Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.
Analysis of the modulatory effects of amyloid-beta and age on microglial activation.

Julie-Ann O'Reilly

A thesis submitted to
Trinity College Dublin
for the degree of
Doctor of Philosophy

Supervisor: Prof. Marina Lynch
Trinity College Institute of Neuroscience
2009
1. Declaration

This thesis is submitted by the undersigned for the degree of Doctor of Philosophy at the University of Dublin. I declare that this thesis is entirely my own work. This work has not been submitted in whole or in part to this or any other university for any other degree. The author gives permission to the library to lend or copy this work upon request.

Julie-Ann O'Reilly

30th October 2009
II. Abstract

Microglia are the resident immune cells of the brain, and in the healthy brain they are believed to be responsible for the clearance of amyloid-beta (Aβ). Age is a primary risk factor for Alzheimer's disease (AD) and the age-related proinflammatory environment is thought to contribute to the pathogenesis of AD. In AD, activated microglia have been shown to be associated with insoluble Aβ plaques, but whether they play a role in phagocytosis of Aβ in this context is not known. Both inflammatory molecules and Aβ can trigger neurodegenerative changes and both exert a negative impact on memory. Therefore it is believed that both play a crucial role in the pathogenesis of AD.

In this study, microglial activation was assessed in vitro, and in brain tissue prepared from young and aged rats, and in a transgenic mouse model of AD. Following development of an assay to assess phagocytic activity using quantum dots (QD), Aβ1-42 was shown to have no affect on QD uptake but it increased IL-1β secretion in vitro. H₂O₂ and NaF, two known inhibitors of phagocytosis, decreased QD uptake and increased IL-1β secretion, and H₂O₂ also decreased OX-6 expression, in primary rat microglia and bone marrow-derived macrophages. Aβ1-42 increased, while H₂O₂ and NaF decreased, the number of CD11b⁺ cells that took up QD and expressed CD68, an indicator of phagocytic activity. These data indicate that there is a direct relationship between expression of OX-6 or CD68 and phagocytic activity.

Phagocytic activity was increased in CD11b⁺ cells prepared from aged, compared with young, rats and this was associated with increased expression of OX6 on these cells. Aβ1-42 and H₂O₂ increased phagocytic activity in cells isolated from the brains of young rats but Aβ1-42 induced no change, and H₂O₂ decreased phagocytic activity of cells isolated from the brains of aged rats. These data demonstrate the differential age-related effects of Aβ1-42 and H₂O₂ which indicate that microglia in the aged brain may be defective in their ability to clear Aβ, possibly contributing to the development of plaques in AD.

As previously reported, APPswe/PS1dE9 mice which overexpress amyloid precursor protein (APP) and presenilin 1 (PS1), develop Aβ plaques with age and, at 7 months of age, these were associated with an impairment in spatial learning. These changes were paralleled by an increase in microglial activation as indicated by increased expression of MHC II on CD11b⁺ cells isolated from the brains of these mice. However, no change in phagocytosis was identified. Rosiglitazone, a PPARγ agonist, attenuated the genotype-dependent impairment in learning and reduced the Congo red-positive plaque load as well as the genotype-dependent increase in insoluble Aβ1-42. These data indicate that the increased Aβ burden in the brains of APPswe/PS1dE9 transgenic mice is associated with increased microglial activation and impaired spatial learning which can be attenuated by rosiglitazone.

The data presented in this thesis demonstrate that microglial activation is altered in the brain of aged animals and that this may impact on the role played by microglia in AD. In addition, the data indicate that AD-associated impairments in learning and memory are closely coupled with increased microglial activation and Aβ accumulation. It is concluded that modulation of microglial activation could represent a potential therapeutic target in the treatment of AD.
Finally, I have made it! There are a great number of people who helped me to reach this day and I would like to thank them. Firstly, and most importantly, I would like to thank my supervisor, Prof. Marina Lynch. In spite of her very busy schedule, she has always been very accessible to me. I have probably talked to, or bothered her, on most days for the last four years and I appreciate everything she has done for me. I have benefited greatly from interacting directly with her and from her close mentoring style. She is someone I truly admire; I don’t know how she manages to do everything she does.

I would like to thank Barry Moran and Orla Hanrahan for their technical expertise, and for their friendship. Barry, you saved me on many occasions, and you saved the FACS machine from me! Denise Coyne is also someone I went to for assistance who has now become a friend. She was never too busy to help me out, even when she was six months pregnant and in the midst of writing her own thesis.

To all the members of the MAL Lab, you really are a great crew! Thanks for the help, the fun and the knowledge, that you have all shared with me. It went a long way to getting me through my thesis. In particular, I would like to thank Aine Murphy who was my co-supervisor in first year, and who I have shared everything with since, you rock murph! Belinda, thank you so much for your help printing this thesis, I don’t think I could have done it without you. Yesterday, like on many other days during my PhD, you kept me going! To Tara, Stephanie and Ita, the fourth year students who helped/hindered this PhD – I’m not quite sure which is true! Either way it was a lot of fun! To my cousin Samantha and to Evanna Mills, who both very kindly helped with analysis, I really appreciated it and I hope I haven’t put you off science for life.

To all the members of my HRB group: Joe, Kim, Kev, Dana, Joanne and Stephen – we really were the best year! We had some really great times in first year and I’m glad to say we’ve stuck together throughout. You are all people I respect and admire. I hope we will remain lifelong friends.

To my friends Sandra, Karen, Emma, Amy, Aisling, Catherine and Helena, although some of you may be near at hand and some of you may be far, far away, you have all supported me whenever I needed you. I am very lucky to have friends like you! To my sister Majella who often assisted a ‘poor student’,
you are a star and don’t you forget it! You make me laugh when I want to cry and keep me going when times are hard. To my brothers Lindsay and Ronan, my sister-in-law Sarah and my beautiful new niece Ruby, thank you all for your love and support.

To Kate, Mark, Tara and my god-daughter Roisin – your home was my solace, I could always find comfort, and distraction from my worries, there. Thank you for all the kind things you have done for me. Katie, you really are the big sister I’ve always wanted. To my dear Uncle Patrick who I remember and miss daily; I know you’ve been helping me.

To Dr. Susan Carpenter, aka hudie, whose been by my side through all of this – you have always been there for me. Can you believe it’s been 11 years? We really are getting old! I will miss you when you and Panda, aka Dr. Andy Crawford, move to the States. Our home has been a happy one!

Lastly, and most importantly, I would like to thank my parents, Liam and Paula, for their endless support, both emotional and financial, this could never have happened without you. I will be forever grateful for your constant generosity, encouragement and belief in me. You truly are amazing people.
CHAPTER 1 INTRODUCTION.............................................1

1.1 The Central Nervous System.........................................................2

1.2 Glial Cells of the CNS.................................................................2
  1.2.1 Microglia..............................................................................3
  1.2.2 Astrocytes...........................................................................4
  1.2.3 Oligodendrocytes and ependymal cells...............................6

1.3 The Immune System.................................................................7
  1.3.1 Innate immunity...............................................................7
  1.3.2 Adaptive Immunity........................................................12

1.4 Inflammation in the CNS.........................................................13
  1.4.1 Microglial Activation.......................................................14
  1.4.1.1 Cytokine Production.....................................................17
  1.4.1.2 Expression of Cell Surface Markers of Activation..........19
  1.4.1.3 Phagocytic Activity.....................................................22
  1.4.1.4 Modulating microglial activation.................................25

