification was performed using the Mascot software (Matrix Science), searching the human subset of SwissProt, with the following settings: cleavage enzyme: trypsin; precursor mass tolerance: 15 ppm; fragment mass tolerance: 0.05 Da; Missed cleavage sites: 2; static modifications: Carbamidomethylation; dynamic modifications: oxidation. Percolator scoring was used, and validation was performed using the target-decoy approach, using 1% false discovery rate as cut-off for peptide identifications.
Table 4-1 Exosome samples used

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Pooled samples</th>
</tr>
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<tr>
<td>Cv 1</td>
<td>SFC 9/4/19</td>
</tr>
<tr>
<td>Cv 2</td>
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</tr>
<tr>
<td></td>
<td>ND 10/3/19</td>
</tr>
<tr>
<td>Cv 3</td>
<td>CTRL1 1/3/19</td>
</tr>
<tr>
<td></td>
<td>ND 9/3/19</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>SFC 9/3/19</td>
</tr>
<tr>
<td>Cv LPS 1</td>
<td>SFC 24/4/19</td>
</tr>
<tr>
<td></td>
<td>CTRL1 29/3/19</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>SFC 29/3/19</td>
</tr>
<tr>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>SFC 23/3/19</td>
</tr>
<tr>
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<td>SFC 6/6/19</td>
</tr>
<tr>
<td>Cv apop 2</td>
<td>SFC 19/6/19</td>
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</tr>
<tr>
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<td></td>
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<tr>
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</tr>
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<td>ADRC 26.15 10/4/19</td>
</tr>
<tr>
<td></td>
<td>ADRC 26.15 6/3/19</td>
</tr>
</tbody>
</table>
Figure 4-1 LC-MS workflow

Workflow of sample collection and LC-MS. The steps from protein digestion to normalisation were performed by Dr Johan Gobom in the laboratory of Professor Henrik Zetterberg. Due to the number of samples, the tagging and separation were performed in two separate experiments.
<table>
<thead>
<tr>
<th>Sample name</th>
<th>TMT Set</th>
<th>TMT reporter</th>
</tr>
</thead>
<tbody>
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<td>126</td>
</tr>
<tr>
<td>Cv LPS 1</td>
<td>1</td>
<td>127N</td>
</tr>
<tr>
<td>Cv apop 1</td>
<td>1</td>
<td>127C</td>
</tr>
<tr>
<td>R47H 1</td>
<td>1</td>
<td>128N</td>
</tr>
<tr>
<td>R47H LPS 1</td>
<td>1</td>
<td>128C</td>
</tr>
<tr>
<td>R47H apop 1</td>
<td>1</td>
<td>129N</td>
</tr>
<tr>
<td>Cv 2</td>
<td>1</td>
<td>129C</td>
</tr>
<tr>
<td>Cv LPS 2</td>
<td>1</td>
<td>130N</td>
</tr>
<tr>
<td>Cv apop 2</td>
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</tr>
<tr>
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</tr>
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<td>Cv 3</td>
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<tr>
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<td>127N</td>
</tr>
<tr>
<td>Cv apop 3</td>
<td>2</td>
<td>127C</td>
</tr>
<tr>
<td>R47H 2</td>
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<td>128N</td>
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<tr>
<td>R47H LPS 2</td>
<td>2</td>
<td>128C</td>
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<tr>
<td>R47H apop 2</td>
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<td>129N</td>
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<tr>
<td>R47H 3</td>
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<td>130C</td>
</tr>
<tr>
<td>Reference</td>
<td>2</td>
<td>131N</td>
</tr>
</tbody>
</table>
4.2.4 Analysis

I analysed the relative abundances, normalised to the reference sample, provided by Dr Johan Gobom, using MATLAB and tested the results for a normal distribution and the potential influence of the two different experimental runs (Figure 4-2). Following from this, the abundances were normalised to the individual reference sample for each experimental run, accounting for the overall differences of number of proteins detected in each experimental run. In addition to this, for further analysis, the abundance ratios were log_{10}-transformed to generate a normal distribution, allowing standard statistical tests to be used for the data (Figure 4-5).

The results from the database search were analysed using MATLAB, using code described in the appendix (Appendix 7.1). Analysis of the functional effect of the different proteins was conducted using FunRich and STRING analysis. For functional FunRich analysis, the enrichment analysis was performed against the UniProt database as reference background.

4.2.5 Protein co-expression network analysis (ProCoNA)

Modules in the samples were analysed using the previously published weighted gene co-expression network analysis (Zhang and Horvath, 2005; Langfelder and Horvath, 2008; Gibbs et al., 2013). The Cv and R47H^{het} samples respectively were pooled and treatments used as traits (Figure 4-3), generating two networks, one for Cv and one for R47H^{het} samples.

The entire code run in RStudio is included in the appendix (Appendix 7.2) and is based on the tutorial provided with the package. Briefly, topological overlap matrices based on the correlations between different proteins in the sample were generated, before they were converted into a dissimilarity measure. Subsequently, based on this dissimilarity measure, the proteins were clustered. This signed network was then raised to a power function, based on the assumption of a scale-free network. The power function was chosen through plotting scale independence and mean connectivity (Figure 4-4) and selecting the powers of 30 for both the Cv and R47H^{het} networks to meet the assumption of a scale-free model. Following this, proteins were clustered into discrete branches. To merge closely related modules, 30 proteins was set as a minimum module size and a threshold of 0.25 chosen to merge closely clustered modules. Grey modules represent proteins that did not cluster with any other modules.
Module preservation was done based on previous publications (Langfelder and Horvath, 2008; Langfelder et al., 2011; Carbajosa et al., 2018). Briefly, using the `modulePreservation` command, 200 permutations were run for both the Cv and R47H\textsuperscript{het} network to generate `medianRank` and `Zsummary` values for the different modules (Figure 4-18). In addition, the correlation between the kME, also called module membership, between the two different networks was plotted (Figure 4-19 and Figure 4-20).

After the modules were identified, the association of the individual modules with the different treatments, NT, LPS and apop neurons, was plotted and functional annotation was provided through the `GOenrichmentAnalysis` package, with a Bonferroni correction being applied to the p value to account for multiple comparisons. Functional annotation information is included in the appendix (Table 7-1 and Table 7-2). Finally, the similarity between the modules identified in the Cv and R47H\textsuperscript{het} networks was plotted.
Figure 4-2 Quality controlling LC-MS results

Following the database search, the raw abundances were plotted in a heatmap, revealing overall differences in the two different experiments run, with the second experiment (boxed in green) displaying overall a lower abundance of proteins in comparison with the first experimental run. This meant that abundances were normalised to the scrambled sample for each experiment, rather than the mean of both experiments to account for the differences in protein abundances.
Figure 4-3 Sample dendrograms

The dendrograms reveal clustering of the samples used for building of the Cv network (A) and R47H<sup>het</sup> network (B). The samples clustered according to the treatments the iPS-Mg received prior to exosome collection, which was indicated below.
Figure 4-4 Testing underlying ProCoNA assumptions

The networks built through the ProCoNA technique assume scale independence and low mean connectivity. To achieve these assumptions, a range of soft threshold powers was modelled for the Cv network (A) and the R47H\textsuperscript{het} network (B) to determine which power achieves that assumption. For the Cv network, scale independence is achieved with a power of 30, whilst the R47H\textsuperscript{het} network does not achieve true scale independence. Therefore, the power of 30 was chosen, since the R\textsuperscript{2} for the scale independence was highest for this modelled power. However, the R47H\textsuperscript{het} network achieves similar mean connectivity to the Cv network.
4.3 Results

4.3.1 Quality-controlling the data

To normalise the data properly and to ensure the results are reliable for further analysis, several quality-control steps were taken. First, the distribution of the data was plotted to reveal a non-normal distribution of the relative abundances of proteins (Figure 4-5A). As most statistical tests assume normal distribution, the data was log_{10} transformed, as is common for these kinds of analysis (Silva et al., 2012; Benitez et al., 2014; Bonham et al., 2019) to achieve a normal distribution (Figure 4-5B).

Database searches were conducted considering both no PTM (called Baseline) and phosphorylation as a potential modification (P-PTM). On one hand, not considering any PTM could lead to more robust data, on the other hand previous publications have suggested that phosphorylated proteins can be found within exosomes (Properzi et al., 2015; Moreno-Gonzalo et al., 2018). Comparing both datasets revealed that only 11 proteins were identified in the P-PTM dataset that the baseline dataset did not identify, with the large majority of proteins detected in both datasets (Figure 4-6A). As additional PTM can introduce uncertainty and therefore less reliable data, histograms were plotted for both datasets to compare how the data clustered. Individual replicates were clustered less closely in the P-PTM dataset (Figure 4-6B, C). As considering potential phosphorylation during the database search did not seem to add additional data to the datasets, the decision was taken to subsequently use the baseline dataset, as the data here appears to be more consistent and reliable.
Figure 4-5 Histogram of protein abundances

Normal distribution is assumed for standard statistical tests, but the normalized data did not display this as plotted in a normal histogram of relative abundance ratios (A). Log\textsubscript{10} transformation using the log\textsubscript{10}(x+1) equation led to a normal distribution in the histogram of abundance ratios (B) and therefore, data normalised in this way was used for subsequent analyses.
Figure 4-6 Comparing different database searches
The different database searches, taking phosphorylation into consideration as PTM (P-PTM) and taking no PTM into account (baseline), were compared. The majority of detected proteins overlapped between both datasets (A) with only 11 proteins detected in the P-PTM dataset, but 113 proteins only detected in the baseline dataset. Heatmap of the P-PTM dataset showed that neither samples from different treatments nor TREM2 variants appeared to cluster (B). Rather, experimental replicates appeared to spread out. In the baseline dataset, however, clustering into both treatment condition and TREM2 variants was observed (C), therefore this dataset was used for subsequent analysis.
4.3.2 This dataset resembles other microglial exosome datasets

To understand the similarity of this dataset to previously published exosomal proteomics, the detected proteins were further analysed. Out of the 3,019 detected proteins, over 70% have been previously identified in exosomes, recorded either in the ExoCarta repository (http://www.exocarta.org/) or a meta-analysis of high throughput exosomal proteomics (Choi et al., 2013) (Figure 4-7A). The other proteins identified in the exosomal fraction from the iPS-Mg could be microglia specific proteins. The proteins identified within the exosomes are highly enriched for proteins previously associated with exosomes, followed by lysosomes, the cellular component with which MVB can fuse, and the cytoplasm (Figure 4-7B). This verifies that the extracted and analysed particles are indeed of exosomal origin.

In addition to analysing overall similarity of the dataset with proteins previously associated with exosomes, the presence of known exosomal proteins was also assessed. Classical exosomal markers were identified through LC-MS, such as CD63, CD81 and ALIX (Table 4-3). Similar to previous studies (Drago et al., 2017; Murgoci et al., 2020), microglia specific markers such as TREM2 and osteopontin were also found in the fractions. In addition to this, CD13, distinguishing between microglial exosomes and exosomes from other hematopoietic cells (Potolicchio et al., 2005; Prada et al., 2013), was also found (Table 4-3). In addition to these markers, negative markers (Witwer et al., 2013), such as calnexin, an ER marker, and HSP90, a mitochondrial protein, were also identified in the exomes through LC-MS (Table 4-3).
Over 70% of the detected proteins in the dataset (iPS-Mg exosomes) have been previously linked to exosomal proteins (A). 2147 have been previously published in a meta-analysis of high-throughput proteomics of exosomes (Choi et al., 2013), whilst 475 proteins have been reported into the online repository of exosomal proteins, ExoCarta (red). This is also verified when analysing the proteins for their cellular compartment of origin (B). The proteins are
significantly associated with exosomes, followed by the lysosomes and cytoplasm relative to the annotated control genome. *** $p \leq 0.001$. 
<table>
<thead>
<tr>
<th>Name</th>
<th>Importance</th>
<th>Relative abundance (mean ± SEM)</th>
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<td>ALIX</td>
<td>Classical exosome marker</td>
<td>0.37 ± 0.02</td>
<td>(Hooper et al., 2012; Colombo et al., 2013; Andreu and Yáñez-Mó, 2014)</td>
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<tr>
<td>CD63</td>
<td>Classical exosome marker</td>
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<td>(Witwer et al., 2013; Andreu and Yáñez-Mó, 2014)</td>
</tr>
<tr>
<td>CD81</td>
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<td>0.42 ± 0.03</td>
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<tr>
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</tr>
<tr>
<td>Flotillin-1</td>
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<td>0.34 ± 0.01</td>
<td>(Silva et al., 2012; Colombo et al., 2013; Witwer et al., 2013)</td>
</tr>
<tr>
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<td>(Horibe et al., 2018; Huang et al., 2018)</td>
</tr>
<tr>
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<td>(Horibe et al., 2018; Huang et al., 2018)</td>
</tr>
<tr>
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<td>0.34 ± 0.02</td>
<td>(Horibe et al., 2018; Huang et al., 2018)</td>
</tr>
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</tr>
<tr>
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<td>(Drago et al., 2017)</td>
</tr>
<tr>
<td>GPNMB</td>
<td>Microglial marker</td>
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<td>(Murgoci et al., 2020)</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Microglial marker</td>
<td>0.43 ± 0.06</td>
<td>(Gosselin et al., 2017; Murgoci et al., 2020)</td>
</tr>
<tr>
<td>TREM2</td>
<td>Microglial marker</td>
<td>0.41 ± 0.04</td>
<td>(Muffat et al., 2015; Abud et al., 2017; Haenseler et al., 2017)</td>
</tr>
<tr>
<td>α-enolase</td>
<td>Microglial marker</td>
<td>0.38 ± 0.02</td>
<td>(Drago et al., 2017)</td>
</tr>
<tr>
<td>Calnexin</td>
<td>Possible marker for contamination</td>
<td>0.39 ± 0.02</td>
<td>(Witwer et al., 2013)</td>
</tr>
<tr>
<td>Histone H4</td>
<td>Possible marker for contamination</td>
<td>0.22 ± 0.02</td>
<td>(Witwer et al., 2013)</td>
</tr>
<tr>
<td>HSP90-α</td>
<td>Possible marker for contamination</td>
<td>0.38 ± 0.05</td>
<td>(Witwer et al., 2013)</td>
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</table>
4.3.3 R47H<sup>het</sup> exosomes contain a different proteomic profile

The difference between the protein abundances of exosomes extracted from baseline Cv and R47H<sup>het</sup> iPS-Mg was plotted with the use of a volcano plot. The log<sub>2</sub> of the ratio, the fold change, was plotted in the x-axis, whilst the significance of this change, in the form of the log<sub>10</sub> of the p value, was plotted in the y-axis. This plot enabled the identification of proteins that displayed a statistically significant two-fold or larger change, as plotted in the top left and right panel of the graph. Comparing the R47H<sup>het</sup> exosomes with the Cv exosomes, nine proteins were found to be present at significantly higher levels in R47H<sup>het</sup> exosomes in comparison with Cv exosomes (Figure 4-8). The overarching functions of these proteins were explored through STRING analysis, which revealed that the proteins were mainly associated with the negative regulation of transcription and metabolic processes (Figure 4-9A).

In addition to comparing the differentially packaged proteins, the 200 most abundant proteins packaged in both Cv and R47H<sup>het</sup> exosomes were also analysed and compared. 40.5% of the most abundant proteins overlapped at baseline (Figure 4-9B). Functionally, this translated to the most abundant proteins in Cv exosomes fulfilling functions relating to cell growth and/or maintenance, whilst R47H<sup>het</sup> exosomes were enriched for proteins in the energy pathway and metabolism clusters, partially supporting the previous results showing that exosomes from R47H<sup>het</sup> iPS-Mg contain significantly more proteins involved in the negative regulation of metabolic processes (Figure 4-9C).
A

B

Phospholipase D4

<table>
<thead>
<tr>
<th></th>
<th>Cv</th>
<th>R47H^{het}</th>
</tr>
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<tbody>
<tr>
<td>Abundance Ratio log_{10} transformed</td>
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Calreticulin

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<th></th>
<th>Cv</th>
<th>R47H^{het}</th>
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<td>Abundance Ratio log_{10} transformed</td>
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Chitinase-3-like protein 1

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<tr>
<th></th>
<th>Cv</th>
<th>R47H^{het}</th>
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<tbody>
<tr>
<td>Abundance Ratio log_{10} transformed</td>
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Chitotriosidase-1

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<tr>
<th></th>
<th>Cv</th>
<th>R47H^{het}</th>
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</thead>
<tbody>
<tr>
<td>Abundance Ratio log_{10} transformed</td>
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</table>
To discern the difference between the protein concentration of exosomes from Cv and R47H\textsuperscript{het} iPS-Mg a volcano plot was generated to plot the significant 2-fold changes (A). The nine proteins that were found to be increasingly present in exosomes from R47H\textsuperscript{het} were plotted individually (B). $n = 3$, t-test. * $p < 0.05$, ** $p \leq 0.01$.

Figure 4-8 Volcano plots at baseline
Figure 4-9 Functional analysis of proteins
Functional analysis of the nine proteins that were increasingly packaged into exosomes from R47H<sub>het</sub> iPS-Mg was performed using STRING analysis. It revealed that the proteins were mainly involved in the negative regulation of transcription and metabolic processes (A). The 200 most abundant proteins in both Cv and R47H<sub>het</sub> exosomes were compared, showing that out of 200 proteins, 119 were found exclusively in either one or the other (B). These differences are also translated in different functions being assigned to the proteins. Whilst Cv exosomes were enriched for proteins falling in the “cell growth and/or maintenance” cluster, R47H<sub>het</sub> exosomes contained significantly more exosomes relating to energy pathways and metabolism. n = 3 with ** p ≤ 0.01, *** p ≤ 0.001.
4.3.4 LPS activation of iPS-Mg change exosomal content

Other research has revealed that exosomes from microglia have a different protein content following activation of the cells, with LPS for example (Yang et al., 2018). To verify this and investigate the effect of TREM2 variants on this process, the differences in exosomal proteins were plotted following activation of iPS-Mg with 100ng/ml LPS for 24hrs. Again, volcano plots were used to plot the changes in exosomes from LPS-treated Cv iPS-Mg in comparison to Cv exosomes, and the changes in R47Hhet exosomes following LPS activation in comparison to baseline R47Hhet exosomes (Figure 4-10A and B respectively). Some proteins of interest were circled in the volcano plots and plotted individually (Figure 4-10C). Inflammatory markers such as CXCL5, CCL22 and IL-1β were significantly packaged into exosomes from LPS-activated Cv iPS-Mg, with the effect being less pronounced in exosomes originating from LPS-treated R47Hhet iPS-Mg. Components of the complement cascade, such as C1qc, were found to be significantly downregulated in activated exosomes, independent of TREM2 status of the cells. Similar to the sTREM2 levels (Figure 3-6), TREM2 levels in the exosomes also fell with LPS treatment of the iPS-Mg, albeit only significantly in the R47Hhet iPS-Mg.
Figure 4-10 Proteomic differences following LPS treatment
Following activation of iPS-Mg with 100ng/ml LPS for 24hrs, exosomes were extracted and proteomic content analysed using LC-MS. Volcano plots were plotted to compare the differences in exosomes from LPS-treated Cv iPS-Mg to baseline Cv (A). The differences in exosomes from LPS-activated R47het iPS-Mg were compared to baseline exosomes from R47Hhet (B). Proteins of interest were circled and some were plotted individually (C). CXCL5 and TNF-α receptor associated protein 1 (TRAF1) were increased Cv exosomes following LPS activation, but only TRAF1 also increased in the TREM2 variants. CCL22 was only significantly upregulated in the Cv exosomes, but in the R47Hhet exosomes, LPS treatment had no effect. IL-1β, a known cytokine that is increasingly secreted from microglia following activation with LPS, again increased significantly in the Cv exosomes, but not in the R47Hhet. Components of the complement system, such as C1qc, decreased in both TREM2 variants following LPS treatment, whilst TREM2 only decreased significantly in the R47Hhet exosomes following LPS treatment, whilst in the Cv, it was not quite significant. n = 3, two-way ANOVA with Tukey’s post hoc test. * p < 0.05, ** p ≤ 0.01, *** p ≤ 0.001 and n.s. not significant.
4.3.5 Changes in exosomal proteome is stimulus-specific

In addition to investigating the effect of the LPS stimulus on exosomal protein content, the effect of apoptotic neurons on exosomal protein content was also investigated. This is arguably a more physiological stimulus and could stimulate TREM2 directly (Wang et al., 2015; Garcia-Reitboeck et al., 2018; Shirotani et al., 2019). It also provided the opportunity to investigate whether changes in exosomal proteins were stimulus specific.

Performing hierarchical clustering through Euclidian distance calculations, heatmaps were generated for all of the samples analysed through LC-MS. Averaging of the repeats revealed that the samples form stimulus-specific clusters, but that at baseline exosomes from Cv and R47H\textsuperscript{het} iPS-Mg did not form a cluster, but rather the R47H\textsuperscript{het} exosomes were more closely clustered with exosomes from LPS-treated iPS-Mg (Figure 4-11).

4.3.5.1 Changes in the most abundant proteins

Analysis of the most abundant proteins after either LPS or apoptotic neuron treatment of iPS-Mg revealed that a subset of proteins (14% and 19% respectively) were stimulus-specific changes, independent of TREM2 status (Figure 4-12Ai, Bi). The proteins that were found in both Cv and R47H\textsuperscript{het} exosomes following LPS stimulation did not overlap with the proteins found following treatment with apoptotic neurons, suggesting a stimulus specific shift in exosomal profile. However, the majority of abundant proteins were found specific for either Cv or R47H\textsuperscript{het} exosomes, suggesting a TREM2 specific response to both LPS and apoptotic neuron treatment (Figure 4-12Ai, Bi). Functional analysis suggested that the cell growth/maintenance cluster decreased in both Cv and R47H\textsuperscript{het} exosomes following LPS treatment, whilst the immune cluster was only significantly increased in Cv exosomes following LPS treatment (Figure 4-12Aii). The high contribution of the metabolism cluster to R47H\textsuperscript{het} exosomes also decreased in these cells following LPS treatment (Figure 4-12Aii). However, treatment with apoptotic neurons increased proteins involved in protein metabolism in both Cv and R47H\textsuperscript{het} exosomes (Figure 4-12Bii). Additionally, the energy pathway cluster significantly increased in R47H\textsuperscript{het} exosomes following treatment with apoptotic neurons, whilst displaying no changes in Cv exosomes (Figure 4-12Bii). The specific functions of the most abundant proteins in the exosomes appeared to change depending on TREM2 status and treatment of iPS-Mg.
4.3.5.2 PCA revealed cluster of samples

With over 3,000 proteins identified through LC-MS, to only analyse the changes in the 200 most abundant proteins could mean some information is lost. To be able to consider changes in abundances across all identified proteins, dimensionality reduction was performed in the form of PCA (Figure 4-13). In this type of analysis, principal components (PC) were generated to visualise the variance in the data. Plotting the data points against the first and second PC confirmed that at baseline, Cv exosomes were different from exosomes extracted from treated iPS-Mg (Figure 4-13A). Treatment with LPS of the iPS-Mg led to the formation of a separate cluster of samples, the “LPS cluster” (Figure 4-13, indicated in blue). This cluster also contains the samples extracted from LPS-treated R47Hhet iPS-Mg, but interestingly also the baseline R47Hhet exosomes. This further confirmed the trend from the heatmap (Figure 4-11), that at baseline R47Hhet exosomes were very similar to LPS-activated exosomes. This also meant that in response to LPS stimulation, the protein content changes less in the R47Hhet, possibly explaining the subdued response in the R47Hhet iPS-Mg in response to LPS stimulation (Figure 4-10).