1.5 The Ageing Brain.................................................................28
2.2 Preparation of Amyloid-beta
2.2.1 Aggregation of Aβ1-42
2.2.2 Confirmation of the fibrillar nature of Aβ1-42
2.3 In vitro Treatment Protocols
2.4 Animals
2.5 Preparation of glial cells from the brains of adult rats
2.6 Preparation of mononuclear cells
2.7 Flow Cytometry
2.7.1 Analysis of cell surface markers of activation
2.7.2 Development of the phagocytic assay
2.7.3 Analysis of phagocytic activity
2.7.4 Optimising antibodies
2.8 Oral Administration of Rosiglitazone
2.9 Genotyping of APP/PS1dE9 mice
2.9.1 Isolation of genomic DNA from tail snips
2.9.2 Polymerase Chain Reaction for APPswe and PS1dE9 genes
2.10 Behaviour
2.10.1 Motor Tests: Hangwire and inverted screen
2.10.2 Footprint Analysis
2.10.3 Morris Water Maze Apparatus
2.10.4 Morris water maze training
2.10.5 Morris water maze reversal training
2.10.6 Morris water maze statistical analyses ........................................... 68

2.11 Cytokine Analysis ................................................................................. 69
2.11.1 Analysis of IL-1β concentration ......................................................... 69

2.12 Immunocytochemistry ......................................................................... 70
2.12.1 Confocal analysis of CD11b, OX-6, CD68 and GFAP expression, and QD uptake, by mixed glial cells .................................................. 70

2.13 Immunohistochemistry ........................................................................ 71
2.13.1 Preparation of tissue sections for immunohistochemistry ............. 71
2.13.2 Immunohistochemical staining for Aβ plaques using Congo red .... 72
2.13.3 Quantification of cerebral amyloid load ........................................... 72
2.13.4 Fluorescent immunohistochemical staining for Aβ plaques .......... 73
2.13.5 Double immunofluorescent labelling: Analysis of microglial activation and Aβ plaque deposition .................................................. 74
2.13.6 Microscopy ....................................................................................... 74

2.14 Live Cell Imaging of QD uptake by mixed glial cells ....................... 76

2.15 Analysis of Cell Viability in vitro ......................................................... 76
2.15.1 Propidium Iodide Assay ................................................................. 76
2.15.2 MTS Assay ................................................................................... 77

2.16 Real-time Polymerase Chain Reaction (PCR) ................................ 77
2.16.1 Preparation of tissue for RNA isolation ......................................... 77
2.16.2 Reverse transcription for cDNA synthesis ...................................... 78
2.16.3 Real-time PCR ............................................................................... 78
CHAPTER 3 DEVELOPMENT OF AN ASSAY TO ASSESS PHAGOCYTOSIS USING QUANTUM DOTS

3.1 Introduction...................................................................................................................84
3.2 Results..............................................................................................................................86
  3.2.1 Identifying the cell population of interest...............................................................86
  3.2.2 Choosing a Quantum Dot.......................................................................................86
  3.2.3 Choosing antibodies for flow cytometry...............................................................87
  3.2.4 Assessing potential modulators of phagocytosis...................................................87
  3.2.5 Assessment of Aβ_{1-42} aggregation state and effect on phagocytic activity of CD11b^+ cells...............................................................88
  3.2.6 Comparison of QD and fluorescent latex particles...............................................88
  3.2.7 Assessment of cell viability following treatment..................................................89
  3.2.8 Assessment of internalisation of QD.................................................................89
3.3 Discussion...................................................................................................................116
CHAPTER 4 ANALYSIS AND MODULATION OF ACTIVATION STATES OF MIXED GLIA AND BMDM

4.1 Introduction ......................................................................................................123

4.2 Results ................................................................................................................ 125

4.2.1 H₂O₂ and NaF significantly inhibited QD uptake by CD11b⁺ BMDM. 125

4.2.2 H₂O₂ significantly reduced expression of OX-6 by BMDM.............125

4.2.3 Aβ₁-42, H₂O₂ and NaF significantly increased IL-1β release by BMDM..................................................126

4.2.4 H₂O₂ and NaF significantly inhibited QD uptake by mixed glia.......126

4.2.5 Effect of Aβ₁-42 on expression of cell surface markers of activation and phagocytosis by CD11b⁺ microglia..............................................................................127

4.2.6 Aβ₁-42 and H₂O₂ significantly increase IL-1β release by mixed glia... 128

4.2.7 Comparison of mixed glial cell activity in the presence of commercially available fluorescent latex particles or in the presence of QD.....................128

4.3 Discussion ..........................................................................................................150

CHAPTER 5 ASSESSMENT AND MODULATION OF MICROGLIAL ACTIVATION IN AGEING

5.1 Introduction ......................................................................................................157

5.2 Results ................................................................................................................ 159

5.2.1 Ageing increased phagocytic activity of CD11b⁺ cells.......................159

5.2.2 Ageing increased OX-6 expression by CD11b⁺ cells.........................159
5.2.3: Ageing increased the proportion of CD11b+ cells that were expressed OX-6 and took up QD........................................................................................................160
5.2.4: Aβ1-42 increased QD uptake by CD11b+ cells isolated from the brains of young rats..................................................................................................................................................160
5.2.5: H2O2 increased QD uptake by CD11b+ cells isolated from the brains of young, but not aged, rats.............................................................................................................................161
5.2.6: Aβ1-42 or H2O2 had no effect on expression of cell surface markers of activation by CD11b+ cells..............................................................................................................................................161
5.2.7: Expression of cell surface markers of activation and cytokines is increased at the mRNA level in tissue isolated from the brains of aged rats..162

5.3 Discussion..............................................................................................................................................178

CHAPTER 6 INVESTIGATION INTO THE EFFECT OF ROSIGLITAZONE ON MICROGLIAL ACTIVATION, PLAQUE DEPOSITION AND BEHAVIOUR IN THE APPswe/PS1dE9 MOUSE

6.1 Introduction............................................................................................................................................186
6.2 Results...................................................................................................................................................188
6.2.1: APPswe/PS1dE9 mice were unimpaired in muscular strength and coordination..................................................................................................................................................................................188
6.2.2 Wild-type and APPswe/PS1dE9 mice showed significant learning during the acquisition phase of the Morris water maze.........................................................................................................................189
6.2.3 APPswe/PS1dE9 mice had a significantly longer pathlength to the platform during reversal training.........................................................................................................................................................190
6.2.4 Oral administration of rosiglitazone significantly improved performance of APPswe/PS1dE9 mice during the reversal phase of the Morris water maze.....190
6.2.5 Insoluble $\beta_1$-$\beta_2$ concentration was significantly greater in the brains of APPswe/PS1dE9 mice .................................................................191

6.2.6 Oral administration of rosiglitazone significantly decreased the number of plaques found in the cortex of APPswe/PS1dE9 mice ..............................................191

6.2.7 A pro-inflammatory environment existed in the brains of APPswe/PS1dE9 mice ........................................................................................................192

6.2.8 $\beta_1$-$\beta_2$ significantly increased expression of IA/IE by CD11b$^+$ cells isolated from the brains of wild-type and APPswe/PS1dE9 mice ...........................................193

6.2.9 $\beta$ plaques were visible in the hippocampus and cortex of control- and rosiglitazone-treated APPswe/PS1dE9 mice .........................................................194

6.3 Discussion .................................................................................................................230

CHAPTER 7 GENERAL DISCUSSION

7.1 General Discussion ..............................................................................................238

7.2 Future Work .........................................................................................................247

CHAPTER 8 BIBLIOGRAPHY ........................................................................248

Appendix I Publications...........................................................................................267

Appendix II Materials ..............................................................................................269

Appendix III General Products ..............................................................................274

Appendix IV Company Addresses ..........................................................................278
V. List of Figures

CHAPTER 1 INTRODUCTION
Figure 1.1: Ramified Microglia and Amoeboid Microglia
Figure 1.2: An astrocyte in association with a blood vessel
Figure 1.3: An oligodendrocyte extending processes to three axons
Figure 1.4: Phagocytosis pathways
Figure 1.5: Schematic representation of microglial activation by a stressor
Figure 1.6: (A) Relationship between size and emission wavelength for CdTe QD.
(B) Schematic of a QD nanocrystal core with stabilising TGA attached to the cadmium atoms on the surface of the QD

CHAPTER 2 METHODS
Figure 2.1: Timeline of study involving wildtype and APPswe/PS1dE9 mice
Figure 2.2: Hangwire apparatus
Figure 2.3: Footprint Analysis Apparatus
Figure 2.4: Morris Water Maze Apparatus
Figure 2.5: Visual Cues used in Morris Water Maze

CHAPTER 3 DEVELOPMENT OF AN ASSAY TO ASSESS PHAGOCYTOSIS USING QUANTUM DOTS
Figure 3.1: Cell populations were identified based on forward and side scatter characteristics.
Figure 3.2: Analysis of a FACS dot plot.

xiv
Figure 3.3: Expression of CD11b by mixed glia.

Figure 3.4: QD uptake by mixed glia.

Figure 3.5: QD uptake by BMDM.

Figure 3.6: QD uptake varies with preparation.

Figure 3.7: Uptake of QD varies with charge on the particle.

Figure 3.8: Effect of potential modulators of phagocytosis on the percentage of CD11b+ cells.

Figure 3.9: Effect of LPS on QD uptake by mixed glia.

Figure 3.10: Measurement of \( \Lambda \beta_{1-42} \) aggregation by thioflavin T assay.

Figure 3.11: Effect of \( \Lambda \beta_{1-42} \) on QD uptake by CD11b+ cells.

Figure 3.12: Effect of \( \text{H}_2\text{O}_2 \) and NaF on uptake of QD and fluorescent latex particles.

Figure 3.13: Effect of \( \Lambda \beta_{1-42} \) on uptake of QD and fluorescent latex particles.

Figure 3.14: Assessment of cell viability following treatment.

Figure 3.15: Assessment of QD internalisation by microglia.

Figure 3.16: Assessment of QD internalisation by astrocytes.

Figure 3.17: QD are internalised by microglia and astrocytes.

Figure 3.18: Live cell imaging time series analysis of QD uptake by mixed glia.

CHAPTER 4 ANALYSIS AND MODULATION OF ACTIVATION STATES OF MIXED GLIA AND BMDM

Figure 4.1: \( \text{H}_2\text{O}_2 \) significantly inhibited QD uptake by BMDM

Figure 4.2: NaF significantly inhibited QD uptake by BMDM

Figure 4.3: \( \text{H}_2\text{O}_2 \) significantly reduced \( \text{OX}-\text{6} \) expression by BMDM

Figure 4.4: \( \Lambda \beta_{1-42}, \text{H}_2\text{O}_2 \) and NaF significantly increased IL-1\( \beta \) release
Figure 4.5: H2O2 significantly inhibited QD uptake by mixed glia

Figure 4.6: NaF significantly inhibited QD uptake by mixed glia

Figure 4.7: Aβ1-42 increased expression of cell surface markers of activation in a concentration-dependent manner

Figure 4.8: Aβ1-42 had no effect on the percentage of CD11b+ cells that expressed cell surface markers of activation and took up QD

Figure 4.9: Co-localisation of OX-6 expression and QD fluorescence

Figure 4.10: Aβ1-42 and H2O2 significantly increased IL-1β release by mixed glia

Figure 4.11: Aβ1-42 significantly increased expression of CD68 and phagocytosis by CD11b+ mixed glia

Figure 4.12: H2O2 and NaF significantly reduced expression of CD68 and phagocytosis by CD11b+ mixed glia

Figure 4.13: Cytochalasin B did not significantly reduce phagocytosis by mixed glia

Figure 4.14: Cytochalasin B significantly reduced expression of CD68 and phagocytosis by CD11b+ mixed glia

CHAPTER 5 ASSESSMENT AND MODULATION OF MICROGLIAL ACTIVATION IN AGEING

Figure 5.1: QD uptake was increased in CD11b+ cells prepared from the brains of aged rats.

Figure 5.2: OX-6 expression was increased on CD11b+ cells prepared from the brains of aged rats.

Figure 5.3: CD86 expression was unchanged on CD11b+ cells prepared from the brains of aged rats.
Figure 5.4: The number of CD11b+ cells that expressed OX-6 and took up QD was increased in the brains of aged rats.

Figure 5.5: Aβ1-42 increased QD uptake by CD11b+ cells isolated from the brains of young rats.

Figure 5.6: H2O2 increased QD uptake by CD11b+ cells isolated from the brains of young rats but reduced QD uptake by CD11b+ cells isolated from the brains of aged rats.

Figure 5.7: Aβ1-42 or H2O2 had no effect on expression of OX-6 by CD11b+ cells isolated from the brains of young and aged rats.

Figure 5.8: Aβ1-42 or H2O2 had no effect on expression of CD86 by CD11b+ cells isolated from the brains of young and aged rats.

Figure 5.9: Aβ1-42 or H2O2 had no effect on the number of CD11b+ cells that expressed OX-6 and took up QD.

Figure 5.10: Aβ1-42 or H2O2 had no effect on the number of CD11b+ cells that expressed CD86 and took up QD.

Figure 5.11: Expression of cell surface markers of activation was increased at the mRNA level in tissue isolated from the brains of aged rats.

Figure 5.12: Expression of cell surface markers of activation was increased at the mRNA level in tissue isolated from the brains of aged rats.

Figure 5.13: Expression of IL-1β mRNA was decreased in tissue isolated from the brains of aged rats.

CHAPTER 6 INVESTIGATION INTO THE EFFECT OF ROSIGLITAZONE ON MICROGLIAL ACTIVATION, PLAQUE DEPOSITION AND BEHAVIOUR IN THE APPswe/PS1dE9 MOUSE
Figure 6.1: Genotyping of APPswe/PS1dE9 mice

Figure 6.2: Front- and hind-paw strength were not impaired in APPswe/PS1dE9 mice

Figure 6.3: Motor co-ordination and balance was not impaired in APPswe/PS1dE9 mice

Figure 6.4: Oral administration of maple syrup or maple syrup containing rosiglitazone did not significantly affect the body weights of the mice

Figure 6.5: There is no significant difference in pathlength to the platform during acquisition of the MWM between wild-type and APPswe/PS1dE9 transgenic mice

Figure 6.6: There was no significant difference in latency to the platform between wild-type and APPswe/PS1dE9 transgenic mice

Figure 6.7: Oral administration of rosiglitazone did not significantly effect learning in the Morris water maze

Figure 6.8: Performance in the probe trial of the Morris water maze was unaffected by genotype or rosiglitazone

Figure 6.9: Wild-type mice had a significantly shorter pathlength to the platform than APPswe/PS1dE9 mice during the reversal phase of the MWM

Figure 6.10: Wild-type mice had a significantly shorter latency to the platform than APPswe/PS1dE9 transgenic mice

Figure 6.11: Oral administration of rosiglitazone significantly reduced the length of the path taken to the platform by APPswe/PS1dE9 mice

Figure 6.12: Oral administration of rosiglitazone significantly reduced the latency to the platform by APPswe/PS1dE9 mice

Figure 6.13: There was no significant difference in soluble Aβ1-38, Aβ1-40 and Aβ1-42 concentrations in cerebellar tissue isolated from wild-type or APPswe/PS1dE9 mice
Figure 6.14: There was significantly greater insoluble Aβ1-42 in cerebellar tissue isolated from APPswe/PS1dE9 mice.