Stimulation of iPS-Mg with apoptotic neurons led the exosomal proteins to cluster in another cluster, indicated in red called the “apop cluster” (Figure 4-13A). This apop cluster contained the samples from exosomes secreted from both Cv and R47Hhet iPS-Mg treated with apoptotic neurons. Due to the spread in the apop cluster, the samples were plotted on the third PC, relative to the first PC, and this made the difference between Cv apop exosomes and R47Hhet apop exosomes clear (Figure 4-13B). Therefore, whilst R47Hhet exosomes display a shift in protein abundances in response to treatment, this response was different from the response of Cv exosomes.

4.3.5.3 Functional analysis of identified clusters

To better understand the underlying functional meaning of the different clusters identified during the PCA analysis, further analysis was performed on the PC themselves. Each of the proteins detected in the LC-MS experiment contributed to a certain extent to each PC. That meant that some proteins were strongly associated with the first PC and others less so. Understanding the functions of these proteins could elucidate the functional meaning of the shifts from one cluster to another.
Out of the 100 proteins that contribute the most to the three PC, only proteins that were exclusive to each PC were analysed. The analysis revealed that the proteins in each PC appeared to fulfil slightly different roles (Figure 4-14).

The first PC broke the samples up into three distinct clusters: untreated Cv, the LPS cluster, containing the untreated R47H$^{\text{het}}$ exosomes, and the apop cluster (Figure 4-13A). The functions of the proteins contributing to it were mainly related to the regulation of the response to stress and metabolic processes (Figure 4-14B). This meant that differences between the LPS and apop cluster in comparison with the untreated Cv exosomes were related to metabolic processes and the responses to stress.

Whilst it was less obvious, the second PC separated the samples into four different clusters, comprising of baseline Cv exosomes, Cv apop exosomes, baseline R47H$^{\text{het}}$ and R47H$^{\text{het}}$ apop in one and the final one containing both Cv and R47H$^{\text{het}}$ LPS exosomes (Figure 4-13A). The functions of the proteins contributing to this PC were cytoskeletal organization and response to external stimuli (Figure 4-14C).

As previously discussed, the third PC separated the R47H$^{\text{het}}$ apop samples from the Cv apop samples (Figure 4-13B), which clustered with the LPS-treated samples and baseline Cv samples, with protein functions differing in metabolic processes and cellular biogenesis (Figure 4-14D).

Overall, the untreated Cv proteins were separated from the other samples in the first and second PC (Figure 4-13), suggesting they were unique in metabolic processes, the regulation of cell responses and cytoskeletal organization. On the other hand, untreated R47H$^{\text{het}}$ exosomes were similar to LPS-treated samples in the first PC, suggesting similarities in the response to stress, and similar to apop-treated samples in the second PC, again in the response to external stimuli. This would suggest that the distinct feature of proteins packaged into R47H$^{\text{het}}$ exosomes are the function they fulfil in response to external stimuli, and how this response to stress is modulated.

Following LPS treatment, both exosomes from Cv and R47H$^{\text{het}}$ iPS-Mg were very similar to each other, not separating in any of the three PC (Figure 4-13). In line with literature (Yang et al., 2018) and the previous in-depth analysis (Chapter 4.3.4), the proteins packaged were involved in the response to external stimuli, such as
inflammatory cytokines and chemokines. The apop cluster was seen along the second PC (Figure 4-13), indicating changes in the response to external stimuli and the cytoskeleton in both Cv and R47H^{het} exosomes. However, the R47H^{het} apop exosomes were separated out from the other samples along the third PC (Figure 4-13), indicating changes in the biogenesis function of these proteins.

4.3.5.4 Individual proteins

To further understand these changes along the clusters, individual proteins, which contributed the most to each PC, were plotted separately (Figure 4-15). The proteins that contributed the most to the first PC could play a role in the cellular stress response (Figure 4-14B). In line with this, exosomal ApoD (Figure 4-15Ai), a key contributor to both the first and second PC, was shown to protect neurons against ROS (Pascua-Maestro et al., 2019) and increased stress resistance in an animal model (Muffat et al., 2008). Additionally, it is also implicated in playing a protective role in AD (Dassati et al., 2014). S100P on the other hand (Figure 4-15Aii), is a calcium binding protein, which can bind to RAGE products to induce cell proliferation and activate MAPK pathways (Penumutchu et al., 2014). Reductions in these proteins following treatment or because of the R47H^{het} variant could impair cell stress responses in target cells.

As shown in Figure 4-14A, there is considerable overlap between proteins contributing to the first and second PC, with ApoD contributing the most to both components. Other proteins that contribute significantly to the second PC are keratin 6A (KRT6A) and small proline rich protein 1B (SPRR1B, Figure 4-15B). Both Keratin and SPRR1B are associated with the cytoskeleton. Structural changes in the cytoskeleton, caused by keratin mutations have been implicated in inducing neurodegeneration in a model of Down syndrome (Perluigi et al., 2014), whilst the SPRR1B paralog, SPRR1A, was associated with actin in membrane ruffles to facilitate dendrite outgrowth following peripheral nerve damage, making it a regeneration-associated gene (Bonilla et al., 2002; Starkey et al., 2009).

The proteins significantly contributed to the third PC were LDH-A and translin (TSN) (Figure 4-15C). LDH-A has been shown to reduce mitochondrial sensitivity to ROS, in addition to being decreased in murine models of AD (Newington et al., 2013). TSN on the other hand is a RNA binding protein playing a role in the interaction between metabolism and sleep (Murakami et al., 2016). Specifically targeting the miRNA pathway to induce early miRNA degradation, TSN was linked to the decrease
in the miRNA pathway in PD, another neurodegenerative disease (Fu et al., 2016; Vinnikov and Domanskyi, 2017).

4.3.5.5 Influence of TREM2 variations on specific responses to different treatments

The analysis so far has revealed that exosome proteome content shifts depending on the stimulus the iPS-Mg have been exposed to. Differences in the clusters following LPS and apoptotic neuron treatment has revealed that after treatment with apoptotic neurons, Cv and R47H<sup>het</sup> exosomes differ, due to changes in proteins involved biogenesis and metabolism (Figure 4-14). This is reflected in increases in LDH-A and TSN in R47H<sup>het</sup> exosomes (Figure 4-15C), involved in ROS sensitivity and miRNA degradation. However, PCA, on which this analysis was based, was performed on all samples at once, which could hide subtle differences. To investigate whether there are specific differences in the LPS and apop cluster, based on TREM2 status, volcano plots were generated to compare the proteomic profile of Cv and R47H<sup>het</sup> exosomes following each treatment (Figure 4-16). This has the advantage that only two conditions at once are compared, making the test more sensitive to specific distinct changes.

No differences were observed in the LPS condition (Figure 4-16A), supporting previous results showing a clear LPS cluster and few differences between Cv LPS exosomes and R47H<sup>het</sup> LPS exosomes (Figure 4-13). However, five proteins were found to be increasingly present in exosomes from apoptotic neuron treated Cv iPS-Mg (Figure 4-16Bi). MAP1A interacts with microtubules and mutations on it can cause structural defects. Due to its location in the somatodendritic compartment, it is also linked with synaptic protein modulation (Liu et al., 2015). The TNF-α induced protein 8 like 2 protein (TNFAIP8L2) is involved in immune homeostasis through inhibiting the PI3K-Rac pathway. Additionally, increased levels have been shown to be associated with severity of PD (Kouchaki et al., 2018). The differential expression of ubiquilin-2 is particularly interesting due to the role ubiquitin-protease system plays in a host of neurodegenerative disorders (Daoud and Rouleau, 2011). Missense mutations of UBQLN2 are associated with some cases of familial ALS as ubiquilin-2 fails to deliver ubiquitinated proteins to the proteasome (Daoud and Rouleau, 2011). Decreased levels of ubiquilin-2 are also reported in the brains of AD patients (Zhang et al., 2014). Overall, these differentially packaged proteins between Cv and R47H<sup>het</sup> exosomes following stimulation of iPS-Mg with apoptotic neurons involved cytoskeletal changes, the immune system and the proteasome.
Plotting the Euclidian distance between the averages of the three repeats in a heatmap showed that the samples cluster depending on the stimuli the iPS-Mg were exposed to. At baseline however the Cv and R47H\textsuperscript{het} samples appear not to fall into the same cluster, with previously discussed differences (4.4.3) likely to explain this. \( n = 3 \).

*Figure 4-11 Heatmap of exosomal proteome following different iPS-Mg treatments*
The changes in the 200 most abundant proteins following LPS treatment (A) or treatment with apoptotic neurons (B) were observed and compared. Despite the clustering observed in Figure 4-11, only 28 proteins were shared between the TREM2 variants following LPS stimulation, with the majority of the changes in proteins unique to either Cv or R47H^het exosomes (Ai). Comparing this shift functionally showed an enrichment in the cell growth and/or maintenance
module in Cv exosomes, which decreased in response to LPS treatment (Aii). The enrichment for the metabolism module observed in R47H\textsuperscript{het} exosomes also decreased with LPS activation. On the other hand, following LPS treatment, iPS-Mg secreted exosomes enriched for proteins involved in the immune response, with Cv exosomes showing a bigger increase in this module than R47H\textsuperscript{het} exosomes (Aii). Following treatment of iPS-Mg with apoptotic neurons, a similar shift in the most abundant proteins packaged within exosomes was observed with over 70% changing in response to the treatment in both the Cv and R47H\textsuperscript{het} exosomes (Bi). However, the 38 proteins found in exosomes from both TREM2 variants following treatment did not overlap with the proteins found exclusively in the exosomes following LPS treatment. Functionally, the most abundant proteins in the Cv exosomes from apop treatment show a higher proportion in the cell growth/maintenance module (Bii) than following LPS treatment. Treatment with apoptotic neurons induced an increased packaging of proteins involved in protein metabolism and in case of the R47H\textsuperscript{het} iPS-Mg, energy pathways. n = 3, ** p ≤ 0.01, *** p ≤ 0.001
A

Second Principal Component

First Principal Component

B

Third Principal Component

First Principal Component
Figure 4-13 PCA analysis

A plot of the first PC (explaining 32% of the variance) and the second PC (25.4% of the variance, A) showed that on average the control samples cluster (circled in grey) far away from the other conditions. Upon activation with LPS, the protein abundances shift down on the first component, close to the baseline R47H^het samples (circled in blue). This indicates, that even at baseline, R47H^het samples are very similar to LPS-treated Cv exosomes. Activation of R47H^het with LPS did not appear to shift the samples. On the other hand, treatment with apoptotic neurons led exosomes to form their own cluster (circled in red). The response of R47H^het iPS-Mg to apoptotic neurons in terms of changes in exosome protein abundances was subdued in comparison, seen best when plotting the first PC and the third PC (explaining 12.4%, B), with Cv exosomes (circled in yellow) being different from the R47H^het exosomes (circled in green). PCA analysis with n = 3 shown.
Figure 4-14 Functional analysis of PC

The proteins contributing the most to each of the three PC were analysed to elucidate their functions within cells. There was some overlap between the 100 proteins contributing the most to each PC (A), particularly between the first and second PC. Therefore, only proteins that were found to contribute exclusively to one PC were further analysed. STRING analyses of the first PC (B), the second PC (C) and the third PC (D) were plotted to elucidate potential functions of these proteins.
Proteins of interest that contribute the most to each PC were plotted individually, with proteins contributing to PC1 (A) and PC2 (B) showing significant decreases in exosomes from treated iPS-Mg and iPS-Mg carrying the R47H<sup>het</sup> variant in comparison to NT Cv exosomes. ApoD contributed the most to both the first and second PC. In contrast to this, proteins contributing to the third PC (C), LDH-A and TSN increased following treatment of iPS-Mg with apoptotic neurons, in particular in the R47H<sup>het</sup> exosomes. n = 3, two-way ANOVA with Tukey’s post hoc test, * p < 0.05.
The contribution of the R47H*het variant on exosomal proteins following treatment of iPS-Mg with either LPS or apoptotic neurons was analysed through volcano plots. Comparing the
proteome of exosomes from LPS-treated Cv iPS-Mg to LPS-treated R47H^{het} iPS-Mg revealed no differentially packaged proteins (A), however the same graph for the apoptotic neuron treatment (Bi) showed that five proteins were significantly more packed in exosomes from Cv iPS-Mg treated with apoptotic neurons than in exosomes from R47H^{het} iPS-Mg treated with apoptotic neurons. These proteins were individually plotted (Bii). n = 3, two-way ANOVA with Tukey's post hoc test and * p < 0.05.
4.3.6 ProCoNA

Based on the assumption that proteins, whose changes in protein levels within exosomes are similar, are functionally related, the proteins identified in the LC-MS were clustered into functionally relevant modules. This was done separately for the Cv and R47H\textsuperscript{het} samples. Similar approaches have been used in the past to identify modules, clusters and trends in big data (DiLeo \textit{et al.}, 2011; Gibbs \textit{et al.}, 2013).

For both networks, 14 different modules were identified, with the final 15\textsuperscript{th} grey module representing proteins that did not cluster with any other modules (Figure 4-17). To assess whether the modules detected were indeed real modules or were only detected because of noise in the data, a range of module preservation analyses were performed with each measure representing a different mathematical approach (Langfelder \textit{et al.}, 2011). A high \textit{medianRank}, based on observed preservation statistics, suggests a less well preserved module, whilst modules with a \textit{Zsummary} higher than 10 are considered well preserved. For values below 2, the modules are assumed to not be preserved at all, and above 5, they are moderately well preserved (Langfelder \textit{et al.}, 2011; Carbajosa \textit{et al.}, 2018).

Based on the \textit{Zsummary}, the salmon and greenyellow modules in the Cv network are not preserved, whilst the red, black, purple, tan, pink, cyan and magenta modules may only be moderately preserved (Figure 4-18A). However, the \textit{Zsummary} measure is influenced by the size of the analysed modules, giving smaller modules lower values, making the second preservation readout of the \textit{medianRank} necessary. Based on the \textit{medianRank}, with a low score indicating module preservation, the pink, red, black, purple, tan and cyan Cv modules have a lower score than the brown Cv module, which has a \textit{Zsummary} of above 10, however the problem with this measurement is the lack of a clear cut-off point. This would indicate that the salmon, greenyellow and magenta Cv modules were less well preserved.

For the R47H\textsuperscript{het} network, no module had a \textit{Zsummary} score of below 2, but the yellow, magenta, black, red, purple, cyan, tan, salmon and greenyellow modules had a score below 10 (Figure 4-18B). Again, the \textit{medianRank} suggests that cyan, purple, salmon and tan modules may be preserved, as indicated by a low score, indicating only moderate preservation for the yellow, magenta, black, red and greenyellow R47H\textsuperscript{het} modules.
Due to the lack of cut-off point for the medianRank and the size dependency displayed in the Zsummary plots, another measurement of module preservation was investigated in addition. The kME is a measurement of the proteins with the module eigennode, effectively representing module membership of proteins. Correlating the kME of module-specific proteins in the Cv network with the kME of the same proteins in the R47Hhet network is reflective of whether Cv module-specific proteins behave similarly to the other network, again representing module preservation. These correlations were plotted for modules with a Zsummary below 10 to further understand their preservation. Based on this, the purple, black, red and cyan Cv modules were deemed to be preserved, owing to their kME correlations (Figure 4-19) and relatively low medianRank scores (Figure 4-18A). The pink and magenta modules were considered to be moderately preserved, whilst the salmon and greenyellow modules in the Cv network, displaying a low Zsummary score, also showed no significant kME correlation.

For the R47Hhet network, the kME correlations were also plotted for modules with a Zsummary below 10. Based on the significant correlations (Figure 4-20), the yellow, magenta, black, purple and cyan R47Hhet modules, paired with a low medianRank score (Figure 4-18B), were considered to be preserved, whilst the red, tan and salmon module were only moderately preserved. The greenyellow module on the other hand did not appear to be preserved.

Following on from the module preservation analysis, the relationship between the identified modules and the treatments the iPS-Mg experienced before exosomes were collected were analysed (Figure 4-21). This revealed that modules did not have a significant positive correlation with more than one treatment. The modules that were associated with the NT condition in the Cv network (Figure 4-21A) were the brown, black and purple modules. Functional annotation of the modules (Table 7-1) revealed that these modules were enriched for proteins associated with differentiation and development, signalling receptors and activity of kinases respectively, representing a wide range of functions exosomes can support. The turquoise module, associated with the LPS treatment (Figure 4-21A), was enriched for proteins involved in the immune response, whilst the modules associated with the apoptotic neuron treatment, blue, cyan and green, were linked to metabolism, RNA binding and protein translocation.
For the R47H\textsuperscript{het} network, the modules appeared to be more exclusively separated into modules associated with either treatment (Figure 4-21B), in contrast to the Cv network which showed some overlap between modules associated with the NT and LPS treatment. The tan and turquoise modules in this network were associated with the NT condition and were linked to actin organization and differentiation and development (Table 7-2). The modules most strongly associated with the LPS treatment were the purple and blue modules (Figure 4-21B), linked to RNA binding and protein translocation respectively. On the other hand, the apoptotic neuron treatment was associated with the yellow and pink modules and their function of metabolism and proteasome activity.

To study the correspondence between the modules identified in both the Cv and R47H\textsuperscript{het} network, the overlap between the two networks was plotted, with the number in the matrix representing the number of shared proteins between the modules of the two networks and the colour being a reflection of the significance of the overlap (Figure 4-22). What this matrix showed is that some modules of the two different networks overlap very highly, whilst some modules identified in the Cv network were not identified in the R47H\textsuperscript{het} network and vice versa. Whilst overlap suggests a shared function between the two networks, modules that are not shared could highlight differences between the two genotypes. The overlap between the brown module of the Cv network and the turquoise module of the R47H\textsuperscript{het} network, both associated with the NT condition and differentiation and development, showed that this function appears to be shared between the TREM2 variants at baseline. On the other hand, the black and purple Cv modules, also linked to NT, were not shared with the R47H\textsuperscript{het} network and in turn, the tan R47H\textsuperscript{het} network was not represented in the Cv network. This suggests that at baseline, there are subtle differences between exosomes from Cv and R47H\textsuperscript{het} iPS-Mg with Cv exosomes more involved with signalling receptors and kinase activity whilst the R47H\textsuperscript{het} exosomes had an influence on actin reorganization.

The module associated with the LPS treatment in the Cv network, the turquoise module, was split across two different modules in the R47H\textsuperscript{het} network, the blue and green modules (Figure 4-22). Whilst the turquoise Cv module is linked to an immune response (Table 7-1), the blue R47H\textsuperscript{het} network is linked to protein translocation (Table 7-2), indicating potentially diverging functionalities despite the overlap in proteins. On the other hand, the green R47H\textsuperscript{het} module that the turquoise Cv module also overlapped with, was also linked to the immune response, however, this green
R47H\text{het} module was not significantly correlated to the LPS treatment, despite a positive correlation (Figure 4-21B).

The modules in both networks associated with the apoptotic neuron treatment also display some overlap. The blue Cv module strongly overlapped with the brown R47H\text{het} module, which was strongly, but not quite significantly (p = 0.06), correlated to the treatment. Both modules were strongly linked to metabolic proteins. The green Cv module, also linked to the treatment with apoptotic neurons, overlapped with the yellow R47H\text{het} module. Whilst the green Cv module was enriched for proteins involved in the protein translocation to the mitochondria, the yellow R47H\text{het} module was linked to processes of metabolism. The other modules associated with this treatment in both the Cv and R47H\text{het} network shared no overlap with the other, suggesting that whilst there was a strong overall link to metabolism in the exosomes following iPS-Mg treatment with apoptotic neurons, there were some subtle \textit{TREM2} specific differences.
The proteins in the Cv and R47H<sup>het</sup> samples were clustered based on their correlation matrix to form modules. These modules are indicated for the Cv and R47H<sup>het</sup> samples in A and B respectively by colour with the height representing the distance between the individual clusters.

*Figure 4-17 Network building in ProCoNA*
Figure 4-18 Module preservation in the two networks

To assess module preservation in both the Cv (A) and R47Hhet networks (B) both medianRank and $Z_{summary}$ were plotted for the 14 identified modules, excluding the grey module. A low medianRank suggests the module to be preserved, whilst modules with a $Z_{summary}$ of above 10, indicated by the red line, are considered highly preserved. A $Z_{summary}$ of above 5, indicated by the black line, indicates moderate module preservation, whilst a score of below 2, indicated by the blue line, suggests no module preservation.
Figure 4-19 kME correlations for Cv modules
For modules in the Cv network with a Zsummary below 10 (Figure 4-18A), the correlation of the kME of the corresponding genes between the Cv and R47H^het network was plotted, with both the correlation and significance shown for each module. The modules shown represent both preserved modules (A), moderately preserved modules (B) and less preserved modules (C).
Figure 4-20 kME correlations for R47H^het modules
For modules in the R47Hhet network with a \textit{Zsummary} score below 10 (Figure 4-18B), the correlation of the kME of the corresponding genes between the Cv and R47Hhet network was plotted, with both the correlation and significance shown for each module. The modules shown represent both preserved modules (A), moderately preserved modules (B) and less preserved modules (C).
Figure 4-21 Module treatment relationships
The interaction between the modules identified in the Cv network (A) and the R47H^het network with the different treatments were analysed. Modules that are likely not preserved are marked in grey. The correlations are indicated in the box with the p value in parentheses. The direction of the correlation is indicated in the colour legend with positive correlations indicated in red and negative correlations in blue.
The overlap between the modules identified in the Cv and R47H\textsuperscript{het} networks was plotted in this correspondence matrix. The proteins shared between the different modules were displayed in the boxes with the –log of the p value colour coded. The stronger the red colour, the more significant is the overlap between the two modules. Again, modules that appear to be less preserved are marked in grey.
4.3.7 Effect on exosomal proteins on specific cellular functions

4.3.7.1 Exosomal protein changes could influence synapse function in neurons

The analysis so far has revealed global differences in exosomal protein content after treatment of iPS-Mg with LPS and apoptotic treatment and the influence \textit{TREM2} status had on this. In addition to changes in inflammatory cytokines and the potential effect this could have on neighbouring cells, previous studies have suggested that exosomes from microglia may also be able to influence synaptic functioning (Antonucci \textit{et al.}, 2012; Drago \textit{et al.}, 2017; Murgoci \textit{et al.}, 2020).

The significant drop in C1qc following LPS treatment in both Cv and R47H\textsuperscript{het} exosomes (Figure 4-10) suggests that treatment could induce changes in the ability of the extracted exosomes to support synaptic functioning. To further investigate the changes in proteins involved in synaptic pruning and strength, a targeted analysis of proteins of interest was undertaken (Figure 4-23).

C1qc is one component of the complement pathway involved in tagging aberrant synapses for phagocytosis by microglia (Stevens \textit{et al.}, 2007). The staggered significant decreases in C1qc levels was mirrored by similar trends in C1qa and C1qb (Figure 4-23A). This suggested that following treatment, exosomes could tag fewer synapses for degradation and that there could be differences between LPS and apoptotic neuron treatment, with apop treatment decreasing C1q levels even further. \textit{TREM2} status however did not appear to have an effect. This could suggest less synaptic pruning occurring when neurons are exposed to exosomes from LPS-activated iPS-Mg and even further reductions in pruning following iPS-Mg exposure to apoptotic neurons.