Figure 6.15: There was a significantly greater number of Congo red-positive Aβ plaques in the hippocampus of APPswe/PS1dE9 mice.

Figure 6.16: Oral administration of rosiglitazone significantly decreased the number of Congo red-positive Aβ plaques in the cortex of APPswe/PS1dE9 mice.

Figure 6.18: Expression of GFAP and CD68 mRNA significantly increased in the hippocampus of APPswe/PS1dE9 transgenic mice.

Figure 6.19: IL-1β, TRL-2 and TLR-4 mRNA expression was significantly increased in the hippocampus of APPswe/PS1dE9 transgenic mice.

Figure 6.20: Expression of IA/IE is significantly increased on CD11b+ cells isolated from the brains of APPswe/PS1dE9 mice.

Figure 6.21: Rosiglitazone significantly increased expression of IA/IE on CD11b+ cells from the brains of wild-type mice.

Figure 6.22: Aβ1-42 significantly increased expression of IA/IE by CD11b+ cells isolated from the brains of wild-type and APPswe/PS1dE9 mice.

Figure 6.23: H2O2 significantly reduced QD uptake by CD11b+ cells isolated from the brains of APPswe/PS1dE9 mice that received rosiglitazone by oral administration.

Figure 6.24: Aβ plaques are visible in the dentate gyrus and hippocampus of control- and rosiglitazone-treated APPswe/PS1dE9 mice.

Figure 6.25: Aβ plaques are visible in the cortex of control- and rosiglitazone-treated APPswe/PS1dE9 mice.

Figure 6.26: Aβ plaques in the cortex and hippocampus of APPswe/PS1dE9 mice.
VI. List of Tables

CHAPTER 2 METHODS

Table 2.1: *In vitro* treatment protocols.

Table 2.2: Fluorochrome labeled antibodies used in these studies.

Table 2.3: Primers used for DNA amplification

Table 2.4: Antibodies used in fluorescent staining

Table 2.5. Primers used in this study

CHAPTER 3 DEVELOPMENT OF AN ASSAY TO ASSESS PHAGOCYTOSIS USING QUANTUM DOTS

Table 3.1: Characteristics of QD preparations.

Table 3.2: Uptake of QD varies in mixed glia and BMDM.

Table 3.3: QD and fluorescent labels suitable for use in the phagocytic assay.

Table 3.4: Antibodies used in flow cytometry.

Table 3.5: Potential modulators of phagocytosis.
### VII. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ₁₋₄₂</td>
<td>Amyloid β₁₋₄₂ peptide</td>
</tr>
<tr>
<td>ACM</td>
<td>Astrocyte conditioned medium</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>APPswe</td>
<td>APP gene containing the ‘swedish’ mutation.</td>
</tr>
<tr>
<td>BACE</td>
<td>β-APP cleaving enzyme</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone marrow cells</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophages</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CB</td>
<td>Cytochalasin B</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CdTe</td>
<td>Cadmium Tellurium</td>
</tr>
<tr>
<td>cDMEM</td>
<td>Complete DMEM</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>dE9</td>
<td>Deletion on exon 9</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DPX</td>
<td>Depex polystyrene</td>
</tr>
<tr>
<td>E</td>
<td>East</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporters</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FS/SS</td>
<td>Forward Scanner/Side Scanner</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidise</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule 1</td>
</tr>
<tr>
<td>icv</td>
<td>Intracerebroventricularly</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>ihc</td>
<td>Intrahippocampally</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lection</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris Water Maze</td>
</tr>
<tr>
<td>N</td>
<td>North</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum cutting temperature compound</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS/FCS</td>
<td>Phosphate buffered saline containing 3% foetal calf serum</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG-1,000</td>
<td>Polyethylene glycol-1,000</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear cell</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion Protein</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>QD</td>
<td>Quantum dots</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation Endproducts</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>sAPPα</td>
<td>Soluble amyloid precursor protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>S</td>
<td>South</td>
</tr>
<tr>
<td>TGA</td>
<td>Thioglycolic acid</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor Alpha</td>
</tr>
<tr>
<td>TREM2</td>
<td>Triggering receptor expressed on myeloid cells2</td>
</tr>
<tr>
<td>Trypsin-EDTA</td>
<td>Trypsin-ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>TWB</td>
<td>Tris wash buffer</td>
</tr>
<tr>
<td>W</td>
<td>West</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
INTRODUCTION

1.1 The Central Nervous System

The central nervous system (CNS) contains billions of cells that can be broadly classified into two main types: nerve cells and glial cells. Neurons are outnumbered 10 to 1 in the CNS by glial cells which were originally thought to play a secondary supportive role to the neurons (Kandel et al., 2000). More recently, it is emerging that glia play diverse and essential roles - much more complex than previously imagined ((Moore and Thanos, 1996). Within the CNS, neurons and glia interact in a dynamic and co-operative fashion and this intricate relationship is altered in ageing and neurodegenerative disease, resulting in detrimental neuroinflammation (Moore and Thanos, 1996; Stevens, 2008).

1.2 Glial Cells of the CNS

Glia, named after the Greek word for ‘glue’, were discovered more than 150 years ago and have been shown to play roles in metabolic signalling cascades, regulation of synaptic strength and modulation of cellular signals and neurotransmitters (Klein, 2009; Perea et al., 2009; Haydon et al., 2009). Glia have four main established functions: provision of structural support to neurons, maintenance of homeostasis, protection of the CNS from invading pathogens and cell debris, and modulation of neurotransmission (Kandel et al., 2000). Glial cells can play dual protective and destructive roles within the CNS (Minagar et al., 2002). There are four main types of glial cells: microglia, astrocytes, oligodendrocytes and ependymal cells.
1.2.1 Microglia

Microglia are the resident immune effector cells of the CNS (Olson and Miller, 2004) and are of myeloid lineage, displaying similar characteristics to the macrophages of the peripheral nervous system (PNS) (Mezey et al., 2000). Microglia are key players in modulation of the immune system in the CNS playing a role in both the innate and adaptive immune responses (Olson and Miller, 2004). They are believed to protect the brain from infection and injury, and are the key cellular mediators of neuroinflammatory processes (Streit et al., 2004).

Microglia can exist in at least two distinct morphological forms, known as ramified and amoeboid microglia (See Figure 1), which serve different functional roles and change state in response to pathological stimuli. Ramified microglia exhibit a characteristic morphology comprising of a small cell body and long, extended, branching processes similar to dendrites. In the mature adult brain they constitute approximately 10-20% of the total glial cell population (Vaughan and Peters, 1974; Banati, 2003). Classically, ramified microglia were considered inactive under normal physiological conditions; however, it is now known that ramified microglia are not dormant but function as a surveillance system for the brain, maintaining homeostasis and monitoring the well-being of other brain cells (Booth and Thomas, 1991; Thomas, 1992; Fetler and Amigorena, 2005). Ramified microglia show minimal expression of molecules associated with macrophage function such as MHC II (Venneti et al., 2009).
1.2.2 Astrocytes

Astrocytes are involved in maintenance of homeostasis and are crucial for neuronal survival providing glycogen for conversion to lactate and use as an energy supply for neurons during intense neuronal activity (Teeling and Perry, 2009; DeKeyser et al, 2008). They play a role in the removal of neurotransmitters such as glutamate, gamma-aminobutyric acid (GABA), norepinephrine, dopamine, serotonin, and acetylcholine from the synaptic cleft and can prevent excitotoxicity in this way (DeKeyser et al, 2008). Astrocytes modulate neuronal transmission by releasing chemical transmitters, expressing a wide range of receptors such as glutamate transporter 1 (GLT1) and glutamate aspartate transporter (GLAST) and producing a variety of trophic factors such as brain-derived neurotrophic factors, glial-derived neurotrophic factor, nerve growth factor, neurotrophins, and insulin-like growth factor (Haydon et al, 2009; DeKeyser et al, 2008). They are involved in cross-talk with neurons and are
Chapt er 1 Introduction

thought to mediate the extent of the microglial immune response through release of ATP (DeKeyser et al., 2008; Bianco et al., 2005).