Annexin A2 is a membrane binding protein that resides in lipid rafts (Gauthier-Kemper \textit{et al.}, 2018). In neurons, it was found in growth cones and axonal branches and is capable of anchoring tau to axons (Zhao and Lu, 2007; Gauthier-Kemper \textit{et al.}, 2018). In addition to this, it was also found on the surface of EV (Stewart \textit{et al.}, 2016). In previous research, annexins have been shown to mediate binding of exosomes to target cells (Drago \textit{et al.}, 2017). The drop of exosomal Annexin A2 levels in exosomes originating from iPS-Mg treated with apoptotic neurons (Figure 4-23B) could suggest that these exosomes bind less to synapses, since A2 is increasingly found there.
Cathepsins are enzymes normally enclosed in the lysosome to degrade proteins (Hook et al., 2015). Cathepsin B was implicated in improving the outcome of animal models of TBI (Hook et al., 2015), but it was also linked to Aβ levels in models of AD (Embury et al., 2017), as cathepsin B can degrade this protein. Cathepsin B has also been found to be secreted from activated microglia and can induce apoptosis in CGC (Kingham et al., 1999; Kingham and Pocock, 2001). Dendritic spin collapse and axonal swelling were both processes linked to increases in cathepsin B functions at the synapse, during excitotoxicity for example (Hook et al., 2015). This was further corroborated with a double KO of cathepsin B and L, highlighting the role these enzymes play in the processes of axonal outgrowth and synapse formation (Stahl et al., 2007). Cathepsin B and L inhibitors were also protective against N-methyl-D-aspartic acid (NMDA)-induced spine loss, which was linked to localisation of these enzymes specifically to synapses (Graber et al., 2004). The drop in cathepsin levels, both B and L1 in exosomes from iPS-Mg treated with either LPS or apoptotic neurons (Figure 4-23C) could suggest potential protection against spine collapses in response to synaptic activity. Interestingly, R47H\textsuperscript{het} exosomes also contain lower levels of cathepsin L1 (Figure 4-23Ci). Overall, this could suggest an increase in spine density both in neurons treated with exosomes from treated iPS-Mg and untreated R47H\textsuperscript{het} iPS-Mg.

Translationally-controlled tumour protein (TCTP) is one of the many proteins found within exosomes associated with the cytoskeleton and microtubules in particular. Primarily localised to the axon terminals, TCTP is a protein that stabilized microtubules and is hypothesized to maintain synaptic structures (Bae et al., 2017). It contributed to the development of axonal projections and was found to be particularly important in synaptogenesis (Gouveia Roque and Holt, 2018), most likely through its ability to affect cytoskeletal reorganization through a range of downstream signalling pathways, such as the mTOR and β-catenin pathway (Gouveia Roque and Holt, 2018; Mishra et al., 2018). Decreases in exosomal TCTP following treatment of iPS-Mg with either LPS or apoptotic neurons (Figure 4-23Di) suggests that synaptic structures may be less supported when neurons are exposed to these exosomes.

Pigment epithelium-derived factor (PEDF) promoted neurite outgrowth of retinal ganglion cells in addition to promoting survival of cerebellar granule neurons and developing motor neurons (Tanimoto et al., 2006). In addition to supporting outgrowth, it can also protect neurons from excitotoxicity (Gettins et al., 2002). The significant drop in PEDF in exosomes following LPS treatment (Figure 4-23Dii)
suggests that in addition to other deficits, these exosomes may be less able to support neurite outgrowth. The markedly lower levels of PEDF in R47H\textsuperscript{het} exosomes from iPS-Mg treated with apoptotic neurons in comparison to Cv apop exosomes (Figure 4-23Dii) suggested differences in how exosomes from iPS-Mg treated with apoptotic neurons could support neurite outgrowth.

In conclusion, the reduction in exosomal C1q and cathepsin B/L in response to treatment of iPS-Mg with either LPS or apoptotic neurons could indicate reductions in synaptic pruning and proteolysis neurons experience when exposed to these exosomes. The reductions in cathepsin levels at baseline R47H\textsuperscript{het} exosomes could also indicate a differential effect of different TREM2 variants. Reductions of Annexin A2 levels suggest that following treatment with apoptotic neurons, exosomes may be less efficient in binding to synapses and delivering their cargo. The LPS-specific drop in PEDF could suggest that neurite outgrowth is impaired in neurons exposed to these exosomes, with the deficit only partially recovered in R47H\textsuperscript{het} exosomes from iPS-Mg treated with apoptotic neurons. TCTP, involved in axonal projection development and synaptogenesis, also decreased in exosomes from treated iPS-Mg. Overall, exosomal proteins associated with both synaptic pruning and axonal outgrowth are differentially affected by both TREM2 status and treatment of the iPS-Mg they originate from.

C1q, Cathepsin L1 and B and Annexin A2 appear to be specifically downregulated in exosomes from Cv exosomes originating from iPS-Mg treated with apoptotic neurons, which in most cases is mirrored by exosomes from R47H\textsuperscript{het} iPS-Mg, indicating that specifically this treatment could influence synapse pruning in neurons. Neurite outgrowth, linked to PEDF and TCTP, however appear to be influenced by LPS treatment, perhaps even more than apoptotic neurons, as indicated by PEDF, which could mean that LPS could affect specifically this aspect of neuronal functioning and development.

4.3.7.2 R47H\textsuperscript{het} exosomes contain more DAM-related proteins

One change in microglia that is associated with disease progression and TREM2 is the development of the DAM gene signature (Keren-Shaul et al., 2017).

To test whether DAM-related proteins were found in the exosomes, the identified proteins were compared with a list of DAM-related proteins (Keren-Shaul et al., 2017; Butovsky and Weiner, 2018; Rangaraju et al., 2018). This revealed that a range of proteins associated with DAM was found within the exosomes (Table 7-3),
at varying levels. This raised the interesting prospect that exomes may be able to either communicate the DAM status of their cell of origin or induce the DAM status in recipient cells.

To assess whether one condition displayed significant changes in the exosomal DAM-related proteins, the log_{10} transformed abundance of the identified DAM-related proteins was plotted (Figure 4-24A). The heatmap revealed that following treatment with either LPS or apoptotic neurons, the exosomes from Cv and R47H^{het} iPS-Mg cluster together, as previously observed (Figure 4-11). At baseline though, there appeared to be a subset of DAM-associated proteins that specifically increased in R47H^{het} exosomes in comparison to Cv exosomes (Figure 4-24A, highlighted in purple). This subset of proteins was specifically enriched for proteins identified in the TREM2-dependent second DAM stage (Keren-Shaul et al., 2017). To verify a specific increase in stage 2 DAM-related proteins in R47H^{het} exosomes, the relative abundances of stage 2 DAM-related proteins found within exosomes were plotted and again, a significant increase was observed in exosomes from R47H^{het} iPS-Mg (Figure 4-24B). As observed in the overall analysis, R47H^{het} exosomes contained higher levels of these three proteins in comparison to the other conditions.

Whether these changes were specific to exosomes, suggesting increased packaging of these factors into exosomes, or were reflecting differences in the R4H^{het} iPS-Mg, the expression of DAM genes, including GPNMB, APOE, TYROBP, TREM2, GRN, ALX and CSF1R, was tested in iPS-Mg (Figure 4-25). For the tested DAM-related genes, R47H^{het} iPS-Mg displayed elevated expression levels for over half of them, suggesting that the increases in DAM-related proteins in R47H^{het} exosomes (Figure 4-23) was reflective of changes in the iPS-Mg themselves.
Figure 4-23 Exosomes contain proteins influencing synaptic activity

Changes in specific proteins found in exosomes involved in both synaptic pruning (A-C) and neurite outgrowth (D) were plotted. Levels of exosomal C1qa, b and c were all significantly
decreased following treatment of iPS-Mg with LPS or apoptotic neurons (A). Annexin A2, hypothesized to support binding of exosomes to synapses, also showed a significant decrease in response to apoptotic neuron treatment of Cv iPS-Mg (B). Cathepsin L1 showed a significant decrease in Cv exosomes from iPS-Mg treated with apoptotic neurons, whilst even at baseline, levels in R47H\textsuperscript{het} exosomes were lower than Cv (Ci). Cathepsin B showed a similar trend, albeit with larger error bars and a significant decrease in Cv apop exosomes (Cii). TCTP was decreased in exosomes from both LPS and apoptotic neuron treated iPS-Mg, independent of TREM2 status (Di). PEDF, associated with neurite outgrowth, was similarly decreased in Cv exosomes from LPS-activated iPS-Mg, which was also seen in LPS-treated R47H\textsuperscript{het} exosomes (Dii). Treatment with apoptotic neurons recovered PEDF levels in Cv exosomes but only partially in R47H\textsuperscript{het} exosomes (Dii). n = 3, two-way ANOVA with Tukey’s post hoc test and * p < 0.05, ** p ≤ 0.01, *** p ≤ 0.001.
Figure 4-24 DAM related proteins were found in exosomes

A number of proteins implicated in the DAM signature were found in exosomes. The log$_{10}$ transformed abundance of these proteins in the different conditions was displayed in a heatmap (A). The levels of a particular subset of proteins (indicated in purple) appeared to be increased in the R47H$^{het}$ exosomes. These proteins appear to correspond to the TREM2-dependent second DAM stage (Keren-Shaul et al., 2017) and therefore, the log$_{10}$ transformed...
abundances normalised to the Cv condition of these proteins were plotted individually (B). R47H\textsuperscript{het} exosomes contained significantly increases in a range of these proteins, such as CSF1, lipoprotein lipase (LPL) and osteopontin. n = 3. two-way ANOVA with Tukey’s post hoc test (B). * p < 0.05.
The expression of DAM-related genes was analysed in the Cv and R47H\textsuperscript{het} iPS-Mg. This showed a significant influence of the iPS-Mg genotype on the expression of these genes, with R47H\textsuperscript{het} iPS-Mg displaying elevated levels of all tested genes, apart from GRN. The data represents the fold change relative to GAPDH and relative to Cv expression. \( n = 4 \), with the data for APOE, TREM2 and CSF1R supplemented with normalised data from the gene array (Figure 3-4). Two-way ANOVA with Tukey’s post hoc test and * \( p < 0.05 \), ** \( p \leq 0.001 \).
4.4 Discussion

To better understand the differences between the content of exosomes secreted from Cv and R47Hhet iPS-Mg and the effect treatments had on these contents, high throughput LC-MS was used to understand the changes on a large scale. Analysis of these experiments revealed a range of different insights.

4.4.1 Sample size and quality controlling the dataset

Due to time constraints and costs, only exosomes from the R47Hhet TREM2 variant were compared to Cv, without studying exosomes from the R47Hom or the T66Mhom TREM2 variants. This decision allowed the focus on a TREM2 variant that is both disease-relevant in the AD context and is found in patients. This also meant that more clones from each cell line could be pooled for the experiments, making the data more robust.

The pooling was performed randomly to reduce potential artefacts caused by this. The repeats from the same condition displayed some variability (Figure 4-3, Figure 4-13). The sample dendrograms (Figure 4-3) showed that whilst there were some samples that did not fall within the same dendrograms arm as the other two of the same condition, this did not appear to be correlated with the number of samples pooled. As expected, this sample variation was also represented in the PCA analysis (Figure 4-13). The multiplex labelling, dividing the samples into two separate experiments (Table 4-2) also did not appear to influence the variation seen in the samples, with samples from both experimental runs showing variation. Overall, this appears to support the idea that the spread of the data is down to biological variation rather than experimental setup. However, the small number of repeats run in this experiment, meant that despite the variability found within the replicates, none were excluded for analysis.

The dataset was also probed for markers that are either associated with exosomes or have been used as negative markers, indicating possible contamination of the exosomal fraction with other particles or cell debris. When analysing specific exosomal markers (Table 4-3) next to classical exosomal markers, markers specifically associated with microglia were found in the samples, in line with previous literature (Potolicchio et al., 2005; Prada et al., 2013; Drago et al., 2017; Murgoci et al., 2020). Next to these markers, negative markers were also found to be present at
comparable levels to classical exosome markers (Table 4-3). Whilst calnexin has been used to show no ER contamination was present in the extracted exosomes in the previous chapter (Figure 3-8), it was picked up by LC-MS. The extreme sensitivity of this experimental method could mean that markers, which are below detection in traditional methodologies like WB, could be detected. And indeed, other datasets have reported histones, mitochondrial and ER-associated proteins in their exosomal fractions when their proteomics were analysed (Potolicchio et al., 2005; Hooper et al., 2012; Trotta et al., 2018; Yang et al., 2018). Overall, this begs the question whether ‘negative exosome markers’ truly exist, whether this is more a matter of sensitivity or whether the current negative markers are in fact not accurate negative markers.

4.4.2 Different analysis techniques have different strengths

With a large dataset like this, many different analysis techniques can be used to understand the underlying trends in differences. The relative small number of repeats with many different conditions meant that care had to be taken when analysing the changes in the 3,019 identified proteins to avoid the multiple testing problem.

To reduce the number of proteins to a manageable number, volcano plots can be useful to compare two different conditions with each other (Figure 4-8, Figure 4-10 and Figure 4-16). This allowed significantly changed proteins to be filtered out over a certain fold change threshold, however, setting this threshold can introduce a non-physiological filter to the data (DiLeo et al., 2011; Dalman et al., 2012). For most biological systems under physiological conditions, only small variations in protein expression is observed and to only consider proteins that experience a twofold change, could cause more physiologically changed proteins to be ignored (Norris and Kahn, 2006). In addition to this, changes in the cut-offs have the ability to change the interpretation of the dataset (Dalman et al., 2012).

To compare more than two datasets, PCA can be a useful tool to explore complex data in a relatively simple manner (Figure 4-13). Using this dimensionality reduction technique allows identification of clusters of samples in a straightforward manner. However, to assign any functionality to these clusters is difficult through PCA. Whilst the proteins contributing the most to each component can be determined, again, the risk exists that proteins with only a moderate influence on the first few PC are overlooked, but could nonetheless carry a biological function. In addition, PCA
considers only clusters in the dimensions in which the data shows the biggest variance (Zhao et al., 2010).

In order to overcome the limitations of both the volcano plots and PCA, ProCoNA was also employed to analyse the dataset, as this network analysis carries certain advantages. Through unsupervised clustering, modules containing proteins that only change moderately can influence the results and be identified. Furthermore, by assessing the link between modules and treatments, the multiple testing problem can be circumvented (DiLeo et al., 2011). One caveat of this type of analysis is, that it can be quite sensitive to noisy data, in particular in small datasets, but this will be discussed further later (Chapter 4.4.5).

To understand both broad trends and small changes in this multivariate dataset, a range of different analysis techniques was employed. This has allowed for the limitations of each technique to be taken into account and for a more holistic understanding of this dataset and functional changes it represents.

### 4.4.3 Differences at baseline

Differences between exosomes from Cv and R47Hhet iPS-Mg at baseline were revealed through different analyses.

At baseline, nine proteins were found to be increasingly packaged in R47Hhet exosomes in comparison to Cv exosomes (Figure 4-8). Functional analysis revealed that this could translate to a negative regulation of transcription and metabolic processes (Figure 4-9). One of the proteins, HIST1H4A, was also implicated in a recent study, showing that changes in microglial metabolism correlate with AD progression (Johnson et al., 2020). HIST1H4A was one of the proteins identified in the microglial metabolism cluster.

Apolipoprotein C2, another one of the identified proteins (Figure 4-8), can work with LPL, an enzyme used for the metabolism of lipoproteins, to generate free fatty acids (Wolska et al., 2017). Fatty acids in the brain, particularly in neurons, can depolarize the inner membrane of mitochondria upon accumulation, inducing a decreased calcium retention in the organelles (Schönfeld and Reiser, 2013). Another indication that exosomes from R47Hhet iPS-Mg could disrupt calcium homeostasis in target cells comes from the increased packaging of calreticulin in R47Hhet exosomes.
(Figure 408). Calreticulin is a calcium sensor located in the ER, where it is involved in storing and buffering calcium (Roderick et al., 1998).

In addition to these differentially packaged proteins, less than 50% of the most abundant proteins were shared between Cv and R47H\textsuperscript{het} exosomes (Figure 4-9B), translating to increases in metabolism in R47H\textsuperscript{het} exosomes in comparison to Cv exosomes. Based on this, exosomes from R47H\textsuperscript{het} iPS-Mg could influence the metabolism and perhaps the calcium homeostasis of recipient cells, however this will be further investigated in the next chapter.

### 4.4.4 Effects of treatment

#### 4.4.4.1 Changes following LPS treatment

Following treatment of iPS-Mg with LPS, the extracted exosomes contained higher levels of inflammatory cytokines, such as IL-1β (Figure 4-10), a finding which is in line with previously published results (Yang et al., 2018). Whilst this dataset found increases in IL-1β, CXCL5 and CCL22, the increase in IL-6 following LPS treatment was not significant (Figure 4-10), whilst Yang et al, 2018 did not detect differences in IL-1β. This could be due to different sensitivities as they detected the cytokines by ELISA or differences between the BV2 and the iPS-Mg used in this study. Interestingly, whilst TNF-α was not detected in the exosomes, TNF receptor associated proteins, such as TRAF1, were found (Figure 4-10). Previous research has suggested that in addition to being found within exosomes, cytokines such as TNF-α, could bind to receptors found at the surface of exosomes (Hawari et al., 2004). This suggested that extracellular TNF-α could bind to exosomes and in turn activate target cell receptors. In this study, it was not further tested whether the detected cytokines in the exosomal fraction were bound to the surface or were encapsulated within exosomes, but this would be an interesting next step. Interestingly, in addition to the presence of expected inflammatory cytokines in the exosomes of activated iPS-Mg, LC-MS did not detect any neurotrophins such as BDNF or nerve growth factor that microglia have been shown to release to support neuronal functioning (Parkhurst et al., 2013; Crotti and Ransohoff, 2016).

Similarly to the decrease in sTREM2 secretion following LPS activation (Figure 3-6), a decrease in exosomal TREM2 was also observed, at least in the R47H\textsuperscript{het} exosomes following LPS treatment (Figure 4-10). A drop in sTREM2 was also described in the brain of AD patients and patients with Down syndrome (Raha-
Chowdhury et al., 2018). This study also hypothesized that TREM2 was secreted from microglia in the form of exosomes and could be neuroprotective when taken up by neighbouring neurons (Raha-Chowdhury et al., 2018).

The fact that R47H\textsuperscript{het} exosomes showed smaller changes following activation of the iPS-Mg with LPS was also shown in the PCA analysis (Figure 4-13), explaining the subdued and less significant changes that were observed in some proteins in R47H\textsuperscript{het} exosomes following treatment with LPS (Figure 4-10). This suggested that at baseline, R47H\textsuperscript{het} exosomes contained a more inflammatory profile, whilst the LPS cluster is TREM2 independent after the iPS-Mg were exposed to LPS. Previous studies have suggested that deficient Trem2 or Dap12 macrophages react to even low levels of TLR agonists with high levels of inflammatory cytokines (Hamerman et al., 2006; Turnbull et al., 2006), raising the possibility that these cells already experience higher levels of activation, which this dataset also supports with the R47H\textsuperscript{het} exosomes clustering with exosomes originating from LPS-treated iPS-Mg (Figure 4-13).

4.4.4.2 Difference between LPS and apoptotic neurons as treatments
Comparing both exosomal profiles after LPS and apoptotic neuron treatments gave the opportunity to contrast the exosomal response to a ubiquitous and commonly used stimulus, in the form of LPS, and a physiological, TREM2-activating stimulus, in the form of apoptotic neurons.

Other studies have shown that microglial exosomes displayed a shift in proteomics (Hooper et al., 2012; Yang et al., 2018) following treatment with different stimuli, however comparing the response to two different stimuli in one study has not been done yet. Exosomal protein content displayed a stimulus specific shift with the samples performing mainly a shift along the second PC in response to LPS and along the first PC in response to apop treatment (Figure 4-13). Functionally, this suggested that in response to LPS, exosomes experienced changes in the proteins involved in cytoskeletal organization and response to external stimuli, whilst treatment with apoptotic neurons changed metabolic proteins within exosomes (Figure 4-14). TREM2 had an effect on the ability of the iPS-Mg to shift their exosomal profile in response to treatment with apoptotic neurons, with differences being revealed both through PCA (Figure 4-13) and analysis of the most abundant proteins (Figure 4-12).
Apoptotic neurons, displaying PS on their surface, can bind specifically to TREM2, activating the TREM2/DAP12 signalling cascade (Wang et al., 2015; Garcia-Reitboeck et al., 2018; Shirotani et al., 2019; Cosker et al., 2020). The R47Hhet TREM2 variant specifically disrupts the binding site of TREM2 (Sudom et al., 2018), which could explain the differences in exosomal profile following treatment with apoptotic neurons. Functionally, the differences are reflected in changes in proteins involved in biogenesis (Figure 4-14).

One question that this dataset does not answer is whether the proteins packaged into exosomes are secreted to remove unwanted material from the iPS-Mg or whether their primary function is to induce specific signalling in neighbouring recipient cells, such as neurons. At baseline, the R47Hhet exosomes mainly contained proteins associated with negative regulation of metabolism and transcription (Figure 4-9). Due to the metabolic deficits these cells have been shown to experience (Piers et al., 2020), it is likely that these proteins are increasingly upregulated in the iPS-Mg and secreting them could alleviate the deficit. As there is more research focussing on the response of microglia to activation, induced for example through LPS treatment, here it is easy to suggest that rather than a removal mechanism, increased concentration of inflammatory cytokines within exosomes are likely a communication tool with the environment.

4.4.5 Network analysis

The first thing that has to be considered when building networks using the ProCoNA approach is to ensure that the modules identified are actual modules and not just created by noise in the data (Langfelder et al., 2011). To reduce the possible influence of noise in the data, the number of samples was increased by tagging the treatment condition as “traits”, combining the different treatment conditions into the same dataset. Additionally, a range of module preservation analyses were run, namely medianRank, Zsummary and kME correlation. Additionally, the overlap between the modules in the Cv and R47Hhet networks was also assessed, as suggested in a previous publication (Langfelder et al., 2011). This suggested that the majority of the observed modules are indeed preserved between the two different networks and trends observed in the data are indeed down to real changes in the data. The specific modules of interest which were further discussed (Chapter 4.3.6) were all well preserved.
When exosomes were collected from untreated iPS-Mg, they largely contained proteins involved in the differentiation and development of cells (Figure 4-21), which matched the previous observation that the most abundant proteins packaged into exosomes are involved in both cell growth and/or maintenance (Figure 4-12). One of the main functions of microglia is to support the differentiation and development of neurons (Morgan et al., 2004; London et al., 2013; Miyamoto et al., 2016), which they continue to do throughout their lifespan. Exosomes appear to be one pathway through which microglia fulfil these functions, which several analyses of this dataset supports.

However, some modules associated with exosomes from untreated iPS-Mg did not show a great overlap between the Cv and R47H\textsuperscript{het} exosomes (Figure 4-22), which could indicate that even at baseline, there were differences in how microglial exosomes communicate with neighbouring cells and fulfil the basic functions of microglia, with the differences appearing to focus around kinase activity, signalling receptors and actin reorganization.

In comparison, following activation of iPS-Mg with LPS, the modules associated with the LPS response overlapped between the two different networks (Figure 4-22). As expected and previously reported (Prada et al., 2013; Paolicelli et al., 2018; Yang et al., 2018; Grimaldi et al., 2019), exosomes from Cv iPS-Mg challenged with LPS contained an immune response cluster, which included the cytokine response. Whilst the immune cluster is also observed in the R47H\textsuperscript{het} network, the modules most strongly associated with this treatment focus on protein translocation (Figure 4-21B and Table 7-2), which is also represented in the Cv immune cluster (Figure 4-21A and Figure 4-22). This could suggest that not every aspect of the exosomal response to LPS is mirrored between the different TREM2 variants.

Modules that are linked to the response of exosomes to apoptotic neuron treatment of iPS-Mg were also shared between the two different networks (Figure 4-22), highlighting the involvement of proteins linked to metabolism in these exosomes. Again, subtle differences between the modules were identified. This overlapped with the PCA result that small differences between exosomes from apoptotic neurons treated iPS-Mg from different TREM2 variants could be found (Figure 4-13).
4.4.6 Implications for neighbouring cells

Exosomes have been shown to be able to transport their contents to recipient cells and elicit a response in these cells, depending on the cargo (Hooper et al., 2012; Choi et al., 2013; Yang et al., 2018). Therefore, observing changes in the exosomal content, both dependent on TREM2 status of the secreting iPS-Mg and the treatment these iPS-Mg received prior to exosome collection, could lead to specific changes in recipient cells.

The increases in inflammatory cytokines found in exosomes from iPS-Mg activated with LPS could indicate that they could elicit a response in target cells. Previous research has suggested that cytokines found in exosomes can be active, independent of whether they are found within the vesicles or at the exosomal surface (Fitzgerald et al., 2018). Additionally, exosomes could be a more concentrated delivery system for cytokines to target cells (Fitzgerald et al., 2018), which could elicit a response in either neurons or other microglia exposed to these exosomes.

The changes in proteins involved in synaptic pruning and neurite outgrowth suggest that exosomes can have a specific effect on neuronal development and functioning. C1q components tagging synapses for degradation (Stevens et al., 2007; Hong et al., 2016), cathepsin enzymes degrading synapses (Graber et al., 2004; Stahl et al., 2007; Hook et al., 2015) and Annexin A2, possibly binding to synapses (Stewart et al., 2016; Drago et al., 2017; Gauthier-Kemper et al., 2018), were all decreased in exosomes, when they originated from iPS-Mg treated with apoptotic neurons, independent of TREM2 status of the iPS-Mg (Figure 4-23). This could decrease synaptic pruning of neurons exposed to these exosomes. LPS treatment of iPS-Mg however appeared to have an effect on exosomal PEDF and TCTP (Figure 4-23), a protein supporting neurite outgrowth and a component of the cytoskeleton involved in synaptogenesis respectively.

Levels of C1q were studied in various TREM2 models, with TREM2 on alveolar macrophages shown to suppress production of C1q, causing an increase in C1q levels in the absence of TREM2 (Sharif et al., 2014; Gawish et al., 2015). On the other hand, Dap12 deficiency was shown to induce a drop in C1q levels independent of TREM2, which was associated with improved learning outcomes (Audrain et al., 2019). However, in microglial exosomes, C1q levels did not appear to be affected by the R47H\textsuperscript{het} variant but rather the treatment (Figure 4-23).
Overall, this indicated that the R47H<sup>het</sup> variant did not appear to have an influence on exosomal abilities to influence synaptic pruning or neurite outgrowth. Previous studies focussing on the effect of TREM2 on synapse pruning showed that Trem2 KO mice models show fewer synapses in phases of synapse pruning through impairing astrocytic synapse pruning (Jay et al., 2019). On the other hand, aged Trem2 KO mice show higher levels of synapses, which is linked to a microglial impairment to phagocytose synapses (Filipello et al., 2018) and changes in Cq1 levels (Linnartz-Gerlach et al., 2019). This indicates the effect of TREM2 on synaptic densities in these animal models at least in part relate to the interaction of microglia with neurons and astrocytes.

In animal models and in post mortem brain samples of AD patients, microglia with specific gene signatures were identified. Microglia expressed a DAM signature in response to the AD environment and research suggested this occurs specifically in the environment of Aβ plaques (Keren-Shaul et al., 2017; Butovsky and Weiner, 2018). The transition from the homeostatic microglial gene signature to the DAM has been shown to occur in two phases, with the first one being independent of TREM2 and the second dependent on TREM2 (Keren-Shaul et al., 2017). Due to the implication of TREM2 in this process, changes in DAM-related proteins were analysed in the exosomal samples (Figure 4-24). Interestingly, a large subset of DAM-related genes were detected in exosomes extracted from iPS-Mg and whilst iPS-Mg treatment with either LPS or apoptotic neurons appeared to have little effect on these proteins, R47H<sup>het</sup> exosomes appeared to contain higher levels of a wide range of different DAM-related proteins, in line with DAM microglia themselves (Figure 4-24). In particular, stage 2 DAM-related proteins were found at higher levels in R47H<sup>het</sup> exosomes, highlighting the influence of TREM2 on this stage. Whether the DAM signature enables microglia to increase phagocytosis to alleviate the Aβ burden in AD to be protective or is involved in further disease progression is not yet known, making it difficult to predict what the effect of this DAM signature in R47H<sup>het</sup> exosomes could be. The increase in the DAM like signature in R47H<sup>het</sup> exosomes appears to be reflected in an increase in the expression of these genes in the iPS-Mg themselves (Figure 4-25), suggesting that the differences observed between the exosomes from the TREM2 variants is due to changes in the cells themselves rather than packaging. A recent study has also suggested that microglia-like cells derived from a human embryonic stem cell line carrying the R47H<sup>hom</sup> TREM2 variant adapt a more DAM like state (Liu et al., 2020a), matching the results obtained here.
4.4.7 Future work

For future work, it would definitely be interesting to increase the sample number for this experiment, to reduce the spread in the data and to elucidate perhaps some more subtle changes occurring in the exosomal proteins. In addition, comparing this proteomic data to exosomes originating from R47Hhet and T66Mhet iPS-Mg could provide a better understanding towards the role of TREM2 in these differences.

In addition to proteins, other research has also found RNA, including mRNA and miRNA within exosomes (Valadi et al., 2007). There has been a big body of research focussing on the changes in these miRNA, capable of influencing RNA transcription on the target cell, in response to inflammatory stimuli (Bellingham et al., 2012; Pinto et al., 2017). Proteomic analysis of exosomal content could therefore only be one approach to understand the functions of exosomes and the packaged messages.

Exosomes originating from R47Hhet iPS-Mg revealed a disturbed proteomic profile with differences still being apparent after treatment with apoptotic neurons. However, how exactly the R47Hhet variant plays into these differences is not known. One theory could be that a lack of energy, as described previously (Ulland et al., 2017; Piers et al., 2020) could prevent and limit packaging of proteins into exosomes, however analysis revealed no overall difference in protein number or abundance within exosomes, only differences in certain proteins. Cytoskeletal abnormalities (Phillips et al., 2018) are also reported in TREM2 KO, however whether this specifically influences the packaging of certain proteins into exosomes is not known. Further analysis of the role TREM2 plays in the packaging of proteins into exosomes would definitely elucidate this further, as would proteomic analysis of exosomes secreted from R47Hhet iPS-Mg that were supplied with an alternate form of energy to elucidate which changes in exosomal protein are energy dependent and which ones are caused by PS found on the surface of apoptotic neurons.

Finally, the functional effect of the identified protein networks would be very interesting to assess. The changes in exosomal protein could influence the functioning of neighbouring cells receiving these exosomes, such as astrocytes and neurons, meaning that microglial exosomes could form a specific communication pathway to relay microglial state specific messages to other cells. Some of these exosomal functions on neurons will be investigated in the next chapter (Chapter 5).
4.4.8 Conclusion

In conclusion, independent of secretion rate, at baseline the proteomic content of exosomes extracted from Cv and R47H<sup>het</sup> iPS-Mg was different. The profile of R47H<sup>het</sup> exosomes resembled that of exosomes originating from LPS-activated iPS-Mg in addition to containing more proteins involved in the negative regulation of transcription and metabolism.

Following LPS activation, exosomes displayed an increase in inflammatory cytokines and a decrease in components of the complement system, a response that is more subdued in exosomes from R47H<sup>het</sup> iPS-Mg, as at baseline, these exosomes already resemble the LPS exosomes. Treatment of iPS-Mg with apoptotic neurons revealed that changes in the exosome profile is stimulus dependent and that even with this stimulus, differences between Cv and R47H<sup>het</sup> exosomes can be observed, such as differences in energy metabolism (summarised in Figure 4-26).

Based on their proteomic makeup, exosomes from iPS-Mg could differentially influence synaptic pruning and neurite outgrowth, depending on the specific stimulus iPS-Mg receive, whilst without treatment, exosomes from R47H<sup>het</sup> iPS-Mg showed an increase in DAM-related proteins, suggesting that dependent on TREM2 status and treatment, exosomes could convey a range of different complex messages to neighbouring cells. Whether the changes observed in the exosome packaging is mainly a mechanism for iPS-Mg to remove proteins or whether they are capable of inducing a specific response in neighbouring cells, such as neurons, will in part be studied in the next chapter.
Expanding on the previously shown diagram (Figure 3-21), the findings of the proteomic analysis of exosomal content were summarised here.
Chapter 5 Effect of exosomes on neuron-like models

5.1 Introduction

Following the proteomic analysis, I sought to investigate the effect of iPS-Mg on neuronal functions.

5.1.1 Modelling neurons in vitro

Several neuronal models could be used for this project. Similar to microglia, primary human neurons are not easily available and come with ethical restrictions. Therefore, different models have been developed.

5.1.1.1 Immortalized cell lines

There are a range of immortalized cell lines available, both from rodents and human. One commonly used cell line is the SH-SY5Y cell line. Originally obtained from a human metastatic bone tumour biopsy (Kovalevich and Langford, 2013), the cells are believed to be a neuroblastoma population, presumably originating from neural crest cells (Forster et al., 2015). As they are a human cell line, SH-SY5Y have been used commonly as a neuronal model and therefore are well characterised (Jahn et al., 2017). The cell line expresses both dopaminergic and adrenergic properties, characterised by expression of tyrosine hydroxylase for example, which is needed for the production of both dopamine and noradrenaline. Another advantage is that the SH-SY5Y can be further differentiated to obtain the morphology and characteristics of more mature neurons. A range of differentiation protocols has been published, but RA is often used. RA is a vitamin A derivative, pushing SH-SY5Y into a more mature neuronal phenotype, specifically cholinergic neurons (Encinas et al., 2000; Kovalevich and Langford, 2013). In a differentiated state, SH-SY5Y display a more neuron-like morphology, characterised by long processes, and neuronal protein expression, including microtubule association protein tau (MAPT), receptors and synaptic proteins (Jahn et al., 2017). Differentiated SH-SY5Y also display a metabolic profile more resembling neurons, with increases in ATP levels (Forster et al., 2015).

5.1.1.2 iPS-neurons

With the advent of inducing somatic cells to become iPS cells, human neurons could be generated from easily accessible samples, such as fibroblasts.
Neurons can be differentiated out from iPSC using a range of different protocols. Particularly when studying neurodegenerative diseases, one disadvantage of iPSC-neurons is that reprogramming of fibroblasts into iPSC resets the epigenetic memory of the cells, restoring the cells into an embryonic-like state (Campos et al., 2014). This means that initially iPSC-neurons resemble functionally immature neurons, as observed in embryonic development (Sposito et al., 2015; Verheyen et al., 2018). Support for this comes from studying tau phosphorylation in iPSC-neurons (Kopach et al., 2020). Up until one year in culture, iPSC-neurons display an immature, embryonic tau phosphorylation pattern, masking the effect of genetic mutations altering tau phosphorylation that have been associated with AD (Sposito et al., 2015). To study age-specific changes in iPSC-neurons, the cells therefore have to be maintained over long periods of time.

One way around the epigenetic reset that occurs during iPSC induction of fibroblasts could be to directly convert fibroblasts into neurons, generating directly induced neurons (Traxler et al., 2019). Transfecting fibroblasts with TF can induce the expression of endogenous pro-neuronal signals in the cells. The advantage of this strategy is that hallmarks of ageing and epigenetics are preserved. The selection of TF is essential in this procedure, as their potency can be cell-type and species specific. The induction rate of cells to become neuronal cells appears to be influenced by the age of the donor and a high percentage of fibroblasts die in this procedure, due to the need of the fibroblasts to shift from aerobic glycolysis to oxidative phosphorylation, the preferred energy pathway for neurons (Traxler et al., 2019). Depending on the downstream application and use of the cells, either iPSC-neurons or directly induced neurons could be more appropriate.

5.1.2 Neuronal stress pathways

One pathological hallmark of AD is neuronal death. Several pathways that can work together and independently of each other to induce neuronal stress and subsequent neuronal death have been identified.

5.1.2.1 Unfolded protein response (UPR)

Build-up of misfolded proteins, such as Aβ and phosphorylated tau in AD, can induce the UPR in the ER of neurons. For example, it has been shown in vitro that cells exposed to Aβ display an increase in ER stress caspases and a subsequent induction of apoptosis (Halliday and Mallucci, 2014). Over short periods, UPR can
promote survival by reducing the working load for the cell, however over long periods it can also promote cell stress (Darling and Cook, 2014). The UPR is induced by ER stress, as the ER is the site for protein modification and folding. Three different proteins in the ER can sense ER stress and induce a stress response.

Inositol requiring enzyme 1 (IRE1) is a transmembrane ER protein, which when activated can contribute to the protein load within the organelle. Cytoplasmic HSP72 and HSP90 can activate IRE1 signalling, so can components of the apoptosis pathway Bax and Bak (Darling and Cook, 2014). This suggests that IRE1 can act as a direct link between UPR and core apoptotic components.

Protein kinase RNA-like endoplasmic reticulum kinase (PERK), the second key ER stress sensor, functions through decreasing protein synthesis. Through one of its downstream signalling partners, Nrf2, PERK can also induce the transcription of antioxidant genes, increasing cell survival. Through phosphorylation of eIF2α, translation is reduced (Darling and Cook, 2014). PERK activation in particular has been associated with post mortem AD brain, particularly in the temporal cortex (Halliday and Mallucci, 2014; Huang et al., 2015). Phosphorylated eIF2α also coincides with phosphorylated tau in the brain of patients, suggesting that activation of the PERK-eIF2α pathway could induce apoptosis in AD (Chang et al., 2002). On the other hand, PERK KD in animal models enhanced Aβ toxicity, as eIF2α activation was reduced (Huang et al., 2015). This highlights the importance of assessing the effect of protein activation in a nuanced way.

PERK activation can also induce activation of activating transcription factor 4 (ATF4), the last stress sensor. ATF4 is a TF that can promote transcription of other proteins that are downstream of the ER stress response such as C/EBP homologous protein (CHOP). CHOP is a protein that is considered to be pro-apoptotic. In addition to its ability to influence transcription, such as decreasing pro-survival proteins, CHOP can also activate the ER IP3 receptor leading to an increase in calcium release from the ER (Darling and Cook, 2014). The ER is a key intracellular calcium store and calcium release from the ER is a key component of the stress response. Decreases in CHOP in a diabetes mouse model decreases neuronal death and functionally improves the condition of the mice (Wang et al., 2016b).

Release of calcium from ER stores in response to ER stress and UPR is mainly mediated through CHOP and PERK. PERK has been shown to play a role in
mitochondria-associated ER membranes (Verfaillie et al., 2012) through establishing physical contacts between the membranes of both organelles.

### 5.1.2.2 MAPK

Three main MAPK that are relatively well conserved are ERK1/2, C-Jun N-terminal kinase (JNK) and p38 MAPK. ERK1/2 is activated in response to extracellular stimuli, such as growth factors, and TREM2 activation in microglia. Phosphorylation of JNK occurs in response to cell stresses and is generally believed to lead to cell death (Darling and Cook, 2014). One way through which JNK can induce cell stress is through enabling IRE1 activation (Salminen et al., 2009).

In response to pro-inflammatory cytokines, p38 MAPK is activated in neurons. In addition to this, CHOP can also increase and modify the activity of p38 (Darling and Cook, 2014), indicating the interplay between ER stress, the UPR and more general cell stress pathways such as p38 MAPK. MAPK can also influence synapses in neurons and their functions. Through downstream signals, ERK1/2 can regulate LTP (Kohsuke and Hidenori, 2002). In contrast, IL-1β can induce p38 MAPK to impair LTP (Vereker et al., 2000).

### 5.1.3 Synaptic functioning influenced by EV

#### 5.1.3.1 Neurite outgrowth

EV from microglia have been shown to induce outgrowth of neurites. Lgals1 found in sEV from primary microglia is amongst one of the factors implicated in outgrowth of dorsal root ganglion (DRG) neurites, when sEV were applied for 24 or 48hrs (Murgoci et al., 2020). Activation of the primary microglia with LPS did not affect the ability of sEV to induce the outgrowth, however the spatial location of the sEV did, with sEV from microglia originating in the spinal cord supporting DRG outgrowth more strongly (Murgoci et al., 2020). This was also shown in primary rat neurons that were treated with microglial EV for 24 or 48hrs, showing a similar timeframe to the other study (Lemaire et al., 2019). EV secreted from primary rat microglia contained members of the Serpin family, which is involved with neurite outgrowth and which could explain the results reported by others (Drago et al., 2017). Another mechanism through which microglial EV can support neurite outgrowth could be mi-RNA-124-3p, which has been shown to induce neurite outgrowth through the inhibition of mTOR signalling (Huang et al., 2018). The importance of the mTOR pathway in inducing neurite outgrowth was supported by an independent study showing that exosomes
from fibroblasts contain Wnt10b and induce mTOR signalling through GSK-3β (Tassew et al., 2017).

5.1.3.2 Synapse pruning

The presence of both C1q and Annexin in exosomes secreted from microglia at baseline (Figure 4-23) suggests that exosomes could deliver these factors to aberrant synapses to tag them for microglial pruning. Annexin binding has previously been shown to allow for EV binding to target cells, which is required for EV to exert their effect on astrocytes (Drago et al., 2017). The actin filaments and lysosomal enzymes, such as cathepsin B, may further mediate the degradation of synapses.

Another study found that in addition to pruning, EV from human macrophages were able to interfere with the propagation of action potentials in primary rat neurons (Vakili et al., 2020). The inflammatory status of these macrophages appeared to have an effect on the conduction velocities of action potential along the neurons (Vakili et al., 2020).

5.1.4 Aims

This part of the project sought to investigate the effect of microglial exosomes on neuron-like cells. Based on other studies and the findings of the proteomic analysis (Chapter 4), specific aspects of neuronal functioning were investigated. The aims of this chapter are:

- Study the effect of exosomes on the viability of neuron-like cells
- Assess changes in cell stress and metabolism induced by exosomes
- Test the effect of exosomes on synapses
5.2 Methods

5.2.1 Differentiation of SH-SY5Y

SH-SY5Y cells, a kind gift from Professor Rohan de Silva, were maintained in DMEM with 10% FBS, penicillin/streptomycin and GlutaMax. The cells were kept for several passages until they reached passage 40 the latest and they were used for experiments at 70-80% confluency. SH-SY5Y cells were differentiated using a previously established protocol (Encinas et al., 2000), using RA (10µM) for 5 days. Subsequently, 50nM BNDF was added in serum-free medium for 7 days (Figure 5-2).

5.2.2 Differentiation of iPS-neurons

iPS-neurons were differentiated by Dr Charles Arber, Department of Neurodegenerative Disease, UCL Queen Square Institute of Neurology. Differentiation was performed following a published protocol to generate cortical glutamatergic neurons (Shi et al., 2012). In brief, confluent iPSC were switched to N2B27 media, supplemented with 10µM SB431542 and 1µM dorsomorphin. N2B27 medium was composed of 1:1 DMEM-F12 and Neurobasal medium, containing 0.5x N2 supplement, 0.5x B27 supplement, 0.5x NEAA, 1mM L-glutamine, 25 U penicillin/streptomycin, 10µM β-mercaptoethanol and 25 U insulin. After 10 days of neural induction, the cells were maintained in N2B27 without SB431542 and dorsomorphin.

5.2.3 Cell treatment

After SH-SY5Y were fully differentiated, they were treated with exosomes extracted from iPS-Mg for 24hrs, unless specified differently. iPS-neurons were differentiated until day 100 and iPS-Mg were fully matured according to the TP1 protocol (see Chapter 2.2.1).

Exosomes were extracted from iPS-Mg treated with either LPS or apoptotic neurons as previously described (see Chapter 2.2.2) or from untreated iPS-Mg and resuspended in PBS. Prior to addition of iPS-Mg exosomes to recipient cells, the cell culture medium was changed and protein concentration of the exosomal samples was determined through BCA assay. This allowed for different amounts of secreted exosomes from different conditions to be taken into account and normalised, so that 6µg of exosomes were added to the cells at a concentration of 1µg/10,000 cells. For
different cell numbers, this was adjusted accordingly. This was based on previous literature (Bhatnagar and Schorey, 2007). Afterwards, the cells were analysed using different techniques (Figure 5-1).
A diagram of the protocol used to differentiate SH-SY5Y into more neuron-like cells based on a previously established protocol (A) and the experimental setup used to treat neuron-like with exosomes extracted from iPS-Mg (B) is shown. iPS-Mg are treated and exosomes extracted from the SN and added to differentiated SH-SY5Y cells, which are analysed for the effect exosomes have on neuron-like cells.

**Figure 5-1 Diagram of SH-SY5Y differentiation and experimental setup**
Figure 5-2 Characterisation of SH-SY5Y differentiation

SH-SY5Y were differentiated out using an established protocol with 10µM RA and 50nM BDNF. Differentiation was shown by staining the cells with Tubb3 (A), PSD-95 and synaptophysin (B), showing an elongated cell morphology in the differentiated SH-SY5Y and presence of synaptic markers in the cell processes. Scale bar represents 10µm.
5.2.4 Exosomal uptake

Exosomes were incubated with vybrant Dil (Thermo Fisher Scientific) following manufacturers’ instructions (1:200) at 37°C for 30mins. The excess unbound dye was removed using 3kDa NMWL columns (Amicon) as previously described (Chapter 3.2.2). PBS treated with Dil was used as a negative sample controlling for potential Dil carryover. Dil-labelled exosomes were then added to SH-SY5Y for 2hrs. As an additional negative control, some SH-SY5Y were treated with CytoD (10µM), CPZ (5µg/ml) or Genistein (200µM) for 30mins prior to addition of exosomes. The concentrations of the inhibitors were based on previous publications and reported IC50 (Rejman et al., 2005; Vercauteren et al., 2010; Wesén et al., 2017; Garcia-Reitboeck et al., 2018; Piers et al., 2020). After incubation, the SH-SY5Y were detached in PBS and the MFI analysed using the FACSCalibur, with analysis completed with the Flowing Software 2 program.

To visualise exosomal uptake, exosomes were stained as stated above with vybrant DiO and added to SH-SY5Y, which were labelled with BioTracker 555 Orange Cytoplasmic Membrane Dye. SH-SY5Y labelling occurred before exosome incubation in the 1hr condition and after exosome incubation in the 24hrs condition. Nuclei were stained with DAPI before being mounted and imaged with a Zeiss LSM710 confocal microscope.