Astrocytes contribute to the structural and functional integrity of the blood brain barrier (BBB) (Dong and Benveniste, 2001). The endfeet of astrocytes wrap themselves tightly around the microvasculature endothelium promoting cell adhesion and reducing the likelihood of pathogen infiltration. The importance of astrocytes in BBB maintenance was demonstrated in a study by Bush and colleagues (1999) where astrocyte ablation by treatment with ganciclovir resulted in a failure of BBB repair leading to cerebral oedema.

GFAP (glial fibrillary acidic protein) forms the main intermediate filament found in astrocytes and during CNS injury, astrocytes undergo proliferation, morphological changes and production of GFAP, a process termed astrogliosis (Kullberg et al., 2001). Astrogliosis, identified by an increase in GFAP, is a common hallmark of many neurodegenerative diseases and is a feature of ageing (Kullberg et al., 2001; Dong and Benveniste, 2001). Like microglial activation, depending on the disease context, astrogliosis can be considered either beneficial or detrimental. Interferon (IFN)-γ is a potent inducer of MHC II expression on astrocytes (Wong et al., 1984) while tumour necrosis factor (TNF)-α enhances the ability of IFN-γ to induce expression of MHC II on astrocytes. Data regarding expression of other cell surface markers by astrocytes is conflicting, but there is general agreement that astrocytes, in CNS disease, can produce interleukin (IL)-1, IL-6, IL-10, IFN-γ and TNF-α (Dong and Benveniste, 2001). For this reason, astrocytes are important mediators of CNS immune and inflammatory responses.
1.2.3 Oligodendrocytes and ependymal cells

Oligodendrocytes insulate axons by wrapping their processes around them forming the myelin sheaths that enable efficient and rapid signalling by neurons. A single oligodendrocyte can myelinate numerous axons (Smythies and Bradley, 1979). Ependymal cells line the ventricular system within the brain and make up the walls of the ventricles (Fleishhauer, 1973). They produce and secrete cerebrospinal fluid (CSF) and circulate this CSF by movement of their cilia.

Figure 1.2: An astrocyte in association with a blood vessel.

Figure 1.3: An oligodendrocyte extending processes to three axons.
1.3 The Immune System

The immune system is a complex network of organs, cells and circulating proteins which functions to protect an organism from invading pathogens. The cells of the immune system recognise foreign materials by the antigens expressed on their surfaces and launch an immune response that results in the elimination of the pathogen from the body. Recognition of an antigen can be relatively non-specific or highly specific.

The non-specific response is mediated by the innate immune system and the specific response is mediated by the adaptive immune system (Vander et al., 1998). The innate immune system is the body’s first line of defence against any invading pathogen; cells of the innate immune system quickly mediate their effector functions and then gradually disappear over hours or days (Sun and Lanier, 2009). The adaptive immune response is antigen-specific and only interacts with the organism that induced the response. Once activated, the cells of the adaptive immune system proliferate to increase the number of effector cells, travel to the site of infection, mediate highly specific anti-pathogen responses and reside in the tissues for months to years (Sun and Lanier, 2009) providing ‘immunological memory’. The innate and adaptive immune responses function in tandem with the innate immune response providing signals that enable the adaptive immune response to select appropriate cells to attack and to determine the best strategy for elimination of the pathogen.

1.3.1 Innate immunity

The innate immune system includes anatomical barriers that must be breached before an antigen can enter the body and stimulate an inflammatory response. Acute inflammation comprises the immediate and early response to an
injurious agent and is a defensive response that precedes repair of the damaged site (Streit et al, 2004). Chronic inflammation results from stimuli that are persistent and can become damaging if it enters a self-perpetuating cycle. Chronic inflammation is implicated in neurodegenerative and neuroinflammatory diseases (Streit et al, 2004).

Cells of the innate immune system originate from the myeloid lineage and include the granulocytes, mast cells, macrophages and dendritic cells. Macrophages are found in nearly all organs and tissues, are monocytic in origin and differentiate into macrophages after they enter the tissues from the circulation. Their primary function is phagocytosis and intracellular killing of infectious or injurious agents but they also express co-stimulatory molecules and MHC II enabling them to process and present antigen to T cells thereby creating a link between the innate and adaptive immune responses (Vander et al, 1998). Dendritic cells are also phagocytic; however, their primary function is not the elimination of pathogen but antigen presentation. The main phagocytic cell of the brain is the microglial cell.

Phagocytosis is the process by which a foreign substance such as an invading pathogen or a damaged or dead cell is ingested and digested by specialised cells called phagocytes. It is an essential process involved in host defense (Tsuboi and Meerloo, 2007). Often, the terms phagocytosis and endocytosis are used interchangeably. However, phagocytosis is one of three types of endocytosis, the other two being pinocytosis and receptor-mediated endocytosis. Pinocytosis is the process used by cells to internalise a liquid. Receptor-mediated endocytosis is the process by which specific molecules, such as low density lipoprotein (LDL), are taken into the cell by specific receptors on the plasma membrane. Phagocytosis is distinct from pinocytosis as it involves the
vesicular internalisation of solid particles whereas pinocytosis involves internalisation of liquids. Pinocytosis is a non-specific process unlike receptor-mediated endocytosis and phagocytosis which are both specific to the substrate being internalised.

There are two main types of phagocytosis: Opsonin-dependent phagocytosis and opsonin-independent phagocytosis (Celli and Finlay, 2002). Opsonin-dependent phagocytosis utilises either the Fcγ receptor or the complement receptors CR1, CR2 and CR3 (Aderem and Underhill, 1999). These two receptor types bind particles that have either IgG or complement bound to their surfaces, respectively. This receptor-mediated recognition of the extracellular particle is a fundamental step in the process of phagocytosis. Opsonin-independent phagocytosis involves the engagement of various cellular receptors that can recognise and bind molecular motifs directly on the surface of pathogens. Examples of these include the mannose receptor, the type A scavenger receptor and the integrins (Celli and Finlay, 2002) and toll-like receptors (TLRs).

Scavenger receptors partake in the binding and uptake of many structurally-unrelated substances including fibrillar Aβ, apoptotic cells and bacteria (Alarcon et al, 2005). TLRs are pathogen recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) on infectious agents. TLR-mediated binding results in phagocytosis and release of proinflammatory cytokines such as IL-1β, TNF and IL-6 by the phagocytic cells (Glezer et al, 2007).

The pathways of phagocytosis are diverse and extremely complex and are described as type 1 and type 2 phagocytosis (Caron and Hall, 1998). Type 1 phagocytosis is Fcγ receptor-mediated. This pathway requires activation of RAC1, Cdc42 and members of the rho family of GTPases amongst other
signalling molecules. When these signalling proteins are activated, polymerisation of F-actin is induced through WASP and the Arp2/3 complex (Ernst, 2000). This leads to extension of the F-actin rich lamellipodia which engulf the particle. Type 2 phagocytosis is CR3-mediated. This type of phagocytosis does not require extension of lamellipodia which suggests that there are mechanistic differences between type 1 and type 2 phagocytosis. However, similar to type 1 phagocytosis, type 2 phagocytosis utilise rho family GTPases and the Arp2/3 complex (Celli and Finlay, 2002). Other phagocytic pathways are awaiting characterisation.