5.2.5 Viability of neuron-like cells

To assess the effect of exosomes on the viability of neuron-like cells, SH-SY5Y were exposed to exosomes for 24hrs and cell death was assessed using PI FC, as previously described (Chapter 2.2.5). Where indicated, SH-SY5Y were simultaneously exposed to exosomes and 100µM H₂O₂ for 24hrs to assess the ability of exosomes to rescue the cells from H₂O₂-induced cell death.

iPS-neurons were treated with 100µM H₂O₂ for 24hrs in addition to exosomes from Cv and R47Hhet iPS-Mg. PI and Hoechst were added at 1µl/well each for 15mins before they were imaged, using PBS containing Ca²⁺/Mg²⁺. Five pictures per coverslip were taken on a Zeiss Axioskop 2 fluorescent microscope, collected with the AxioVision Rel 4.8 software, and analysed using ImageJ. For analysis, a threshold was applied to each separate channel and a mask generated to analyse particle size and area covered by the particles. Overlays were created using the MIN image
calculator equation and particles analysed again to quantify the number of particles and their size of PI positive Hoechst inclusions.

5.2.6 MTT assay

Following incubation with exosomes, 0.5mg/ml MTT solution was added to the SH-SY5Y and incubated for 3hrs at 37°C. Afterwards, the MTT solution was removed and the solvent, isopropanol, was incubated for 15mins on a shaking plate to allow the MTT crystals to dissolve in the solvent. Afterwards, the plate was read on a Tecan 10M plate reader.

To normalise the results to the number of cells, 50µl Crystal violet was added for 20mins. The plate was then thoroughly washed with water and left to dry out over-night. Methanol was added to dissolve the crystal violet the subsequent day, for 15mins on a shaker, and the plate was read on a Tecan 10M plate reader. MTT values were normalised to Crystal violet staining (Figure 5-3).

5.2.7 Staining

For staining, SH-SY5Y or iPS-neurons were fixed with 4% PFA with 4% sucrose for 20mins before being quenched in 50mM NH4Cl for 10mins. After permeabilization with 0.2% Triton X-100 for 5mins, the cells were blocked with 5% normal goat serum. Primary antibodies were incubated overnight at 4°C. Appropriate secondary antibodies were used for 1hr at RT (1:500) before the coverslips were mounted on slides using Vectashield with DAPI. The images were acquired on a Zeiss Axioskop 2 fluorescent microscope using the AxioVision Rel 4.8 software or on a Zeiss LSM710 confocal microscope using the LSM Pascal 5.0 software.

For the Tubb3 and GAP43 staining, 8 ROI were taken per coverslip on a Zeiss LSM confocal microscope. Analysis was undertaken with the Fiji software. For neurite length analysis, the Tubb3 staining was skeletonised, using an adapted version of previously described protocol (Young and Morrison, 2018) with the full macro attached (Appendix 7.5.1). GAP43 levels in iPS-neurons were quantified using a Fiji similar macro to a previously published study (Mallach et al., 2019). Briefly, a threshold was applied to both GAP43 and Tubb3 images before the overlay of both channels was calculated. The area of GAP43 positive staining in Tubb3 positive pixels divided by the total area of Tubb3 positive pixels was plotted. A full macro can be found below (Appendix 7.5.2).
Raw Crystal violet was plotted after SH-SY5Y were treated with exosomes from Cv and R47H\textsuperscript{het} iPS-Mg. When exosomes originated from resting iPS-Mg (Ai) and when comparing them to exosomes from treated iPS-Mg (Aii), the crystal violet raw values did not change significantly, showing that SH-SY5Y did not proliferate significantly in response to exosome treatment. This indicates that the MTT assay is an approximation of metabolism. n = 3, one-way (Ai) and two-way ANOVA (Aii), n.s. not significant.
5.3 Results

5.3.1 SH-SY5Y take exosomes up through endocytosis

Before the effect of exosomes on neuron-like cells could be analysed, the ability of these cells to bind and take up exosomes was analysed. Uptake of exosomes was visualised through staining and quantified through FC analysis. Even after 1hr of exosome incubation, DiO labelled exosomes were found to be co-localised with labelled SH-SY5Y membranes (Figure 5-4). To quantify this, exosome uptake into SH-SY5Y was analysed with FC. After 2hrs of exosome incubation, SH-SY5Y were shown to have taken up exosomes from a range of different TREM2 variants (Figure 5-5). No difference in uptake of exosomes from different TREM2 variants was observed. Interestingly, CytoD did not prevent exosome uptake, suggesting an independent mechanism through which exosomes are taken up into SH-SY5Y. One of these independent mechanisms could be endocytosis, as SH-SY5Y, and neurons in general, have been shown to use endocytosis as a mechanism to take up extracellular particles (Congdon et al., 2013). Inhibitors for both clathrin-mediated endocytosis, CPZ, and caveolin-mediated endocytosis, genistein, were both used to block uptake of exosomes into SH-SY5Y and both were shown to be effective (Figure 5-5).
Uptake of exosomes was analysed through microscopy. DiO-labelled exosomes were seen to co-localise with SH-SY5Y labelled with membrane marker after both 1hr and 24hrs of exosome incubation (A). As a negative control to account for potential DiO carry-over, DiO was incubated with cell tracker and imaged, revealing little carry-over (B). Scale bar represents 10µm.

Figure 5-4 Visualisation of exosome uptake into SH-SY5Y
Quantification of exosomal uptake was quantified with FC (A). Labelled exosomes were increasingly taken up by SH-SY5Y after 2hrs of incubation, with a significant difference being detected between labelled exosomes and the labelled negative PBS control (Bi). Whilst the phagocytosis inhibitor CytoD did not prevent exosome uptake into the SH-SY5Y, uptake was blocked by pre-incubation with the endocytosis inhibitors CPZ and Genistein. n = 4, one-way ANOVA with Tukey’s post hoc test and * p < 0.05 and n.s. not significant.
5.3.2 Effect of exosomes on cell death

Based on the proteomic analysis showing that exosomes from iPS-Mg display differences both in inflammatory cytokines and in proteins involved in metabolism, the effect of exosomes from iPS-Mg on neuronal viability was tested.

Using differentiated cells, SH-SY5Y viability was analysed following addition of exosomes. No difference in PI staining was detected between exosomes originating from different TREM2 variants through FC. Despite treatment with LPS increasing cytokines within exosomes, this did not have an impact on the viability of SH-SY5Y (Figure 5-6). However, interestingly, treatment of SH-SY5Y with exosomes extracted from iPS-Mg treated with apoptotic neurons led to a significant increase in PI staining, indicating an increased rate of cell death, with the effect being the most obvious in exosomes from Cv iPS-Mg (Figure 5-6ii).

As the difference is likely to be subtle and could be difficult to detect in differentiated SH-SY5Y, which are still oncogenic, the ability of exosomes to rescue SH-SY5Y from stress was tested. This was based on a previous study highlighting the ability of exosomes to rescue SH-SY5Y from H$_2$O$_2$-induced stress (Pascua-Maestro et al., 2019). After having determined the optimal H$_2$O$_2$ concentration to induce high levels of cell death (Figure 5-7A), the ability of exosomes to rescue SH-SY5Y from the H$_2$O$_2$-induced cell death was analysed (Figure 5-7C). Only exosomes extracted from Cv iPS-Mg were capable of reducing cell death in H$_2$O$_2$ treated SH-SY5Y, whilst exosomes from TREM2 variants did not have this effect. As observed in cell death analysis in unstressed SH-SY5Y, an effect of iPS-Mg treatment on SH-SY5Y viability was found (Figure 5-8). Whilst LPS treatment of iPS-Mg did not influence the ability of their exosomes to rescue SH-SY5Y from H$_2$O$_2$ induced death, iPS-Mg treatment with apoptotic neurons led to increases in H$_2$O$_2$ induced cell death in SH-SY5Y. This effect was observed in exosomes from Cv and T66M$^{hom}$ iPS-Mg (Figure 5-8B).

To test this in another system, iPS-neurons were also challenged with H$_2$O$_2$ and incubated with exosomes for 24hrs to test cell viability (Figure 5-9). Here, rather than Cv exosomes rescuing the neurons from cell death, R47H$^{het}$ exosomes further exacerbated the cell death experienced by the cells in response to H$_2$O$_2$. 
Cell death was assessed in SH-SY5Y incubated with exosomes for 24hrs through PI FC, with representative histograms shown in Ai, Bi and Ci. At baseline, exosomes from a range of different TREM2 variant iPS-Mg did not significantly influence cell viability (A). Exosomes originating from LPS-treated iPS-Mg also did not impact cell viability (B), but following treatment of iPS-Mg with apoptotic neurons, exosomes from Cv and T66M homo iPS-Mg significantly increased PI staining in SH-SY5Y exposed to these exosomes (C). n = 5. One-way ANOVA (A) and two-way ANOVA (B, C) with Tukey’s post hoc test and * p < 0.05, *** p ≤ 0.001 and n.s. not significant.
Figure 5-7 Cv exosomes can rescue SH-SY5Y from cell death
The concentration of H$_2$O$_2$ to induce cell death, as indicated by PI staining analysed through FC, was determined (A). The concentration for further experiments was chosen to be 100µM, as it caused increases in PI staining, whilst at higher concentration PI staining of the SH-SY5Y appeared to decrease, probably because these high concentrations of H$_2$O$_2$ cause apoptosis to a degree that the cells are no longer identified as cells through FC. Representative FC curves are plotted (B), highlighting the highly PI positive H2-region. Comparing the percentage of analysed cells that fell into the PI positive H2 regions between the different conditions showed that exosomes from Cv iPS-Mg were capable of reducing SH-SY5Y cell death. This effect however was not seen in SH-SY5Y exposed to exosomes from disease-associated TREM2 variants, indicating that only Cv exosomes could be protective against H$_2$O$_2$-induced cell death. A: n = 1, C: n = 4, 1-way ANOVA with Tukey’s post hoc test,* p < 0.05, ** p ≤ 0.01 and n.s. not significant.
Figure 5-8 Exosomes from apoptotic neuron treated iPS-Mg may not be protective

The effect of exosomes extracted from either LPS or apoptotic neuron treated iPS-Mg on rescuing SH-SY5Y from H$_2$O$_2$ induced cell stress was analysed. Whilst exosomes from LPS-activated iPS-Mg did not appear to have an effect of the number of SH-SY5Y staining PI positive (A), exosomes from iPS-Mg treated with apoptotic neurons increased the percentage of dead cells significantly, when they came from Cv and T66M$^{hom}$ iPS-Mg (B). n = 4, two-way ANOVA with Tukey's post hoc test, * p < 0.05 and n.s. not significant.
Figure 5-9 Effect of exosomes on induced cell death of iPS-neurons

Cell death was assessed in iPS-neurons through PI and Hoechst staining (A) following addition of H$_2$O$_2$ and exosomes. The area covered by PI was normalised to overall area covered by Hoechst staining, normalising for potential differences in cell number in the area. Whilst Cv exosomes did not appear to rescue iPS-neurons from H$_2$O$_2$-induced cell stress, exosomes from R47H$^{h}$het iPS-Mg appeared to exacerbate the experienced cell death (B). $n=3$. One-way ANOVA with Tukey’s post hoc test and * $p < 0.05$. 
5.3.3 Effect on cell stress

5.3.3.1 In SH-SY5Y

Besides inducing neuronal cell death, inflammatory cytokines secreted from microglia can also influence neuronal stress pathways (Combs et al., 2001; Li et al., 2003; Guadagno et al., 2013). To understand whether cytokines secreted in association with microglial exosomes could have a similar effect, the influence of exosomes on cell stress pathways was further investigated.

Following exosome incubation for 72hrs, cell stress pathways, as tested through WB, revealed no significant changes in undifferentiated SH-SY5Y (Figure 5-10). PERK levels suggest a decrease following treatment with R47H\textsuperscript{het} exosomes, however phosphorylation of eIF2α, another constituent of the ER stress pathway, was not affected. General stress pathways, probed for by phosphorylation of p38 MAPK could be affected by treatment with R47H\textsuperscript{het} exosomes, however total cytochrome c levels were not affected. Autophagy appeared not to be affected in these cells either as LC3 levels did not change in response to either treatment (Figure 5-10). Undifferentiated SH-SY5Y could be too robust to display changes in stress pathways in response to what could be subtle changes in exosome composition.

To further probe the effects of exosomes from LPS-treated iPS-Mg, more subtle changes in stress pathways were investigated using differentiated SH-SY5Y cells. As in the experiments on undifferentiated SH-SY5Y, total cytochrome c levels were not affected by exosomes (Figure 5-11), however, similar to the previous results, changes in specific ER stress pathways were observed. PERK, and downstream CHOP, were significantly decreased in SH-SY5Y treated with exosomes extracted from LPS-treated Cv iPS-Mg. This showed that in differentiated SH-SY5Y, exosomes from LPS-treated Cv iPS-Mg decreased ER-stress pathways, with exosomes from R47H\textsuperscript{het} iPS-Mg displaying a more subdued effect.

5.3.3.2 In other iPS-Mg

In addition to having an effect on neighbouring neurons, microglial cytokines can also have an effect on other microglia, inducing a self-perpetuating cycle of inflammation (Greer, 2000; Frank-Cannon et al., 2009). To test the ability of exosomes from activated cells to communicate this activation to resting iPS-Mg, iPS-Mg were exposed to exosomes extracted from iPS-Mg treated with either LPS or apoptotic neurons. To make these experiments physiologically relevant, Cv iPS-Mg received
exosomes from other Cv iPS-Mg, whilst R47H<sup>het</sup> iPS-Mg received R47H<sup>het</sup> exosomes (described in Figure 5-12A). A range of inflammatory markers were tested (Figure 5-12). Expression of TNF was significantly increased in iPS-Mg exposed to exosomes from LPS-treated iPS-Mg, with R47H<sup>het</sup> iPS-Mg showing lower levels of TNF induction. Interestingly, exosomes from apoptotic-treated iPS-Mg also increased TNF levels in R47H<sup>het</sup>, whilst this was not the case for Cv iPS-Mg. IL6 and IL1B expression was elevated in Cv iPS-Mg treated with LPS exosomes, whilst exosomes from apoptotic neurons appeared to have no effect (Figure 5-12B).

These effects may be due to LPS, and to a lesser extent apoptotic neurons, carrying over in the exosomal suspension, meaning that the effect could be down to the iPS-Mg reacting to LPS itself and not exosomal cytokines. To account for this, SN from resting iPS-Mg was spiked with either LPS or apoptotic neurons before exosomes were extracted. These suspensions therefore would contain any potential carryover without exosomal changes. Comparing the TNF, IL6 and IL1B responses of iPS-Mg to these spiked exosomes with the response to exosomes from activated cells however shows that iPS-Mg react significantly more to exosomes from activated cells than to spiked exosomes (Figure 5-12C), highlighting that the inflammatory response of iPS-Mg is indeed a real effect, showing that microglial exosomes can function as an autocrine signalling pathway.
Figure 5-10 Cell stress induced by exosomes in undifferentiated SH-SY5Y

Cell stress levels were assessed in SH-SY5Y exposed to exosomes from iPS-Mg for 72hrs. Phosphorylation of p38 MAPK and total PERK levels showed a trend to be decreased in SH-
SY5Y treated with exosomes from R47Hhet iPS-Mg (Bi, Bii), whilst phosphorylation of eIF2α (Biii) and cytochrome c levels were not statistically significantly changed (Biv). LC3 cleavage also was not affected by exosome treatment (Bv). n = 4, two-way ANOVA with n.s. not significant.
Figure 5-11 Cell stress in differentiated SH-SY5Y

Whilst total cytochrome c did not appear to change (Aii), PERK significantly decreased in SH-SY5Y exposed to exosomes from LPS-activated Cv exosomes, whilst LPS R47H\textsuperscript{het} exosomes appeared to not have an effect (Bii). Exosomes from NT R47H\textsuperscript{het} iPS-Mg also reduced PERK levels in comparison with NT Cv exosomes (Bii). CHOP was also decreased in SH-SY5Y exposed to LPS Cv exosomes (Cii), with the trend for R47H\textsuperscript{het} exosomes not significant. \( n = 3 \), two-way ANOVA with Tukey’s post hoc test and \( * p < 0.05 \) and n.s. not significant.
Figure 5-12 Exosomes can induce inflammation in iPS-Mg
Exosomes from iPS-Mg treated with LPS or apoptotic neurons were added to iPS-Mg to assess their ability to induce inflammation, with the experimental setup shown in A. Exosomes from LPS-treated iPS-Mg were shown to increase \textit{TNF}, \textit{IL6} and \textit{IL1B} (Bi, Bii and Biii respectively) expression in both Cv and R47\textsuperscript{Het} iPS-Mg, with the \textit{TNF} response lower in R47\textsuperscript{Het} iPS-Mg in comparison with Cv iPS-Mg (Bi). Additionally, exosomes from apoptotic neurons treated iPS-Mg also increased \textit{TNF} expression in R47\textsuperscript{Het} iPS-Mg (Bi). To control for potential carry-over, SN from resting iPS-Mg was spiked with either LPS or apoptotic neurons before exosomes were extracted and their effect on the expression of \textit{TNF}, \textit{IL6} and \textit{IL1B} was compared to the effect of exosomes from activated cells (Ci, Cii and Ciii respectively). \textit{n} = 3, two-way ANOVA with Tukey's post hoc test and * \textit{p} < 0.05, ** \textit{p} < 0.01, *** \textit{p} < 0.001.
5.3.4 Exosomes have a differential effect on neuronal metabolism

Proteomic analysis revealed changes in proteins involved in metabolism (Figure 4-12 and Figure 4-14). This could ultimately influence neuronal viability, as already assessed (Chapter 5.3.2), but also other measures of metabolism.

Levels of phosphorylated NFκB are linked to metabolic processes, however, levels of phosphorylation were not changed in SH-SY5Y treated with exosomes from iPS-Mg, independent of R47Hhet variant and LPS activation (Figure 5-13Aii). Reduction of MTT to formazan is regulated by NADPH-dependent enzymes and can be used as a measure of metabolic activity of cells (Connelly et al., 2000; Rai et al., 2018). This reduction was assessed with an MTT assay (Figure 5-13B) and revealed an increase in SH-SY5Y metabolism after addition of Cv exosomes, with a similar trend being observed in response to R47Hhet exosomes. As proteomic analysis specifically implicated apoptotic neuron treatment of iPS-Mg in altering the ability of exosomes to influence metabolism, the effect of exosomes collected from treated iPS-Mg on SH-SY5Y MTT metabolism was also tested. Whilst exosomes from apop treated Cv iPS-Mg induced a modest increase in metabolic activity, exosomes from apop treated R47Hhet iPS-Mg induced a significant drop in MTT metabolism in SH-SY5Y in comparison to exosomes from apop treated Cv iPS-Mg (Figure 5-13Bii).

To test whether the changes in MTT metabolism were due to changes in mitochondrial metabolism or other aspects of cellular metabolism, ATP levels were measured in SH-SY5Y following addition of iPS-Mg exosomes and very similar trends to the MTT assay were observed (Figure 5-13C). Addition of exosomes from resting iPS-Mg increased the ATP levels in SH-SY5Y, in particular when the exosomes were extracted from Cv iPS-Mg, whilst the effect for R47Hhet exosomes appeared to be smaller. When the exosomes were extracted from treated iPS-Mg, exosomes from apop treated R47Hhet iPS-Mg displayed lower ATP levels than SH-SY5Y treated with baseline R47Hhet exosomes. Furthermore, following treatment with either LPS or apoptotic neurons, R47Hhet exosomes induced significantly lower ATP levels in comparison to their counterparts extracted from Cv iPS-Mg (Figure 5-13Cii).

The changes in ATP levels in SH-SY5Y following treatment with exosomes indicate that exosomes appear to primarily affect energy metabolism. This is in line with the proteomic data as well (Figure 4-12, Figure 4-21 and Figure 4-22). Neurons in particular rely on mitochondria to supply the necessary ATP for synaptic signalling.
(Cheng et al., 2012) and therefore mitochondrial function was analysed in more detail in neuron-like cells exposed to exosomes.

To assess whether the increase in ATP production (Figure 5-13C) was linked to an increase in mitochondrial biogenesis, PGC-1α was investigated. PGC-1α is a key regulator of mitochondrial biogenesis, acting as a transcriptional coactivator for genes involved in mitochondrial biogenesis (Cheng et al., 2012; Onishi et al., 2014). Through its co-activator nuclear respiratory factor 1 (NRF1), nuclear genes required for mitochondrial biogenesis can be transcribed (Satoh et al., 2013; Kiyama et al., 2018). Phosphorylation of PGC-1α at the S571 site, inhibiting its function (Li et al., 2007; Fernandez-Marcos and Auwerx, 2011), was increased in SH-SY5Y exposed to R47Hhet exosomes (Figure 5-14Bi). Independently of this, Cv exosomes increased total PGC-1α levels in comparison with both untreated SH-SY5Y and SH-SY5Y exposed to R47Hhet exosomes (Figure 5-14Bii). This was mirrored by an increased expression of PPARGC, the gene encoding PGC-1α, in response to Cv exosomes (Figure 5-14Ci), whilst NRF1 expression was unchanged (Figure 5-14Cii).

Next to biogenesis, another mechanism exosomes could influence is mitochondrial functioning. Heat shock protein 60 (HSP60), and its functional regulator heat shock protein 10 (HSP10), are involved in the folding of mitochondrial proteins (Höhfeld and Hartl, 1994; Böttinger et al., 2015; Castro et al., 2018). Abnormalities in HSP60 have been linked to mitochondrial dysfunction (Zhou et al., 2018) and therefore, the levels of HSPD1, encoding for HSP60, and HSPE1, encoding for HSP10, were also probed for. Whilst HSPE1 levels were not affected by exosome treatment (Figure 5-14Di), HSPD1 levels were increased in SH-SY5Y exposed to Cv exosomes (Figure 5-14Dii).

To verify the results that specifically Cv exosomes can increase mitochondrial biogenesis through changes in PPARGC and HSPD1 expression, iPS-neurons were exposed to exosomes. Whilst the overall number of neurons appeared unchanged, as measured through MAPT levels (Figure 5-15A), an increase in PPARGC was observed when iPS-neurons were exposed to Cv exosomes, with a similar trend becoming obvious in iPS-neurons exposed to R47Hhet exosomes (Figure 5-15Bi). NRF1 was specifically increased after exposure of iPS-neurons to R47Hhet exosomes (Figure 5-15Bii), whilst HSPE1 levels were unchanged (Figure 5-15Ci). Similar to NRF1, HSPD1 levels appeared to increase specifically in iPS-neurons exposed to R47Hhet exosomes (Figure 5-15Dii).
Figure 5-13 Effect of exosomes on metabolism

Phosphorylation of NFκB was tested in SH-SY5Y treated with exosomes from Cv and R47H<sup>het</sup> iPS-Mg treated with LPS (A), but no significant changes were observed. Additionally, metabolism was directly measured with an MTT assay, normalised to Crystal Violet, which showed a significant upregulation of cellular metabolism following addition of Cv exosomes to...
SH-SY5Y (Bi), with a similar trend being observed for R47H\textsuperscript{het} exosomes. Furthermore, exosomes from Cv iPS-Mg treated with apoptotic neurons led to a significant increase in MTT metabolism in comparison to exosomes from R47H\textsuperscript{het} iPS-Mg treated with apoptotic neurons (Bii). To complement the metabolic assay, the ATP levels were also measured in the SH-SY5Y, following the addition of exosomes from iPS-Mg (C). Again, addition of Cv exosomes significantly increased ATP levels (Cii). Addition of exosomes from R47H\textsuperscript{het} iPS-Mg that were treated with either LPS or apoptotic neurons did decrease ATP levels in comparison to Cv exosomes (Cii). A: n = 5, B: n = 4, C: n = 3. One-way (Bi, Ci) and two-way ANOVA (Ai, Bii, Cii) with Tukey’s post hoc test and * p < 0.05 and n.s. not significant.
A

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Bi

- **p-PGC-1α**

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Ci

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Di

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To verify the previous results, mitochondrial biogenesis was assessed in SH-SY5Y exposed to iPS-Mg exosomes for 24hrs. Whilst SH-SY5Y exposed to R47H\textsuperscript{het} exosomes showed a higher phosphorylation of PGC-1\(\alpha\) (Bi), the total PGC-1\(\alpha\) levels were increased in SH-SY5Y exposed to Cv exosomes in comparison with both untreated and R47H\textsuperscript{het} exosome treated SH-SY5 (Bii). This was further verified in the gene expression analysis of SH-SY5Y, which again showed that PPARGC, encoding for PGC-1\(\alpha\), was significantly increased in SH-SY5Y exposed to Cv exosomes (Ci), similar to HSPD1, encoding for HSP60 (Dii), whilst other factors involved in mitochondrial biogenesis, such as NRF1 (Cii) and HSPE1, encoding for HSP1 (Di) were not affected. n = 3. One-way ANOVA with Tukey’s post hoc test and * \(p < 0.05\), ** \(p \leq 0.01\) and n.s. not significant.
Figure 5-15 Effect of exosomes on iPS-neuron mitochondrial function
qPCR was performed on iPS-neurons treated with exosomes for 24hrs. Whilst MAPT levels (A) were unchanged, expression of PPARG was increased when cells were exposed to either Cv or R47H^het exosomes (Bi) with NRF1 expression upregulated in iPS-neurons treated with R47H^het exosomes (Bii). Expression of HSPE1, encoding for HSP10, did not appear to be affected by exosome treatment (Ci), but HSPD1, encoding for HSP60, was selectively increased with exosomes from R47H^het iPS-Mg. n = 3. One-way ANOVA with Tukey’s post hoc test and * p < 0.05 and n.s. not significant.
5.3.5 Cv exosomes support neuronal development

Microglia have been reported to support neuronal development (Morgan et al., 2004; London et al., 2013) and since the differentiation/development module was identified in the proteomic analysis of exosomes from iPS-Mg (Figure 4-12, Figure 4-21 and Figure 4-22), the influence of exosomes on the development of iPS-neurons was analysed. iPS-neurons were used for this analysis as they closely recapitulate human neuronal development (Shi et al., 2012; Campos et al., 2014). Due to these cultures containing different cell-types (Shi et al., 2012), Tubb3 staining was used to quantify neurite outgrowth.

The length of the Tubb3-positive projections were shown to increase significantly in cultures exposed to Cv exosomes (Figure 5-16). The same trend however was not observed for iPS-neurons exposed to R47H\textsuperscript{het} exosomes, indicating differences in the ability of exosomes to support neuronal development. These differences could be due to changes in either the number of neuronal cells present in the cultures or in the taken image. However, previous analysis (Figure 5-15A) has suggested the total number of neurons did not change with different treatments. Moreover, when the length of the Tubb3-positive projections was normalised to the overall level of Tubb3 level of staining in the images, the same trend was observed (Figure 5-16Bii), suggesting an increase in the length of Tubb3 positive neurites.

One protein that is involved in neurite outgrowth and found in the growth cones of neurites is GAP43 (Meiri et al., 1998; Korshunova et al., 2007). To verify that the observed changes in neurite length were caused by increased outgrowth linked to GAP43, iPS-neurons were co-labelled for Tubb3 and GAP43. As GAP43 staining also appeared outside of Tubb3-positive areas (Figure 5-17A), most likely due to it being located in other cell types, only GAP43 staining overlapping with Tubb3 staining was quantified. Increased levels of GAP43 were observed in iPS-neurons exposed to Cv exosomes, but not PBS or R47H\textsuperscript{het} exosomes (Figure 5-17B), matching the previous results (Figure 5-16) suggesting that primarily Cv exosomes are capable of supporting neurite outgrowth.
Figure 5-16 Cv exosomes increase neurite length of iPS-neurons
iPS-neurons were treated with exosomes from iPS-Mg for 24hrs and their neurite outgrowth, visualised through Tubb3 staining. Representative images were shown (A) including skeleton images of Tubb3 used for processes length quantification. In iPS-neurons treated with exosomes from Cv iPS-Mg, the overall branches were significantly longer, a trend that was not observed in cells treated with R47H<sup>het</sup> exosomes (Bi). As total Tubb3 staining could skew this result, the overall neurite length was normalised to overall levels of Tub3 straining, but again increases in iPS-neurons treated with Cv exosomes was observed (Bii). Scale bar represents 10µm. n = 3 with 8 ROI analysed per coverslip. One-way ANOVA with Tukey’s post hoc test and * p < 0.05, ** p ≤ 0.01.
A

PBS

Cv

R47H^{het}

B

GAP43 staining

% of Tubb3 positive neurons with GAP43 staining

PBS  Cv  R47H^{het}
Figure 5-17 Cv exosomes induce neurite outgrowth

The levels of GAP43 were quantified in iPS-neurons treated with exosomes from iPS-Mg for 24hrs. Representative images showing the overlap of Tubb3 and GAP43 following the exosome treatment were shown (A). Additionally, the binary image showing GAP43 staining that was overlaid with Tubb3 staining was also shown (B). The quantification of the overlay showed that iPS-neurons contained significantly more GAP43 staining when they were treated with Cv exosomes in comparison with either PBS-treated iPS-neurons or cells treated with R47H<sup>het</sup> exosomes (B). Scale bar represents 10µm. n = 3 with 8 ROI analysed per coverslip. One-way ANOVA with Tukey’s post hoc test and * p < 0.05.
5.3.6  **Exosomes can influence synaptic functioning**

Literature has suggested that EV from microglia can supply synapses with necessary factors to support synaptic activity (Antonucci *et al.*, 2012), whilst proteomic analysis of the exosomes showed that treatment of iPS-Mg could influence synaptic pruning and neurite outgrowth (Chapter 4.3.7.1). Therefore, the effect of exosomes on synapse functioning was assessed through a range of different methods.

5.3.6.1  **Changes in synaptic proteins**

Changes in proteins associated with synapses were investigated in differentiated SH-SY5Y treated with exosomes. No changes in synaptophysin and drebrin were observed (Figure 5-18), which could suggest that the overall number of synapses in SH-SY5Y, as characterised by levels of synaptophysin (Li *et al.*, 2010; Jiang *et al.*, 2015) was not significantly affected by exosomes from Cv or R47H^{het} iPS-Mg independent of activation of iPS-Mg with LPS. Phosphorylation status is important for the localization of postsynaptic density protein 95 (PSD-95), the proteins stabilizing receptor proteins in the postsynaptic site (Nelson *et al.*, 2013; Sen *et al.*, 2016), leading to the investigation of phosphorylation of PSD-95 at the S295 and T19 site. Phosphorylation at T19 is associated with long-term depression and internalization of the protein away from the synapse, ultimately weakening the synapse (Nelson *et al.*, 2013). A decrease in T19 phosphorylation of PSD-95 was observed when SH-SY5Y were treated with exosomes from R47H^{het} iPS-Mg in comparison to exosomes from Cv iPS-Mg (Figure 5-18Cii). Phosphorylation at the S295 site of PSD-95 leads to the stabilization of the protein at the synapse, supporting synaptic signalling and functioning (Sen *et al.*, 2016). S295 phosphorylation appeared to increase following treatment of SH-SY5Y with exosomes from LPS-treated Cv iPS-Mg, however this was not significant (Figure 5-18Dii). This suggests that exosomes from LPS-treated iPS-Mg could support synaptic strength. Additionally, a drop in S295 phosphorylation was also observed when SH-SY5Y were treated with exosomes from R47H^{het} iPS-Mg (Figure 5-18Dii). The combined decrease of T19 and S205 phosphorylation could indicate an overall drop in PSD-95 levels, again indicating weaker synapses.

5.3.6.2  **Effect on voltage gated calcium channels**

As a functional readout, intracellular calcium imaging was performed on differentiated SH-SY5Y following addition of iPS-Mg exosomes. Addition of KCl allowed for the assessment of voltage-gated calcium channels (VGCC) (Barreto-
SH-SY5Y have been shown to express a range of different VGCC including L-type and N-type (Sousa et al., 2013).

NIF, an inhibitor of L-type VGCC (Evans and Pocock, 1999), did not appear to have an effect on intracellular calcium (Figure 5-19), suggesting that the effect seen is not mediated through L-type VGCC but rather N-type VGCC. However, a specific N-type VGCC inhibitor, such as Ω-conotoxin (Pocock et al., 1993; Reuveny and Narahashi, 1993; Harrold et al., 1997) should be used in the future to verify this. The amplitude of the calcium response in SH-SY5Y in response to KCl was analysed, taking into account the amplitude of response and the speed of response. When SH-SY5Y were exposed to exosomes for only half a minute, LPS treatment of iPS-Mg prior to exosome extraction induced a higher/steeper slope, independent of TREM2 status (Figure 5-19Aii), indicating a higher activity of the VGCC, potentially through the N-type VGCC. After prolonged exposure to exosomes, Cv exosomes significantly lowered the response of SH-SY5Y to KCl, whilst LPS treatment had a reduced effect on the behaviour of the calcium trace (Figure 5-19B).

To support this data further in a more neuron-like model, the calcium channels in iPS-neurons were characterised (Figure 5-20). Processes showed a higher response to KCl than cell bodies and ω Agatoxin IVa, blocking the P/Q-type VGCC (Pocock et al., 1993; Sidach and Mintz, 2000; Nimmrich and Gross, 2012), reduced the response to KCl in both processes and cell bodies (Figure 5-20B), suggesting that the iPS-neurons display P/Q type VGCC through which the KCl invoked increases in intracellular calcium concentrations. The responses were decreased in both the cell bodies and processes of the iPS-neurons, a similar response to matured CGC (Harrold et al., 1997). 4-AP depolarised both processes and cell bodies, potentially making it a model to study the role of exosomes in synaptic transmission (Figure 5-20C).
Figure 5-18 Effect of exosomes on synaptic proteins

Synaptic changes in differentiated SH-SY5Y following 72hrs incubation of exosomes were investigated. Both synaptophysin (Ai) and drebrin (Bi) levels were unchanged. The phosphorylation of T19 of PSD-95, leading to an internalization of receptors, was decreased following exposure of SH-SY5Y to exosomes from R47H<sup>het</sup> iPS-Mg (Cii). Phosphorylation of S295 at PSD-95, stabilizing the scaffolding protein at the synaptic site, was decreased again when exosomes from R47H<sup>het</sup> iPS-Mg were added (Dii), with a trend of increased phosphorylation observed when exosomes from LPS-treated Cv iPS-Mg were added (Dii). Aii, Bi, and Cii: n = 4, Dii: n = 3. Two-way ANOVA with Tukey’s post hoc test and * p < 0.05 and n.s. not significant.
Figure 5-19 VGCC changes in response to exosomes
The effect of exosomes on VGCC was tested, both in SH-SY5Y exposed acutely for a minute to exosomes (A) and SH-SY5Y exposed for 72hrs to exosomes (B). Representative curves were shown (Ai, Bi) representing the mean of 20 ROI analysed with the SEM represented by the grey fill. The slope of the response to KCl was analysed and showed that when SH-SY5Y were exposed to exosomes from iPS-Mg treated with LPS acutely, an increased response in response to KCl was seen (Aii and Aiii). Following prolonged exposure to exomes though, the effect of the TREM2 status of the exosome-secreting iPS-Mg influences the result more, with R47Hhet exosomes inducing a bigger KCl response in comparison to Cv exosomes (Bii and Biii). Aii, Aiii: n = 3, Bii, Biii: n = 4. One-way ANOVA (Aii, Biii) and two-way ANOVA (Aiii, Biii) with Tukey's post hoc test and * p < 0.05 and n.s. not significant.
Figure 5-20 Characterisation of VGCC on iPS-neurons

Representative images of calcium imaging performed on iPS-neurons. Response to 30mM KCl and 5μM NIF were assessed in both processes (Ai) and cell bodies (Aii). After pre-incubation with 1μM ωAgatoxin IV for 30 minutes, KCl response was abolished both in the processes (Bi) and in cell bodies (Bii). 125μM 4-AP leads to a sustained depolarization of the processes (Ci) and cell bodies (Cii). Averages were plotted in black with the SEM coloured in grey. At least 3 ROI for each process were analysed (5 for Bi and Ci), with 9 (Ai), 6 (Bi) and 8 (Ci) processes analysed in total.
5.4 Discussion

5.4.1 Exosome treatment

Different exosome concentrations have been used to induce an effect on target cells. Some studies have used significantly more exosomes, adding an equivalent of up to 50µg in comparison with the 6µg used here (Horibe et al., 2018; Chen et al., 2019), but the concentration used here and by others (Bhatnagar and Schorey, 2007) roughly reflects the ratio of neurons to microglia in the brain. On average the 6µg of protein loaded reflects 2% of secreted exosomes from 500,000 iPS-Mg, so it is equivalent to the material secreted from roughly 10,000 iPS-Mg. Previous analysis has shown that 50% of cells in the brain are neurons and that in the cortex, microglia make up between 10% and 5% of the cells (Herculano-Houzel, 2014). Therefore adding exosomes from 10,000 iPS-Mg to 60,000 SH-SY5Y is similar to the cell ratios found in the brain. Even lower concentration of exosomes have been used to elicit a response from target cells, with only 50ng of exosomes used to study aspects of nerve injury (Tassew et al., 2017).

When studying the uptake of exosomes into SH-SY5Y, no difference between exosomes from TREM2 variants was found (Figure 5-5), in line with previous research suggesting that differences in uptake is dependent on the target cells not the secreting cells (Horibe et al., 2018). Inhibition of phagocytosis and macropinocytosis, using the actin inhibitor CytoD, did not prevent exosome uptake into SH-SY5Y (Figure 5-5). On the other hand, CytoD did block exosome uptake into the iPS-Mg (Figure 3-5E). Previous research has suggested that microglia, and other associated macrophages, mainly rely on phagocytosis as an uptake mechanism (Kuhn et al., 2014), whilst neurons appear to rely on clathrin-mediated endocytosis, at least for uptake of antibodies (Congdon et al., 2013). This is supported by the inability of CytoD to prevent exosome uptake into SH-SY5Y and the finding that endocytosis inhibitors CPZ and genistein were effective in blocking exosome uptake (Figure 5-5). This indicated that exosomes were taken up through endocytosis into SH-SY5Y, but since both the clathrin-mediated endocytosis and the caveolin-mediated endocytosis inhibitor blocked exosome uptake effectively, it was not possible to further narrow down the uptake mechanism. Inhibitors for an endocytosis pathway, such as clathrin-mediated or caveolin-mediated, can also impact other endocytic uptake mechanisms (Ivanov, 2008), but this confirms previous studies which suggested that neurons primarily use endocytosis as an uptake mechanism (Congdon et al., 2013).
5.4.2 Cell death and stress

Investigating both cell viability and intracellular stress pathways in SH-SY5Y treated with exosomes revealed several interesting trends. First, SH-SY5Y were found to be relatively robust, showing few changes in stress levels when in an undifferentiated state (Figure 5-10). In a differentiated state, functional effects of different exosomal contents were revealed following treatment of iPS-Mg. Proteomics revealed that upon iPS-Mg treatment, protein packaging into exosomes changes dramatically (Chapter 4.3.5). For the subtle differences between Cv and R47H\textsuperscript{het} exosomes at baseline, and the effect they have on neurons, differentiated SH-SY5Y were further stressed with H\textsubscript{2}O\textsubscript{2} to assess the potential of exosomes to rescue the cells from the induced cell stress. This was based on a previous study, showing that glial exosomes containing ApoD can rescue SH-SY5Y experiencing oxidative stress, such as a H\textsubscript{2}O\textsubscript{2} challenge (Pascua-Maestro \textit{et al.}, 2019). Next to the R47H\textsuperscript{het} variant, the effect of exosomes from iPS-Mg carrying the T66M\textsuperscript{hom} and the R47H\textsuperscript{hom} variants was also analysed on rescuing SH-SY5Y from H\textsubscript{2}O\textsubscript{2}-induced stress. This showed that the Cv exosomes were unique in rescuing the SH-SY5Y and that any of the tested \textit{TREM2} variants proved unable to rescue the cells (Figure 5-7C).

Whilst exosomes from LPS-treated iPS-Mg did not affect neuronal viability (Figure 5-6B), independent of \textit{TREM2} variants, the exosomes appeared to influence cell stress levels, in particular ER stress (Figure 5-11). Exosomes from LPS-activated iPS-Mg, both carrying Cv and R47H\textsuperscript{het} \textit{TREM2} variants, decreased downstream CHOP, whilst only exosomes from LPS-treated Cv iPS-Mg decreased PERK (Figure 5-11). This could be a protective mechanism, as a previous study showed that a PERK KO, and through this reductions in CHOP, reduced cell death in the cells following insults (Verfaillie \textit{et al.}, 2012).

This suggested that the changes in exosomal proteins, such as the increases in inflammatory cytokines within the exosomes, did not affect viability significantly but only intracellular stress levels, when SH-SY5Y were exposed to LPS-treated Cv exosomes. R47H\textsuperscript{het} exosomes on the other hand, displayed a more subdued response when they were extracted from LPS-treated iPS-Mg, with R47H\textsuperscript{het} already decreasing PERK levels before LPS treatment (Figure 5-11Bii). This matched the finding of the LC-MS analysis, which showed that at baseline R47H\textsuperscript{het} exosomes already closely resembled exosomes from LPS-activated iPS-Mg (Figure 4-11 and Figure 4-13).
However, exosomes originating from iPS-Mg treated with apoptotic neurons did increase cell death in SH-SY5Y both at baseline (Figure 5-6) and following H$_2$O$_2$ cell stress (Figure 5-8). This was seen in both Cv and T66M$^{hom}$ iPS-Mg, but not in exosomes originating from R47H$^{het}$ iPS-Mg. The fact that the R47H$^{het}$ exosomes already caused an increase in cell death, when the SH-SY5Y were stressed with H$_2$O$_2$ and the bigger error bars could suggest that exosomes from R47H$^{het}$ iPS-Mg treated with apoptotic neurons could indeed be toxic to SH-SY5Y and that this effect is only masked. However, the fact that neither unstressed SH-SY5Y nor H$_2$O$_2$-stressed SH-SY5Y are not increasingly killed with exosomes from R47H$^{het}$ iPS-Mg treated with apoptotic neurons suggests that this is a true effect and not a statistical error.

Exosomes from Cv iPS-Mg treated with apoptotic neurons were toxic to SH-SY5Y, leading to an increase in cell death both in unstressed and H$_2$O$_2$-stressed cells. This observation implies that a self-perpetuating cycle may occur in the context of neuronal injury, with dead neurons inducing microglia to release exosomes toxic to neurons, which subsequently die and stimulate the microglia further. In contrast to R47H$^{het}$ exosomes, exosomes from iPS-Mg carrying the T66M$^{hom}$ variant have also been shown to be toxic to SH-SY5Y following exposure to apoptotic neurons.

5.4.3 Exosomes as autocrine microglial communication pathway

Due to the presence of cytokines identified in the exosomes extracted from iPS-Mg exposed to LPS (Chapter 4.3.4), the effect of exosomes on communicating inflammation to downstream resting iPS-Mg was tested. This showed that exosomes from LPS-treated iPS-Mg were capable of inducing inflammation in resting iPS-Mg, as characterised by the increased expression of inflammatory genes, $TNF$, $IL1B$ and $IL6$ (Figure 5-12). The upregulation of these genes is in line with microglial responses to LPS (Figure 3-6) (Cosker et al., 2020), however control experiments (Figure 5-12B) indicated that this response is due to exosomal content and not accidental carryover of LPS. Perhaps as expected, exosomes from LPS-treated iPS-Mg induced inflammation but not exosomes from apoptotic neuron-treated iPS-Mg. A recent study has suggested that the ability of EV from an LPS-treated microglia cell line to induce an inflammatory response in resting microglia was down to the packaging of miRNA-146a into the EV (Yang et al., 2020).
Exosomes from LPS-treated R47H<sup>het</sup> iPS-Mg induced a lower TNF response in R47H<sup>het</sup> iPS-Mg than exosomes from LPS-treated Cv iPS-Mg did in Cv iPS-Mg (Figure 5-12Ai). Interestingly, exosomes from R47H<sup>het</sup> iPS-Mg treated with apoptotic neurons were also able to induce an increase in TNF expression (Figure 5-12Ai). This could be due to differences in exosomal content, as proteomic analysis has suggested that exosomes from R47H<sup>het</sup> iPS-Mg treated with apoptotic neurons differed from their Cv counterparts in terms of energy pathways (Figure 4-12Bii). A previous study has shown a reduced TNF-α secretion from LPS/IFN-γ treated R47H<sup>het</sup> iPS-Mg, linking this to metabolic deficits associated with this TREM2 variant (Piers et al., 2020). This would suggest that the decrease in TNF levels in R47H<sup>het</sup> iPS-Mg exposed to LPS exosomes (Figure 5-12Ai) could be due to the reduced ability of the R47H<sup>het</sup> iPS-Mg to respond to the exosomes, rather than intrinsic changes in the content of the R47H<sup>het</sup> exosomes. However, enrichment of proteins linked to energy pathways in exosomes from R47H<sup>het</sup> iPS-Mg treated with apoptotic neurons (Figure 4-12Bii) could potentially supply R47H<sup>het</sup> iPS-Mg with the needed energy to launch an inflammatory response, as seen with the increase in TNF in response to these exosomes (Figure 5-12Ai). This hypothesis however would require further testing, such as supplying R47H<sup>het</sup> iPS-Mg with other sources of energy or testing the effect of R47H<sup>het</sup> LPS exosomes on Cv iPS-Mg to be able to differentiate between cell specific and exosome specific effects.

Overall, the results suggest that in addition to having an effect on neurons, microglial exosomes can also participate in inter-microglial communication, sending signals of inflammation to other microglia.

### 5.4.4 Metabolism

Proteomic analysis of exosomal content has revealed that exosomes from R47H<sup>het</sup> iPS-Mg increasingly contained proteins involved in the negative regulation of metabolism (Chapter 4.3.3) and especially pre-treatment of iPS-Mg with apoptotic neurons increased the contribution of metabolic proteins in the exosomal proteome (Chapter 4.3.5). To better understand the effect of these changes on the metabolism of neuron-like cells, several aspects of metabolism were analysed.

NFκB has been shown to be a link between inflammation and metabolism to coordinate proliferation during immunity and inflammation. Studies have shown that NFκB phosphorylation controls the balance between glycolysis and mitochondrial
respiration (Mauro et al., 2011; Tornatore et al., 2012). However, no difference in NFκB phosphorylation was observed (Figure 5-13Aii).