Activation of type 1 or type 2 phagocytosis results in differential consequences. Type 1 phagocytosis leads to activation of the oxidative burst and initiation of an inflammatory cascade whereas these processes do not occur in type 2 phagocytosis.

Following binding at a phagocytic receptor, activation of downstream signalling cascades must occur to allow rearrangement of the actin cytoskeleton (Koenigsknecht and Landreth, 2004); this rearrangement includes extension of pseudopods to engulf the pathogen forming an intracellular microbe-containing phagosome which fuses with an intracellular lysosome to form a phagolysosome. The lysosomal enzymes break down the microbe’s macromolecules (Vander et al, 1998) and nitric oxide (NO) and hydrogen peroxide (H₂O₂) can be produced which are extremely destructive to the microbe. This process is known as the oxidative burst. The oxidative burst is a critical anti-microbial mechanism which consists of a rapid release of reactive oxygen species (ROS), such as superoxide and hydroxyl radicals, by the phagocyte NADPH oxidase (Graham et al, 2007). Within phagocytic cells, reduction of oxygen leads to the formation of H₂O₂.
which can then combine the chloride ion to form hypochlorite. Hypochlorite, along with other ROS, is responsible for digestion of the ingested particle.

Some particles are not digested but instead are integrated into an MHC molecule and undergo antigen presentation. For antigen presentation to occur, the antigen must be complexed with the body’s own MHC protein and therefore, must be on the plasma membrane of a host cell. There are two classes of MHC proteins: I and II. MHC I proteins are found on the surface of virtually all nucleated cells in the body while MHC II proteins are found only on the surface of macrophages, B cells, and macrophage-like cells (i.e. dendritic cells and activated microglia). MHC II is required for T-cells to recognise antigen and launch an adaptive immune response. Antigen presentation involves phagocytosis, digestion of antigen, association of the fragmented antigen with the MHC II protein and transport to the plasma membrane. The T cell binds to this complex on the cell surface activating the antigen presenting cell (APC) to provide necessary co-stimulatory molecules and cytokines to initiate further T cell activation. Helper T cells produce cytokines that facilitate activation and function of both B cells and cytotoxic T cells (Zenaro et al, 2009; Wynn, 2005). Phagocytes provide a very efficient mechanism of antigen ingestion by antigen-presenting cells (Watts and Amigorena, 2001).

Phagocytic cells can also recruit other phagocytic cells by releasing inflammatory mediators, oxygen derivatives and antimicrobial enzymes into the extracellular fluid (Vander et al, 1998).
Figure 1.4: Phagocytosis pathways

Schematic relating phagocytosis to endocytosis, pinocytosis and receptor-mediated endocytosis. A and B show the two main types of phagocytosis: opsonin-dependent phagocytosis and opsonin-independent phagocytosis. The biological processes activated during phagocytosis, the respiratory burst and antigen presentation, are also shown.

1.3.2 Adaptive Immunity

The cells of the adaptive immune system are the lymphocytes of which there are two principal classes, B cells and T cells (Pocock and Richards, 1999). These cells recognise a specific invader and mount a specific response.

B cells undergo antigen-induced activation and differentiation (LeBien and Tedder, 2008) and produce antibodies that target and destroy antigens identical to those that stimulated their production. Humoral responses have a wide range of
targets and represent a major defense against bacteria, viruses and other microbes (Vander et al, 1998).

T cells can be classified into two main subsets: Cytotoxic T cells which seek out the antigen-bearing cell, bind to the antigen and kill the cell using secreted chemicals and helper T cells which activate other cells.

1.4 Inflammation in the CNS

The CNS is designed to protect itself from infection and immune responses in the periphery and classically, the brain has been described as an immune-privileged organ due to the existence of the blood brain barrier (BBB). The BBB is a specialised structural, transport and biochemical barrier that regulates the movement of compounds, such as immune mediators, and cells between the blood and the brain (Stamatovic et al, 2008; Glezer et al, 2007).

In general, disruption of the BBB is considered a harmful event and it is associated with a range of CNS inflammatory disorders including stroke, meningitis and multiple sclerosis. Increased BBB permeability has also been hypothesised as a contributing factor to increased levels of Aβ accumulating in the brain parenchyma and hence is implicated in Alzheimer’s disease (Stamatovic et al, 2008). The BBB is indispensible for protecting the brain but it is not the only contributor to the privileged immune status of the brain; many CNS-resident cells play immunoregulatory roles and actively maintain the immune-privilege of the brain (Galea et al, 2006). Despite the CNS being almost completely devoid of a lymphatic system, and despite low levels of expression of MHC I and II (Merrill and Benveniste, 1996), the CNS is neither isolated nor passive in its interactions with the immune system. Instead, a highly sophisticated immune system exists within the central nervous system that can act in synchronicity with the immune
Chapter 1 Introduction

system of the periphery (Glezer et al, 2007). Microglia are the prime components of the intrinsic brain immune system (Streit et al, 2004) but both microglia and astrocytes act as the first line of defence by responding to non-specific “danger” signals (Carson and Sutcliffe, 1999). These cells can generate a local immune response by expression of cell surface markers such as CD80/86 and MHC II or by release of cytokines such as IL-1β, IL-6 and TNF-α (Sparkman and Johnson, 2008) and they are the innate immune system of the CNS.

Neuroinflammation is triggered by injury, insult, infection or ischemia in which the cumulative effects of microglial and astrocytic activation contribute to the initial neurodestructive effects (Streit et al, 2004) but when controlled, neuroinflammation can be neuroprotective and lead to the resolution of an infection or injury. Normal aging is associated with a shift towards a proinflammatory brain environment with increased expression of proinflammatory mediators such as MHC II, CD86 and IFN-γ and a decrease in the anti-inflammatory cytokines IL-10 and IL-4 (Sparkman and Johnson, 2008).

1.4.1 Microglial Activation

Microglia become activated following detection of any signal that poses a threat to the integrity of the internal environment of the CNS and these include the presence of bacteria or viruses, a change in the levels of circulating cytokines or antibodies, or the presence of abnormal endogenous proteins (Venneti et al, 2009). They can also become activated if the so-called ‘calming signals’ (Hanisch and Kettenmann, 2007) that help to maintain them in a quiescent state are interrupted by neuronal damage. These signals include the ligand-receptor pairs: CD200-CD200R, CX3CL1-CX3CR1 and SIRPα-CD47 (Hanisch and Kettenmann, 2007).
Following activation, microglia display an amoeboid morphology characterised by shorter, thicker processes and a larger cell body. Originally, it was considered that there were two main states of microglial activation; activated non-phagocytic microglia found in areas of CNS inflammation and reactive phagocytic microglia observed in areas of brain trauma (Minagar et al., 2002). It is now acknowledged that multiple activation states probably exist (Austin et al., 2006; Town et al., 2005). The response of microglia to pathological events is context-dependent and varies as the microenvironment changes (Hanisch and Kettenmann, 2007). Upon stimulation, microglia can increase secretion of cytokines and chemokines (Magnus et al., 2001; Teeling and Perry, 2009), become phagocytic, present antigen, increase expression of cell surface activation markers (Streit et al., 1989), activate the complement system, produce NO and other chemical mediators (Hanisch and Kettenmann, 2007; Benveniste et al., 2001; Lu et al., 2007). Microglial activation has traditionally been associated with neurotoxic and pro-inflammatory downstream effects (Venneti et al., 2009) but it is now evident that microglial activation can have neuroprotective and anti-inflammatory potential (Schwartz et al., 2006; Hanisch and Kettenmann, 2007; Teeling and Perry, 2009). For this reason, the role played by microglia in immune defence is still considered a double-edged sword.