MTT assays can be used to assess cell viability, but through confirming limited changes in cell viability (Figure 5-6) and cell proliferation (Figure 5-3) and by normalising the MTT assay to total cell numbers, the MTT assay was used as a readout of metabolic activity of the cells. This showed that exosomes from resting iPS-Mg could increase metabolic activity of SH-SY5Y, in particular exosomes from Cv iPS-Mg (Figure 5-13Bi). Furthermore, exosomes from Cv iPS-Mg treated with apoptotic neurons were able to increase the metabolic activity of SH-SY5Y, in particular in comparison to exosomes from apop treated R47H\(^{het}\) iPS-Mg (Figure 5-13Bii). The changes observed through the MTT assay could be down to a range of different metabolic processes changing within the SH-SY5Y and to narrow this plethora of metabolic processes, the ATP levels in the SH-SY5Y were also measured to gain an understanding of mitochondrial function (Figure 5-13). The changes in the ATP levels in SH-SY5Y closely follow the changes observed in the MTT assay, with some small differences. Similar to the MTT assay, exosomes from baseline iPS-Mg increased ATP levels, with the effect being stronger for Cv exosomes. The fact that R47H\(^{het}\) exosomes did not induce lower levels of both ATP levels but also of MTT metabolism suggests that this could be a true effect, since it was observed in two different assays testing different metabolic aspects. Therefore, at baseline R47H\(^{het}\) exosomes may be less able to induce increases in SH-SY5Y metabolism, but this subtle effect should be further verified. The TREM2 specific effect became more obvious when the exosomes are extracted from iPS-Mg treated with either LPS or apoptotic neurons. Exosomes from apoptotic neuron-treated Cv iPS-Mg induced significantly higher ATP levels, which is also reflected in the MTT assay, than exosomes from apoptotic neuron-treated R47H\(^{het}\) iPS-Mg (Figure 5-13Cii). Interestingly, this effect is also observed in exosomes from LPS-treated iPS-Mg, with Cv LPS exosomes inducing higher ATP levels than R47H\(^{het}\) exosomes.

PGC-1\(\alpha\) levels were assessed in SH-SY5Y following exposure to exosomes from Cv and R47H\(^{het}\) iPS-Mg (Figure 5-14) to verify that the increase in metabolic activity following exposure to Cv exosomes (Figure 5-13) was due to increased mitochondrial numbers. In response to Cv exosomes, total PGC-1\(\alpha\) levels were significantly increased in SH-SY5Y (Figure 5-14Bii), a trend that was mirrored as an increased expression of PPARGC, encoding for PGC-\(\alpha\) (Figure 5-14Ci). This increase in PGC-1\(\alpha\) in SH-SY5Y exposed to Cv exosomes was not mirrored by an increase in
phosphorylation of the protein at the S571 site (Figure 5-14Bi), suggesting that the increased PGC-1α appeared to be functionally active. On the other hand, increase in PGC-1α phosphorylation in response to the R47H<sup>het</sup> exosomes (Figure 5-14Bi) suggested that in addition too not inducing higher total protein levels, the available protein becomes increasingly inactive.

Next to PPARGC, the expression of HSPD1, encoding for HSP60, was also increased in SH-SY5Y exposed to Cv exosomes. In the past, HSP60 has been shown to protect SH-SY5Y from stress by maintaining ATP production (Veereshwarayya et al., 2006). To verify these results in a more neuron-like model, iPS-neurons were also exposed to iPS-Mg exosomes and changes in mitochondrial function was assessed. MAPT was not altered by exosomes (Figure 5-15A), which could indicate that neuronal numbers themselves are not altered by this treatment. Expression of PPARGC was increased in iPS-neurons exposed to Cv exosomes (Figure 5-15Bi), supporting the SH-SY5Y findings (Figure 5-14Ci), whilst R47H<sup>het</sup> exosomes also induced PPARGC upregulation in iPS-neurons (Figure 5-15Bi). However, both NRF1 and HSPD1, encoding for HSP60, were selectively increased with R47H<sup>het</sup> exosomes, indicating differences between Cv and R47H<sup>het</sup> exosomes in inducing changes in mitochondrial functions. NRF1 has been linked to both mitochondrial biogenesis and proliferation of neuronal progenitor cells (Satoh et al., 2013; Kiyama et al., 2018).

SH-SY5Y and iPS-neurons appear to react slightly differently to R47H<sup>het</sup> exosomes in terms of the changes observed in metabolic markers, such as the expression PPARGC, NRF1 and HSPD1 (Figure 5-14 and Figure 5-15). This could be explained with differences between the two different models used, with iPS-neurons being more sensitive than the neuroblastoma SH-SY5Y line. Additionally, iPS-neurons are also surrounded by other glia cells (Shi et al., 2012), with which the exosomes could also interact with. As the functional readouts for iPS-neurons, namely the ATP and MTT assays, were not performed, due to scarcity of the cells, it could not be verified whether the differences in gene expression translated into differences in ATP production. However, PPARGC upregulation was seen in both models after exposure to Cv exosomes, suggesting that the increase in mitochondrial biogenesis is indeed a real effect.

As the number of exosomes added to both SH-SY5Y and iPS-neurons were normalised to exosomal protein content, accounting for differences in exosome secretion following different treatments, this effect is thought to be independent of
secretion rate but rather down to differences in exosomal content. These findings were in line with the proteomic analysis which suggested that exosomes from baseline iPS-Mg could induce changes in energy production and that following treatment of iPS-Mg with apoptotic neurons, exosomes increasingly contain proteins involved in metabolism and energy production but that there were differences concerning this between Cv and R47H<sup>het</sup> exosomes (Figure 4-12, Figure 4-14 and Figure 4-16).

### 5.4.5 Specific neuronal functions: development and synapses

Exosomes have been reported to support neurite outgrowth (Huang <i>et al.</i>, 2018; Lemaire <i>et al.</i>, 2019; Murgoci <i>et al.</i>, 2020), in line with the proteomic analysis, suggesting differences in both synaptic pruning and neurite outgrowth being dependent on <i>TREM2</i> status and iPS-Mg treatment (Chapter 4.3.7.1). Both functions were subsequently investigated.

Following exposure to Cv exosomes, total neurite length, labelled through Tubb3, significantly increased in iPS-neurons in comparison with both untreated and R47H<sup>het</sup> exosome treated neurons (Figure 5-16). In addition to increased outgrowth, iPS-neurons exposed to Cv exosomes also displayed higher levels of GAP43 staining (Figure 5-17), a protein linked to neurite outgrowth and growth cone development. These results suggest that exosomes from Cv iPS-Mg were in the unique position to support and induce neuronal outgrowth, whilst exosomes from R47H<sup>het</sup> iPS-Mg did not display similar abilities.

Synaptic functioning was also investigated in SH-SY5Y exposed to exosomes. Whilst synaptophysin levels, indicative of the number of synapses (Li <i>et al.</i>, 2010; Jiang <i>et al.</i>, 2015), were unchanged (Figure 5-18Aii), analysis of phosphorylation status of PSD-95 revealed a <i>TREM2</i> dependent effect, with both T19 and S295 phosphorylation being decreased in SH-SY5Y treated with exosomes from R47H<sup>het</sup> iPS-Mg (Figure 5-18Cii, Dii). Overall, this could indicate overall lower levels of PSD-95 in SH-SY5Y treated with exosomes from R47H<sup>het</sup> iPS-Mg. This partially matched the previous findings showing reduced neurite outgrowth in iPS-neurons exposed to R47H<sup>het</sup> exosomes in comparison with Cv exosomes (Figure 5-16 and Figure 5-17).

For a more functional readout, the responses of VGCC on SH-SY5Y were measured. SH-SY5Y were shown to express both L-type and N-type VGCC (Sousa <i>et al.</i>, 2013; Wu <i>et al.</i>, 2013). In these experiments, the L-type VGCC inhibitor NIF,
showed no effect indicating that changes observed were due to the N-type VGCC (Figure 5-19). Two time points were used to gain a better understanding of the effect exosomes have on the functionality of VGCC, representing extremes of a spectrum. After SH-SY5Y were only exposed to exosomes for 30s, exosomes originating from LPS-treated iPS-Mg led to an increased reaction (Figure 5-19Aii) independent of TREM2 status of the iPS-Mg. This showed the speed at which exosomes can either bind to receptors on the SH-SY5Y or even deposit their content. However, this timeframe was chosen, since it was too short for the exosomal proteins to induce downstream signalling cascades. Another reason why this short timeframe was chosen was that it allowed for the imaging of the neuronal response to exosome addition, which revealed that intracellular calcium did not change. This suggested that the signalling pathway through which exosomes influence the functionality of VGCC is calcium-independent.

Incubation of SH-SY5Y with exosomes for a prolonged period, allowing signalling pathways to be induced, revealed differences between Cv and R47H\textsuperscript{het} exosomes (Figure 5-19B). Cv exosomes induced a subdued VGCC response (Figure 5-19Bii), which was shown to increase when the Cv iPS-Mg were treated with LPS prior to exosome collection. The same trend, however, was not seen with R47H\textsuperscript{het} exosomes, potentially because the VGCC response was already increased in SH-SY5Y exposed to these exosomes (Figure 5-19Biii).

N-type VGCC can be found presynaptically and are implicated in the synaptic transmission in the hippocampus (Su \textit{et al.}, 2012) but also in neurite outgrowth (Sann \textit{et al.}, 2008). With Ca\textsubscript{v} 2.2 inhibition being shown to increase the number of nerve terminals (Sann \textit{et al.}, 2008), decreases in VGCC-mediated calcium influx induced by Cv exosomes (Figure 5-19Bii) could increase the number of synapses in comparison to R47H\textsuperscript{het} exosomes, which was also suggested by decreased PSD-95 levels (Figure 5-18Cii, Dii), reductions of neurite length (Figure 5-16) and lower GAP43 staining (Figure 5-17) in response to R47H\textsuperscript{het} exosomes in comparison with Cv exosomes.

\textbf{5.4.6 Future experiments}

There are a range of experiments that could be conducted to increase the robustness of the data in this part of the project.
As a model for neuron-like cells, differentiated SH-SY5Y were primarily used throughout this chapter. In particular in regards to the synaptic function experiments, this model may not be able to fully recapitulate all neuronal functions. Therefore, repetition of some of these experiments, like analysis of PSD-95 phosphorylation status and VGCC activity, using a more neuron-like model, such as iPS-neurons, would be highly beneficial.

The experiments described in this chapter here focussed on exosomes from untreated iPS-Mg and exosomes from LPS-treated iPS-Mg, but as the LC-MS analysis revealed that specifically exosomes from iPS-Mg treated with apoptotic neurons could influence synapses, the question remains what effect these exosomes have on these measures of synaptic functioning. This goes in particular for the analysis of neuronal functions, in particular VGCC analysis.

5.4.7 Conclusion

In conclusion, this work sought to link the findings of the proteomic analysis with functional readouts in neuron-like cells to elucidate the role exosomes play in microglia-neuron communication. After confirmation that exosomes are indeed taken up by neuron-like cells through endocytosis, exosomes from disease-associated TREM2 variants and exosomes from iPS-Mg treated with apoptotic neurons were less capable of maintaining neuronal viability. R47H\textsuperscript{het} exosomes specifically reduces ER stress proteins, similar to exosomes from LPS-treated iPS-Mg, supporting previous findings highlighting the similarity of the exosomal proteome in these different conditions. Proteomic analysis also suggested that exosomes could influence metabolic activity, and here specifically exosomes from Cv iPS-Mg were shown to increase ATP availability through changes in mitochondrial biogenesis. Cv exosomes also proved capable of inducing neurite outgrowth in iPS-neurons. Finally, exosomes from iPS-Mg were also capable of modulating the synaptic response in neuron-like cells (summarised in Figure 5-21).

Overall, based on proteomic analysis, a range of different neuronal functions and the effect of exosomes on them were tested. As a general trend, exosomes influenced cell viability, ER stress pathways, metabolic activity, neurite outgrowth and synaptic functioning, all aspects that were implicated in the previous proteomic analysis. Intriguingly, exosomes were also shown to function as an autocrine
signalling pathway between microglia. Overall, Cv exosomes appear to maintain and support a range of neuronal functions better than R47H$^\text{het}$ exosomes.
Figure 5.21 Summarising diagram of functional effect of exosomes on neighbouring cells

Based on the proteomic analysis, the previous diagram (Figure 4-26) was expanded to summarise the effects exosomes from iPS-Mg, having received a range of different stimuli, were shown to have on neighbouring cells, such as neuron-like cells and other iPS-Mg.
Microglia were first defined as the immune cells of the brain, but the range of functions they fulfil in health and disease in the CNS reveal them to be more than just immune cells, capable of initiating complex responses to a range of different, specific stimuli. Understanding how the plethora of microglial functions interact with different cell types in the CNS is important to understand when studying diseases during which this interaction may be disrupted, such as in AD. Alois Alzheimer already noted abnormal glial pathology in the brain of Auguste Deter (Alzheimer, 1906) and since then, microglia have been implicated in both disease progression and even initiation. Whilst some microglial functions, such as the secretion of inflammatory cytokines, have been thoroughly studied, others, such as clustering around Aβ plaques, are less well understood.

One protein that appears to regulate a whole host of these functions is TREM2. TREM2 has been implicated in energy metabolism, cytoskeleton rearrangement, phagocytosis, migration and mounting an inflammatory response (Glebov et al., 2016; Ulland et al., 2017; Garcia-Reitboeck et al., 2018; Phillips et al., 2018; Nugent et al., 2020; Piers et al., 2020). Given the involvement of TREM2 in these many microglial functions, it is perhaps not surprising that TREM2 variants were identified to increase the risk of developing AD (Guerreiro et al., 2013a; Jonsson et al., 2013), and other neurodegenerative diseases. TREM2 deficits, caused even by reduction of protein binding as in the R47Hhet variant (Kober et al., 2016; Sudom et al., 2018), have shown to disrupt a range of TREM2-related functions. Which one of these functions contributes to the risk of developing AD is not known yet and is difficult to test, since these microglial functions are closely related with one another. Microglial migration to a site of injury is dependent on cytoskeletal rearrangement (Ohsawa et al., 2004; Phillips et al., 2018; Franco-Bocanegra et al., 2019) and so is the phagocytosis of particles at the site of injury (Ohsawa et al., 2004; Kanada et al., 2015; Franco-Bocanegra et al., 2019). Deficits in energy metabolism can impact energy-demanding functions, such as phagocytosis or initiation of an inflammatory response (Piers et al., 2020). Even slight perturbations, either in the microglia through the R47Hhet TREM2 variant, or in the environment, through initial AD-like pathology, can disrupt this highly sensitive network of interactions between microglia, their environment and other cell types.
This thesis provided a novel insight into another microglial function TREM2 can influence, the secretion of exosomes. In this project, exosomes from iPS-Mg carrying the R47H^{het}, T66M^{hom} or R47H^{hom} TREM2 variants were extracted and analysed. At baseline, disease-related TREM2 variants appear to secrete less exosomes, which could be explained by the energy deficit these cells experience.

Independent of deficits in exosome secretion, changes in exosomal content were also identified. At baseline, differences between exosomes from Cv and R47H^{het} TREM2 iPS-Mg suggest differences in the effect these particles have on the functioning of other cells. Cv exosomes displayed a shift in protein content specific to the stimuli iPS-Mg received, whilst this shift was altered in R47H^{het} exosomes, partially as they already resembled exosomes originating from LPS-treated iPS-Mg.

These proteomic differences, both at baseline and following specific treatments, were shown to be functionally relevant. They can affect a range of cellular functions, such as cell death and stress pathways. As exosomes are believed to be an intercellular communication pathway, they have also been shown to affect specific neuronal functions, such as synaptic transmission, and mitochondrial function, a process very important to neurons. In addition to this, exosomes from activated iPS-Mg have been shown to have the potential to activate other iPS-Mg working also as an autocrine communication pathway.

In order to probe the exosomes from microglia cells, a protocol to generate microglia-like cells from iPSC was developed. This was important to accurately model human microglia cells and the specific effects of their exosomes. Difficulties accessing human tissue, the differences between human and murine microglia (Galatro et al., 2017; Gosselin et al., 2017; Zhou et al., 2020) and the quick loss of in vivo microglial properties of primary cells in vitro (Gosselin et al., 2017), in combination with knowledge of the developmental pathway of microglia (Ginhoux et al., 2010) made iPSC an ideal model for human microglia. This protocol has been used to study the effect of a range of TREM2 variants on microglial functions and can be used in a wide range of in vitro studies (Xiang et al., 2018; Cosker et al., 2020; Piers et al., 2020).

Studying microglia, and the effect of TREM2, is particularly important for understanding early disease processes of AD. Treatments for AD so far only temporarily reverses some of the symptoms without targeting the underlying pathology and clinical trials targeting AD pathology so far have proven unsuccessful
(Cummings et al., 2019). This suggests that targeting pathological progression of the disease (that is potentially already far advanced) is not beneficial, whilst treatments targeting disease initiation or early disease processes could be more effective. In order to develop these, early disease processes need to be properly understood. This is where in vitro basic research using human samples becomes important to lay the foundation of knowledge and understanding upon which one can build potential treatments.

One important aspect of understanding AD disease initiation, particularly in regards to the role exosomes play, is the interaction between different cell types. Neurons do not exist in isolation in the brain and their functionality is easily modulated by other cell types, either through direct contact (Stevens et al., 2007; Schafer et al., 2012; Miyamoto et al., 2016; Scott-Hewitt et al., 2020) or secreted factors (Morgan et al., 2004; Mead et al., 2012; Parkhurst et al., 2013; Jebelli et al., 2014; Crotti and Ransohoff, 2016; Colonna and Butovsky, 2017; Liddelow et al., 2017). Therefore, to understand basic disease processes, understanding the interactions between different cell types in the brain is essential. A range of different techniques can be used to study interaction of different cell types, such as exposing cells to conditioned medium, and to all of the different secreted factors from other cells (Kingham et al., 1999; Morgan et al., 2004; Jebelli et al., 2014), using culture inserts to allow cells to share the same medium (Grimaldi et al., 2019) or co-culturing cells together, to also allow for physical interactions between them (Abud et al., 2017; Douvaras et al., 2017). These techniques have revealed that microglia can support neuronal functioning through a range of secreted factors, including EV (Morgan et al., 2004; Antonucci et al., 2012; London et al., 2013; Parkhurst et al., 2013; Grimaldi et al., 2019).

Whilst EV were originally believed to be a pathway through which cells can dispose of waste and unwanted proteins, this view has shifted to appreciate the specific packaging and enrichment of certain proteins and other molecules in these particles (Hawari et al., 2004; Graner et al., 2009; Mathivanan et al., 2010; Gupta and Pulliam, 2014; Record et al., 2014; Baranyai et al., 2015). As the field was emerging, nomenclature was employed to classify the vesicles based on their secretion pathway and to establish markers that can be used to identify them (Witwer et al., 2013; Andreu and Yáñez-Mó, 2014; Lötvall et al., 2014; Villarroya-Beltri et al., 2016; Colombo et al., 2018). However, as the field progressed, limitations to the current nomenclature became increasingly obvious. Different EV extraction protocols have different
efficiencies for isolating different EV types, whilst experimental setup heavily influences the extraction protocol as well (Lötvall et al., 2014; Skotland et al., 2017). Despite EV classification based on secretion type, often other characteristics, such as size or presence of specific proteins, are used by researchers to classify the isolated EV, as reliable, specific inhibitors for each secretion pathway are yet to be developed. This mismatch between theory and practice could lead to EV being misclassified, particularly since cells appear to secrete different types of EV concurrently, which makes differentiating between the different types even more difficult. Different studies have either classified their EV as exosomes based on different size criteria (Mears et al., 2004; Bellingham et al., 2012; Properzi et al., 2015; Hessvik and Llorente, 2018; Mathieu et al., 2019), which ultimately begs the question whether the distinction between exosomes and MV is a useful one. New terminology, with robust positive and negative markers, would ensure comparable and reproducible results in the field. In this project, based on the size criteria and the presence of classical markers, the extracted vesicles were classified as exosomes, however some of the classical exosomal markers can also be found in MV, such as flotillin-1 (Bianco et al., 2005; Kowal et al., 2016; Hessvik and Llorente, 2018), and negative markers were also identified through LC-MS.

Overall, in this project, I developed an optimised protocol to enable the research on human microglia cells and have used this technique to characterise the effect of disease-relevant TREM2 variants on exosome secretion, content and the potential effect this can have on neighbouring neurons.
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Appendix

7.1 Code used in MATLAB for LC-MS analysis

```matlab
%set up
%import ProteinsCleaned as table and Protein names as string array
%sort into the right order:
ProteinsCleaned = movevars(ProteinsCleaned,
'AbundancesGroupedCv2', 'Before', 'AbundancesGroupedCvLPS1');
ProteinsCleaned = movevars(ProteinsCleaned,
'AbundancesGroupedCvLPS2', 'Before',
'AbundancesGroupedCvapop1');
ProteinsCleaned = movevars(ProteinsCleaned,
'AbundancesGroupedCvapop2', 'Before',
'AbundancesGroupedR47H1');
ProteinsCleaned = movevars(ProteinsCleaned,
'AbundancesGroupedCv3', 'Before', 'AbundancesGroupedCvLPS1');
ProteinsCleaned = movevars(ProteinsCleaned,
'AbundancesGroupedCvLPS3', 'Before',
'AbundancesGroupedCvapop1');
ProteinsCleaned = movevars(ProteinsCleaned,
'AbundancesGroupedR47H2', 'Before',
'AbundancesGroupedR47HLPS1');
ProteinsCleaned = movevars(ProteinsCleaned,
'AbundancesGroupedCvapop3', 'Before',
'AbundancesGroupedR47H1');
ProteinsCleaned = movevars(ProteinsCleaned,
'AbundancesGroupedR47HLPS2', 'Before',
'AbundancesGroupedR47Hapop1');
ProteinsCleaned = movevars(ProteinsCleaned,
'AbundancesGroupedR47H3', 'Before',
'AbundancesGroupedR47HLPS1');
ProteinsCleaned = movevars(ProteinsCleaned,
'AbundancesGroupedR47HLPS3', 'Before',
'AbundancesGroupedR47Hapop1');

%Log transformation
array = table2array(ProteinsCleaned);
arrayadded = array + 1;
transformed = log10(arrayadded);

%Generate histogram
Histogram(transformed);

% Calculating the averages of the different conditions
T = array2table(transformed);
T.Properties.RowNames = Accession;
T.Properties.VariableNames = {'Cv1', 'Cv2', 'Cv3', 'Cv LPS 1', 'Cv LPS 2', 'Cv LPS 3', 'Cv apop 1', 'Cv apop 2', 'Cv apop 3', 'R47H1',
'R47H2', 'R47H3', 'R47H LPS 1', 'R47H LPS 2', 'R47H LPS 3', 'R47H apop 1', 'R47H apop 2', 'R47H apop 3'};
```
%saving the averages and the three replicates for each condition in individual files
T.CvAvg = mean(T(:,1:3),2);
T.CvLPSAvg = mean(T(:,4:6),2);
T.CvapopAvg = mean(T(:,7:9),2);
T.R47HAvg = mean(T(:,10:12),2);
T.R47HLPSAvg = mean(T(:,13:15),2);
T.R47HapopAvg = mean(T(:,16:18),2);
TCv = T(:,1:3),2;
TCvLPS = T(:,4:6),2;
TR47H = T(:,10:12),2;
TR47HLPS = T(:,13:15),2;
TCvAvg = mean(T(:,1:3),2);
TCvLPSAvg = mean(T(:,4:6),2);
TR47HAvg = mean(T(:,10:12),2);
TR47HLPSAvg = mean(T(:,13:15),2);
TCvapop = T(:,7:9),2;
TR47Hapop = T(:,16:18),2;
TCvapopAvg = mean(T(:,7:9),2);
TR47HapopAvg = mean(T(:,16:18),2);
Tav = T(:,19:24); %making a new table of just the averages for each condition

%Generate heatmaps
M = table2array(DataNorm);
h = heatmap(M);
h.GridVisible = 'off'
h.ColorScaling = 'scaled';
h.Colormap = hot;
h.XDisplayLabels = {'Cv1','Cv2', 'Cv3', 'Cv LPS 1', 'Cv LPS 2', 'Cv LPS 3', 'Cv apop 1', 'Cv apop 2', 'Cv apop 3', 'R47H1', 'R47H2', 'R47H3', 'R47H LPS 1', 'R47H LPS 2', 'R47H LPS 3', 'R47H apop 1', 'R47H apop 2', 'R47H apop 3'};

%clustergram of all the different samples
cg_s = clustergram(DataNormTrans, 'RowLabels', Proteins, 'ColumnLabels', samples, 'Colormap', hot);
cgAxes = plot(cg_s); % Use the plot function to plot to a separate figure and output the axes
set(cgAxes, 'Clim', [0,2]);

%clustergram of averages
Avg = table2array(Tav);
samples = ["Cv", "Cv LPS", "Cv apop", "R47H", "R47H LPS", "R47H apop"];
cg_s = clustergram(Avg, 'RowLabels', Accession, 'ColumnLabels', samples, 'Colormap', hot);
cgAxes = plot(cg_s); % Use the plot function to plot to a separate figure and output the axes
set(cgAxes, 'Clim', [0,1.5]) % Set colour limit or other axes properties.

%Generate PCA
samples = ["Cv1", "Cv2", "Cv3", "CvLPS1", "CvLPS2", "CvLPS3", 
"Cvapop1", "Cvapop2", "Cvapop3", "R47H1", "R47H2", "R47H3", 
"R47HLPS1", "R47HLPS2", "R47HLPS3", "R47Hapop1", "R47Hapop2", 
"R47Hapop3"];
mapcaplot(transformed, Proteins)

%Generate the volcano plots for the different conditions, 
input the condition of interest first and then the reference 
condition
%Accession = accession value of the identified proteins
PValues = mattest(TR47Hapop, TR47H);
LogTransValue = true;
mavolcanoplot(TR47HapopAvg, TR47HAvg, PValues, 'Labels', Accession, 
'LogTrans', true);

%PCA analysis
%following the set up and log transformation code, the array T 
of the data is used
TranssTrans = T'; %Transposing of the table T so that the 
different rows represent the samples and column1 contains 
abundance of protein1 and column2 contains abundance of 
protein2 for the different samples
S = samples'; %transposing the samples
mapcaplot(TransfTrans, S) %to generate the interactive PCA 
plots

%to plot the first and third principle component. To generate 
a plot of the first and second, the second variable is changed 
to ,2.
sz = 25;
[pc, zscores, pcvars] = pca (TransfTrans);
hold on
scatter(zscores (1:3,1), zscores (1:3,3), sz, [1 0 0], 
'filled')
scatter(zscores (4:6,1), zscores (4:6,3), sz, [0.41 0.84 
0.41], 'filled')
scatter(zscores (7:9,1), zscores (7:9,3), sz, [0.6 0.8 1], 
'filled')
scatter(zscores (10:12,1), zscores (10:12,3), sz, [0.99 0.63 
0.08], 'filled')
scatter(zscores (13:15,1), zscores (13:15,3), sz, [0.79 0.47 
0.99], 'filled')
scatter(zscores (16:18,1), zscores (16:18,3), sz, [0 0 1], 
'filled')
hold off
legend ('Cv', 'Cv LPS', 'Cv apop', 'R47H', 'R47H LPS', 'R47H 
apop', 'Location', 'southeast')
xlabel ('First Principle Component')
ylabel ('Third Principle Component')
title ('PCA analysis')

348
7.2 Code used for ProCoNA analysis in RStudio

```r
# Set up
setwd("\\\ad.ucl.ac.uk/home3/ucbtak3/Documents/PhD_Exosomes/4th Year/Mass Spec/Renormalized 2.0/WGCNA")
getwd()
workingDir = "." 
setwd(workingDir)
library(WGCNA)
options(stringsAsFactors = FALSE)

# Run Log-transformed data and use LPS and apop treatment as traits
# use Cv as example here
Data = read.csv("LogSamples.csv")
datExpr = as.data.frame(t(Data[, -c(1)]))
names(datExpr) = Data$Accession
rownames(datExpr) = names(Data)[-c(1)]
nGenes = ncol(datExpr)
nSamples = nrow(datExpr)

traitData = read.csv("Treatments.csv")
alltraits = traitData
Samples = rownames(datExpr)
traitRows = match(Samples, alltraits$Sample)
datTraits = alltraits[traitRows, -1]
rownames(datTraits) = alltraits[traitRows, 1]
collectGarbage()

# cluster the samples to visualise how "clinical traits" relate to sample dendrogram
sampleTree2 = hclust(dist(datExpr), method = "average")
traitColors = numbers2colors(datTraits, signed = FALSE)
plotDendroAndColors(sampleTree2, traitColors, groupLabels = names(datTraits), main = "Sample dendrogram and treatment heatmap")

# save everything for the next steps
save(datExpr, datTraits, file = "AllSamples_log_traits_ALL_01-dataInput.RData")

# build network
lnames = load(file = "AllSamples_log_traits_01-dataInput.RData")
lnames

# choose soft thresholding power
powers = c(c(1:10), seq(from=12, to=30, by=2))
```
sft = pickSoftThreshold(datExpr, NetworkType = "signed", powerVector = powers, verbose = 5)

sizeGrWindow(9,5)
par(mfrow = c(1,2))
cex1 = 0.9
plot(sft$fitIndices[,1], -sign(sft$fitIndices[,3])*sft$fitIndices[,2], xlab="Soft Threshold(power)", ylab="Scale Free Topology Model Fit, signed R^2", type="n", main = paste("Scale independence"))
text(sft$fitIndices[,1], -sign(sft$fitIndices[,3])*sft$fitIndices[,2], labels=powers, cex=cex1, col="red")
abline(h=0.9,col="red")

plot(sft$fitIndices[,1], sft$fitIndices[,5], xlab="Soft Threshold (power)", ylab="Mean Connectivity", type="n", main = paste("Mean connectivity"))
text(sft$fitIndices[,1], sft$fitIndices[,5], labels=powers, cex=cex1, col="red")

# input the power determined in the previous step here in
power = net = blockwiseModules(datExpr, power = 20, NetworkType = "signed", TOMType = "unsigned", minModuleSize = 30, reassignThreshold = 0, mergeCutHeight = 0.25, numericLabels = TRUE, pamRespectsDendro = FALSE, saveTOMs = TRUE, saveTOMFileBase = "AllTOM", verbose = 3)
table(net$colors)
mergedColors = labels2colors(net$colors)
plotDendroAndColors(net$dendrograms[[1]], mergedColors[net$blockGenes[[1]]], "Module colors", dendroLabels = FALSE, hang = 0.03, addGuide = TRUE, guideHang = 0.05)

moduleLables = net$colors
moduleColors = labels2colors(net$colors)
MEs = net$MEs
geneTree = net$dendrograms[[1]]
save(MEs, moduleLables, moduleColors, geneTree, file = "AllSamples_log_traits-02-networkConstruction.RData")

# plot dendrogram of eigengenes
MET = orderMEs(MEs)
par(cex = 0.9)
plotEigengeneNetworks(MET, "", marDendro = c(0, 4, 1, 2), marHeatmap = c(3, 4, 1, 2), cex.lab = 0.8, xLabelsAngle = 90)
plotEigengeneNetworks(MET, "Eigengene dendrogram Cv LPS signed", marDendro = c(0, 4, 2, 0), plotHeatmaps = FALSE)

# link the module genes with functions
lnames = load(file = "Cv_log_traits_NT_01-dataInput.RData")
lnames
lnames = load(file = "Cv_log_traits-02-networkConstruction.RData")
lnames

annot = read.csv(file = "Accession_ENTREZID.csv")
probes = names(datExpr)
probes2annot = match(probes, annot$Accession)
allIDs = annot$ENTREZ[probes2annot]
GOenr = GOenrichmentAnalysis(moduleColors, allIDs, organism = "human", nBestP = 10)
tab = GOenr$bestPTerms[[4]]$enrichment
write.table(tab, file = "All samples_all treatments_GOEnrichmentTable_Top10.csv", sep =", ", quote = TRUE, row.names = FALSE)

# Compare 2 different datasets
# set-up
lnames = load(file = "Cv_log_traits-02-networkConstruction.RData")
CvLabels = moduleLables
CvColors = moduleColors
CvTree = geneTree
CvMEs = orderMEs(MEs, greyName = "MEO")

lnames = load(file = "R47H_log_traits-02-networkConstruction.RData")
R47HLabels = moduleLables
R47HColors = moduleColors
R47HTree = geneTree
R47HMEs = orderMEs(MEs, greyName = "MEO")

CvModuleLabels = substring(names(CvMEs), 3)
R47HModuleLabels = substring(names(R47HMEs), 3)

CvModules = labels2colors(as.numeric(CvModuleLabels))
R47HModules = labels2colors(as.numeric(R47HModuleLabels))

nCvMods = length(CvModules)
nR47HMods = length(R47HModules)
pTable = matrix (0, nrow = nCvMods, ncol = nR47HMods)
CountTbl = matrix(0,nrow = nCvMods, ncol = nR47HMods)

for (fmod in 1:nCvMods)
  for (cmod in 1:nR47HMods)
    {
      CvMembers = (CvColors == CvModules[fmod])
      R47HMembers = (R47HColors == R47HModules[cmod])
      pTable[fmod, cmod] = -log10(fisher.test(CvMembers,
                                         R47HMembers, alternative = "greater")$p.value)
      CountTbl[fmod, cmod] = sum(CvColors == CvModules[fmod] &
                                 R47HColors == R47HModules[cmod])
    }
# truncate p values smaller than sth
# pTable[is.infinite(pTable)] = 1.3*max(pTable[is.finite(pTable)])
# pTable[pTable>50] = 50
CvModTotals = apply(CountTbl, 1, sum)
R47HModTotals = apply(CountTbl, 2, sum)
sizeGrWindow(10,7)
par(mfrow = c(1,1))
par(cex = 1.0)
par(mar=c(8, 10.4, 2.7, 1)+0.3)
labeledHeatmap(Matrix = pTable, xLabels = paste(" ",
 R47HModules), yLabels = paste(" ", CvModules), colorLabels =
 TRUE, xSymbols = paste("R47H ", R47HModules, ": ",
 R47HModTotals, sep=""), ySymbols = ", Cv ", Cv Modules ", ": ",
 CvModTotals, sep=""), textMatrix = CountTbl, colors =
 greenWhiteRed(100)[50:100], main = "Correspondence of Cv
 modules to R47H modules", cex.text = 1.0, cex.lab = 1.0,
 setStdMargins = FALSE, keepLegendSpace = FALSE)

#set up Module Preservation analysis
setwd("\\ad.ucl.ac.uk\home3\ucbtak3\Documents\PhD Exosomes/4th Year/Mass Spec/Renormalized 2.0/WGCNA")
getwd()
workingDir = "."
setwd(workingDir)
library(WGCNA)
options(stringsAsFactors = FALSE)

#work with 2 datasets
nsSets = 2

#load CV data
lnames = load(file = "Cv_FINAL-01-dataInput.RData")
lnames
CvData=data.frame(datExpr)

#load R47H data
lnames = load(file = "R47H_FINAL-dataInput.RData")
lnames
R47HData=data.frame(datExpr)

#load Cv module data
lnames = load(file = "Cv_FINAL-02-networkConstruction.RData")
lnames
moduleColorsCv=moduleColors

#load R47H module data
lnames = load(file = "R47H_FINAL-02-
networkConstruction.RData")
lnames
moduleColorsR47H=moduleColors

#set up calculation
#this saves only the Cv module colors
multiExpr = list()
multiExpr[[1]] = list(data = CvData)
multiExpr[[2]] = list(data = R47HData)

setLabels = c("Cv", "R47H")

# this saves both Cv and R47H module colors
colorList = list(moduleColorsCv, moduleColorsR47H)
names(colorList) = setLabels

# Module Preservation
system.time(
    mp = modulePreservation(multiExpr, colorList,
                            referenceNetworks = 1,
                            nPermutations = 200, randomSeed = 1,
                            quickCor = 0, verbose = 3)
)

save(mp, file = "modulePreseveration_Cv.RData")
load(file = "modulePreseveration_Cv.RData")

# Display
ref = 1
test = 2

Obs.PreservationStats = mp$preservation$observed[[ref]][[test]]
Z.PreservationStats = mp$preservation$Z[[ref]][[test]]

modColors = rownames(Obs.PreservationStats)
moduleSize = Obs.PreservationStats$moduleSize
selectModules = !(modColors %in% c("grey", "gold"))
point.label = modColors[selectModules]
medianRank = Obs.PreservationStats$medianRank.pres
Zsummary = Z.PreservationStats$Zsummary.pres
par(mfrow = c(1,2), mar = c(4.5, 4.5, 2.5, 1))
plot(moduleSize[selectModules], medianRank[selectModules], col = 1,
     bg = modColors[selectModules], pch = 21, main = "medianRank Preservation",
     cex = 2, ylab = "medianRank", xlab = "Module size", log = "x")
labelPoints(moduleSize[selectModules], medianRank[selectModules],
             point.label, cex = 1, offs = 0.03)

plot(moduleSize[selectModules], Zsummary[selectModules], col = 1,
     bg = modColors[selectModules], pch = 21, main = "Zsummary Preservation",
     cex = 2, ylab = "Zsummary", xlab = "Module size", log = "x")
labelPoints(moduleSize[selectModules], Zsummary[selectModules],
            point.label, cex = 1, offs = 0.03)
# Add threshold lines for Zsummary
abline(h=0); abline(h=5, col = "blue", lty = 2); abline(h=10, col = "red", lty = 2)

#run it to compare R47H modules in Cv network

#load everything again
lnames = load(file = "Cv_FINAL-01-dataInput.RData")
CvData=data.frame(datExpr)
lnames = load(file = "R47H_FINAL-dataInput.RData")
R47HData=data.frame(datExpr)

#load Cv module data
lnames = load(file = "Cv_FINAL-02-networkConstruction.RData")
moduleColorsCv=moduleColors

#load R47H module data
lnames = load(file = "R47H_FINAL-02-networkConstruction.RData")
moduleColorsR47H=moduleColors

#set up calculation
#this saves only the Cv module colors
setLabels = c("R47H", "Cv")
multiExpr = list(R47H = list(data = R47HData), Cv = list(data = CvData))
multiColor = list(R47H = moduleColorsR47H)

system.time({
  mp = modulePreservation(multiExpr, multiColor,
    referenceNetworks = 1,
    nPermutations = 200, randomSeed = 1,
    quickCor = 0, verbose = 3)
})

save(mp, file = "modulePreseveration_R47H.RData")

ref = 1
test = 2

Obs.PreservationStats= mp$preservation$observed[[ref]][[test]]
Z.PreservationStats=mp$preservation$Z[[ref]][[test]]

modColors = rownames(Obs.PreservationStats)
moduleSize = Obs.PreservationStats$moduleSize
selectModules = !(modColors %in% c("grey", "gold"))
point.label = modColors[selectModules]
medianRank=Obs.PreservationStats$medianRank.pres
Zsummary=Z.PreservationStats$Zsummary.pres
par(mfrow=c(1,2),mar = c(4.5,4.5,2.5,1))
plot(moduleSize[selectModules],medianRank[selectModules],col=1,
  bg=modColors[selectModules],pch = 21,main="medianRank Preservation",
  cex = 2, ylab ="medianRank",xlab="Module size", log="x")
labelPoints(moduleSize[selectModules],medianRank[selectModules ],point.label,cex=1,offs=0.03)
plot(moduleSize[selectModules], Zsummary[selectModules], col = 1, 
    bg=modColors[selectModules], pch = 21, main="Zsummary Preservation", 
    cex=2, ylab = "Zsummary", xlab = "Module size", log = "x")
labelPoints(moduleSize[selectModules], Zsummary[selectModules], 
    point.label, cex=1, offs=0.03)
# Add threshold lines for Zsummary
abline(h=0); abline(h=5, col = "blue", lty = 2); abline(h=10, 
    col = "red", lty = 2)

# Plot kME correlations
setwd("\\\ad.ucl.ac.uk/home3/ucbtak3/Documents/PhD_Exosomes/4th Year/Mass Spec/Renormalized 2.0/WGCNA")
getwd()
workingDir = "."
setwd(workingDir)
library(WGCNA)
options(stringsAsFactors = FALSE)

# Work with 2 datasets
nSets = 2

# Load CV data
lnames = load(file = "Cv_FINAL-01-dataInput.RData")
lnames
CvData=data.frame(datExpr)

# Load R47H data
lnames = load(file = "R47H_FINAL-01-dataInput.RData")
lnames
R47HData=data.frame(datExpr)

# Load Cv module data
lnames = load(file = "Cv_FINAL-02-networkConstruction.RData")
lnames
moduleColorsCv=moduleColors

lnames = load(file = "R47H_FINAL-02-networkConstruction.RData")
lnames
moduleColorsR47H=moduleColors

multiExpr = list();
multiExpr[[2]] = list(data = CvData)
multiExpr[[1]] = list(data = R47HData)
setLabels = c("R47H", "Cv")

colorList = list(moduleColorsR47H, moduleColorsCv)
names(colorList) = setLabels

ref = 1
test = 2
refX = 1
# want to look at the blue module
mod = "blue"

modGenes = colorList[[ref]]==mod

mes = list()
for (set in 1:nSets)
{
  mes[[set]] = moduleEigengenes(multiExpr[[set]]$data,
  colorList[[ref]])$eigengenes
}

modx = match(mod, substring(colnames(mes[[refX]]), 3));

kMERef = cor(multiExpr[[ref]]$data[, modGenes], mes[[ref]][, modx], use = "p")
kMETest = cor(multiExpr[[test]]$data[, modGenes], mes[[test]][, modx], use = "p")

ind = 1

verboseScatterplot(kMERef, kMETest,
  xlab = spaste("kME in R47H network"),
  ylab = spaste("kME in Cv network"),
  main = spaste(LETTERS[ind], ". Eigengene-based connect.\n", "in R47H ", mod, 
module\n"), corLabel = "cor.kME",
  cex.main = 1.2, cex.lab = 1, cex.axis = 1,
  cex = 0.6, col = mod)
### 7.3 Functional annotations

**Table 7-1 Functional annotation of modules identified in Cv network**

<table>
<thead>
<tr>
<th>Colour</th>
<th>Size</th>
<th>p value</th>
<th>corrected p value</th>
<th>Fraction of proteins in GO Term</th>
<th>GO Term ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>black</strong></td>
<td>192</td>
<td>1.32E-30</td>
<td>2.38E-26</td>
<td>0.458333</td>
<td>GO:0031224</td>
<td>intrinsic component of membrane</td>
</tr>
<tr>
<td><strong>black</strong></td>
<td>192</td>
<td>9.79E-12</td>
<td>1.77E-07</td>
<td>0.130208</td>
<td>GO:0038023</td>
<td>signaling receptor activity</td>
</tr>
<tr>
<td><strong>blue</strong></td>
<td>580</td>
<td>1.99E-14</td>
<td>3.60E-10</td>
<td>0.5</td>
<td>GO:0046483</td>
<td>heterocycle metabolic process</td>
</tr>
<tr>
<td><strong>blue</strong></td>
<td>580</td>
<td>9.64E-14</td>
<td>1.74E-09</td>
<td>0.49654</td>
<td>GO:0006725</td>
<td>cellular aromatic compound metabolic process</td>
</tr>
<tr>
<td><strong>brown</strong></td>
<td>283</td>
<td>2.19E-43</td>
<td>3.97E-39</td>
<td>0.210714</td>
<td>GO:0030216</td>
<td>keratinocyte differentiation</td>
</tr>
<tr>
<td><strong>brown</strong></td>
<td>283</td>
<td>1.15E-39</td>
<td>2.08E-35</td>
<td>0.225</td>
<td>GO:0043588</td>
<td>skin development</td>
</tr>
<tr>
<td><strong>cyan</strong></td>
<td>44</td>
<td>5.77E-06</td>
<td>0.104425</td>
<td>0.386364</td>
<td>GO:0003723</td>
<td>RNA binding</td>
</tr>
<tr>
<td><strong>cyan</strong></td>
<td>44</td>
<td>0.000644</td>
<td>1</td>
<td>0.045455</td>
<td>GO:0021904</td>
<td>dorsal/ventral neural tube patterning</td>
</tr>
<tr>
<td><strong>green</strong></td>
<td>223</td>
<td>2.54E-09</td>
<td>4.59E-05</td>
<td>0.05</td>
<td>GO:0051205</td>
<td>protein insertion into membrane</td>
</tr>
<tr>
<td><strong>green</strong></td>
<td>223</td>
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### Table 7-2 Functional annotation of modules identified in R47H<sup>het</sup> network

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7.4 DAM-related proteins

Table 7-3 DAM-related proteins in exosomes

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<th>Reference</th>
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<td>(Keren-Shaul et al., 2017; Butovsky and Weiner, 2018; Rangaraju et al., 2018)</td>
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<td>Beta-2-microglobulin</td>
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<td>CD9</td>
<td>CD9</td>
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<td>Macrophage colony stimulating factor 1</td>
<td>CSF1</td>
<td>2</td>
<td>(Keren-Shaul et al., 2017; Butovsky and Weiner, 2018)</td>
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<td>Cathepsin B</td>
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<td>(Keren-Shaul et al., 2017)</td>
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<tr>
<td>Cathepsin D</td>
<td>CTSD</td>
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<tr>
<td>Cathepsin L1</td>
<td>CTSL</td>
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<td>(Keren-Shaul et al., 2017)</td>
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<td>(Butovsky and Weiner, 2018)</td>
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<td>Score</td>
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<td>Dipeptidyl peptidase 2</td>
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<td>TIMP2</td>
<td>2</td>
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<td>Triggering receptor expressed on myeloid cells 2</td>
<td>TREM2</td>
<td>2</td>
<td>(Keren-Shaul et al., 2017; Butovsky and Weiner, 2018)</td>
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<td>(Keren-Shaul et al., 2017; Butovsky and Weiner, 2018)</td>
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7.5 Fiji Macros

7.5.1 Analysis of neurite length

```java
selectWindow("Position1.lsm");
rename("Example1");
run("Split Channels");
selectWindow("C1-Example1");
close();
selectWindow("C3-Example1");
close();
selectWindow("C2-Example1");
run("Unsharp Mask..., "radius=1 mask=0.60");
run("Threshold...");
waitForUser("set the threshold and press OK, or cancel to exit the macro");
run("Convert to Mask");
run("Close- ");
run("Analyze Skeleton (2D/3D)", "prune=none display");
selectWindow("Tagged skeleton");
run("Summarize Skeleton");
selectWindow("Tagged skeleton");
close();
selectWindow("C2-Example1");
close();
```

7.5.2 Analysis of Tubb3 and GAP43 co-localisation

```java
rename("Example");
run("Split Channels");
selectWindow("C1-Example");
run("Remove Outliers...", "radius=1 threshold=50 which=Bright");
run("Threshold...");
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run("Convert to Mask");
selectWindow("C2-Example");
run("Threshold...");
waitForUser("set the threshold and press OK, or cancel to exit the macro");
run("Convert to Mask");
run("Analyze Particles...", "summarize");
imageCalculator("Min create", "C2-Example", "C1-Example");
selectWindow("Result of C2-Example");
run("Analyze Particles...", "summarize");
run("Close All");
```
7.6 List of publications