Following activation, microglia release soluble factors which can induce a pro- or an anti-inflammatory environment and, when appropriate, help to neutralise dangers to the brain. Olson and Miller (2004) reported that microglia do not constitutively express cytokine mRNA at any significant level with the exception of IL-6 but that mRNA expression of IL-1β and TNF-α is upregulated following stimulation with TLR agonists such as LPS and Poly I:C (Olson and Miller, 2004). Pro-inflammatory cytokines have been shown to have both
deleterious and beneficial effects dependent on the inflammatory process occurring when they are produced.

Figure 1.5: Schematic representation of microglial activation by a stressor such as Aβ, ROS or ageing.

(A) Microglia upregulate expression of cell surface markers or increase secretion of chemical mediators of inflammation. (B) Microglia become phagocytic.
1.4.1.1 Cytokine Production

IL-1β is a potent proinflammatory cytokine that is secreted primarily by macrophages and microglia in a regulated manner (Takenouchi et al., 2008). It is synthesised in the cytoplasm as a biologically inactive precursor molecule, pro-IL-1β, and activated caspase 1 converts pro-IL-1β into the biologically active IL-1β that is released (Takenouchi et al., 2008; Miller et al., 1995). IL-1β possesses a wide and varied spectrum of biological activities associated with infection, inflammation and autoimmune processes which include prostaglandin E production, bone marrow release of neutrophils, hepatic acute-phase protein synthesis, thrombocytosis and lymphocyte activation (Dinarello, 2006). It is a key factor in inducing fever which is in part mediated by IL-6 production (Janeway et al., 2001).

In the brain, IL-1β inhibits hippocampal long-term potentiation (LTP), a form of synaptic plasticity that has been extensively studied as a potential mechanism underlying learning and memory (Lynch and Lynch, 2001). In the ageing brain, IL-1β levels are up-regulated and it has been proposed that this contributes to the age-related impairment in LTP that is observed. In AD, Parkinson’s disease, multiple sclerosis and amyotrophic lateral sclerosis, serum and cerebrospinal fluid levels of molecules of the innate immune system, such as IL-1β, IL-6 and TNF-α, are elevated (Glezer et al., 2007). These cytokines, which are associated with inflammation, are elevated in the region of neuritic plaques, a hallmark of AD (Szczepanik et al., 2001).

IL-6 is a proinflammatory cytokine that can be produced by both astrocytes and microglia and it exerts both beneficial and detrimental effects in the CNS. The protective effects of IL-6 in the CNS include promoting neuronal survival, protecting neurons against ischemic damage and modulating
neurotransmitter synthesis (Yamada et al., 1997; Hama et al., 1989). IL-6 also promotes astrocyte proliferation (Selmaj et al., 1990). Dysregulated IL-6 in the CNS can be damaging and cause neurodegeneration, breakdown of the BBB, and increased expression of complement proteins (Barnum et al., 1996) and it has been shown to play an important role in experimental autoimmune encephalomyelitis (EAE), the experimental model of multiple sclerosis, with IL-6 deficient mice being completely resistant to the disease. Over-expression of IL-6 in the CNS induces expression of proinflammatory cytokines such as IL-1β and TNF-α (DiSanto et al., 1996). In ageing, plasma levels of IL-6 are significantly elevated (Albani et al., 2009) and interestingly, increased IL-6 concentration is recognised as a risk factor for many conditions associated with ageing including stroke, type 2 diabetes and dementia (Dugan et al., 2009). In AD, microglia are stimulated by Aβ1-42 to release IL-6 perpetuating the detrimental aspects of the disease and the reflex immune response.

Like IL-1β and IL-6, TNF-α is a proinflammatory cytokine that plays an essential role in orchestrating the immune response in the brain (Blais and Rivest, 2004). Microglia produce TNF-α which acts in an autocrine and paracrine manner to activate the population of immune cells in the brain parenchyma (Nadeau and Rivest, 2000). TNF-α is reported to be upregulated in the brains of AD patients (Kim et al., 2008) and Aβ1-42 has been shown to significantly increase TNF-α production by primary microglia (Casal et al., 2002; Floden and Combs, 2006; Szczepanik et al., 2001). Aside from it’s proinflammatory effects, TNF-α can play an important neuroprotective role within the CNS and blockade of TNF-α in MS patients leads to immune system activation and increased disease activity (Gosselin and Rivest, 2006; Correale and Villa, 2004). Glezer and colleagues (2007) developed an IL-1/TNF double knockout mouse to clarify the role played
by endogenous proinflammatory cytokines in a model of NO-induced neurotoxicity; this double knockout model prevented the possibility of compensatory mechanisms being activated and Glezer and colleagues (2007) found that animals that were deficient in TNF-α displayed a dramatic exacerbation of the NO-induced neurotoxic damage to neurons. It is possible that rapid release of TNF-α in response to acute injury is necessary for activation of innate immune functions of microglia resulting in phagocytosis and resolution of the initial excitotoxic damage, preventing subsequent secondary damage (Glezer et al., 2007).

1.4.1.2 Expression of Cell Surface Markers of Activation

In the normal adult CNS, microglia express minimal MHC II, CD80, CD86 and CD40 (Ponomarev et al., 2005; Vidyadaran et al., 2009). These surface molecules, which are involved in immune processes, are upregulated when microglia are activated and allow the microglia to act as antigen presenting cells (APC).

CD11b is a type I transmembrane protein which is constitutively expressed CD11b in the normal healthy CNS (Ponomarev et al., 2005). It functions as a receptor for complement, fibrinogen or clotting factor X. CD11b is one subunit of the heterodimeric integrin, αMβ2, which is involved in the adhesive interactions of myeloid and lymphoid cells (Corbi et al., 1988); the second subunit of αMβ2 is called CD18. Expression of CD11b is up-regulated in neurodegenerative conditions (Roy et al., 2008).

CD45 is a membrane-bound protein tyrosine phosphatase and it is expressed by microglia and also by leukocytes. CD45 can be used in conjunction with CD11b for differentiating between resident microglia and infiltrating
macrophages in CNS tissue. Resident microglia are CD11b\(^+\), CD45\(^{\text{low}}\) while infiltrating macrophages are CD11b\(^+\), CD45\(^{\text{high}}\) (Henry et al, 2009; Ford et al, 1995; Carson et al, 1998). Expression of CD45 is increased on microglia in the hippocampus and frontal cortex of AD patients (Bamberger and Landreth, 2001) and there is also an age-related increase in expression of CD45 (Stolzing and Grune, 2003).

MHC II proteins are found only on certain lymphocytes, macrophages, monocytes and antigen-presenting cells (Pocock and Richards, 1999). Regulated MHC II expression is essential for presentation of antigen to helper T cells and plays a critical role in induction of immune responses (Dong and Benveniste, 2001), but inappropriate expression of MHC II has been implicated in several autoimmune and inflammatory diseases (Collawn and Benveniste, 1999). In normal ageing, expression of MHC II by microglia is increased in the brain (Henry et al, 2009) which enables potential interactions between T cell receptors and MHC II thereby triggering multiple signalling pathways resulting in production of cytokines, upregulation of cell surface molecules, increased intracellular calcium and activation of protein kinases. The interaction between the T cell receptor and MHC II induces CD40 expression on APC and CD40 ligand (CD40L) expression on T cells (Dong and Benveniste, 2001).

CD40 is constitutively expressed at low levels on microglia and is a co-stimulatory protein involved in antigen presentation (Dong and Benveniste, 2001). The binding of CD40L on helper T cells to CD40 activates APC and induces a variety of downstream effects including secretion of cytokines and upregulation of cell surface markers such as CD80 and CD86 which play a critical role in T cell activation (Sinistro et al, 2008; Dong and Benveniste, 2001). Like MHC II and CD11b, there is an age-related increase in expression of CD40 (Stolzing and
Grune, 2003) and Aβ induces an increase in expression of these 3 molecules on microglia (Tan et al, 1999).

CD80 and CD86 are co-stimulatory molecules that are expressed on the surface of APCs and must be activated for T cell immune response to occur (Vasu et al, 2003). They play a role in down-regulating T cell activation by binding cytotoxic T lymphocyte antigen-4 (CTLA-4) thus controlling the immune response. CD86 has been shown to be upregulated in the brain of aged animals (Stolzing and Grune, 2003).

CD68 is a member of the lysosomal/endosomal-associated membrane glycoprotein (LAMP) family and is a type I integral membrane protein that primarily localises to the lysosomes and endosomes with a smaller fraction circulating to the cell surface (da Silva and Gordon, 1999). CD68 is a member of the scavenger receptor family, members of which typically function to clear cellular debris, promote phagocytosis and mediate the recruitment and activation of macrophages (Wong et al, 2005). Expression of CD68 is increased during normal brain ageing with mRNA and protein levels showing regional-associated increases (Wong et al, 2005). Expression of CD68 is increased in multiple sclerosis, a chronic demyelinating disease of the CNS (Wong et al, 2005). In transgenic mouse models of AD, CD68 positive immunostaining has been shown to be associated with activated microglia adjacent to amyloid plaques (Wong et al, 2005). In this instance, it is possible that CD68 may be an indicator of phagocytosis.

RAGE is the receptor for advanced glycation endproducts and was originally identified as an Aβ-binding protein (Yan et al, 1996). RAGE is expressed on the surface of neurons, microglia and endothelial cells, has the ability to bind advanced glycation endproducts (AGE) and is often classified as a
PRR. Yan et al (1996) showed that in the AD brain, RAGE is expressed on neurons and microglia located near senile plaques. They showed that RAGE could bind both fibrillar and non-fibrillar Aβ but it is unclear whether or not RAGE plays a role in Aβ-mediated neuronal toxicity.

Unstimulated microglia constitutively express TLR1-9 allowing them to recognise PAMPs (Town et al, 2005) and suggesting they are capable of recognising a myriad of microbial infections in the CNS (Olson and Miller, 2004; Iliev et al, 2004; Lehnardt et al, 2002; Alexopoulou et al, 2001). IFN-γ-stimulated microglia show significantly increased mRNA expression of TLRs 3, 6 and 9, and LPS-stimulated microglia show significantly increased mRNA expression of TLRs 2, 4, 6, 8 and 9 (Olson and Miller, 2004). TLR ligands specifically promote phagocytosis (Doyle et al, 2004) but can also upregulate secretion of innate cytokines and pro-inflammatory cytokines that stimulate the adaptive immune response. Babcock and colleagues (2006) showed that TLR2 was upregulated by microglia in the hippocampus in response to transection of axons in the entorhinal cortex while in a model of acute seizures in epilepsy, Turrin and Rivest (2004) found that TLR2 mRNA was increased throughout the forebrain, particularly in the cortex, hippocampus, amygdala, thalamus and hypothalamus.

1.4.1.3 Phagocytic Activity

Phagocytosis is a hallmark indicator of innate immune cell activation (Town et al, 2005); the mechanistic aspects of phagocytosis were discussed in section 1.3.1. Within the CNS, microglia are the principal phagocytes and there are two main types of phagocytosis in which they can be involved; pathogen removal which results in a pro-inflammatory phagocytic response, or removal of...
apoptotic cell debris which stimulates an anti-inflammatory phagocytic response (Neumann et al, 2009). This anti-inflammatory phagocytic function of microglia is one of their major beneficial actions in the CNS. For example, triggering receptor expressed on myeloid cells-2 (TREM2) facilitates debris clearance in the absence of inflammation in vitro and appears to play a critical role in maintenance of CNS tissue homeostasis (Napoli and Neumann, 2009).

In ageing, microglia exhibit cellular senescence and impaired function (Streit, 2006). Zhao and colleagues (2006) reported delayed recruitment of phagocytic cells and decreased myelin clearance in aged, compared with young, rats and have proposed that microglial function is impaired in the aged brain. In AD, microglial activation can have beneficial or detrimental effects; activation of the innate immune system in response to AD may be beneficial as it could result in enhanced clearance of Aβ peptides by microglial phagocytosis (Shie et al, 2005). There is evidence from experimental studies in animals that microglia can phagocytose and degrade amyloid, but it has been suggested that phagocytosis is either ineffective or inadequate in Alzheimer's disease (Frautschy et al, 1992; Weldon et al, 1998).

Analysis of uptake of apoptotic cells by phagocytic cells is one of the most commonly used phagocytic assays. Uptake can be analysed by flow cytometry or light microscopy as apoptotic cells that have been ingested are easily identifiable following staining with a fluorescent maker of apoptosis such as PI or fluorescein-conjugated annexin V (Adayev et al, 1998; Witting et al, 2000; Magnus et al; 2001). A wide variety of other substance including bacteria, IgG coated erythrocytes, zymosan, oxidised LDL and myelin vesicles that microglia and other phagocytic cells have been shown to ingest are also used to assess phagocytic activity of cells (Smith, 2001; Adayev et al, 1998; Witting et al, 2000; Magnus et
The most common phagocytic assay involves the use of commercially-available fluorescent latex beads (Fukasawa et al, 1997; Tsujimoto et al, 2008; Dumrese et al, 2009; Smith, 2001; Koenigsknecht-Talboo and Landreth, 2005; Takeda et al, 1998).

Quantum dots (QD) are positively or negatively charged, fluorescent semiconductor nanocrystals that are produced with characteristic emission wavelengths that are directly related to size of the particle. It was considered that they may be used to assess phagocytosis since they are readily taken up by macrophages (Byrne et al, 2006). The QD used in this study were thioglycolic acid (TGA)-capped cadmium tellurium QD produced by a crystallisation reaction which results in attachment of TGA to the cadmium atoms on the surface of the QD. These QD have a negative charge that is attributed to the TGA capping and the presence of carboxylic groups.

Figure 1.6: (A) Relationship between size and emission wavelength for CdTe QD. (B) Schematic of a QD nanocrystal core with stabilising TGA attached to the cadmium atoms on the surface of the QD. A is adapted from a paper by Yu et al (2003) and B is adapted from a brochure by Sigma Aldrich.
QD show great potential as biolabels due to their stable and narrow emission spectra and the ease with which it can be measured by confocal microscopy; this contrasts with traditional fluorescent dyes whose fluorescence intensity deteriorates following prolonged exposure to excitation light (Byrne et al, 2007). In the human THP-1 monocyte cell line, TGA-stabilised QD have been shown to bind to the cell surface within 5 minutes, to enter the cytoplasm within 10 minutes and to be translocated to the nuclear membrane after 10 minutes (Byrne et al, 2006). Here, QD were used to develop an assay using flow cytometry to assess the phagocytic activity of primary microglial and BMDM cultures as well as microglia isolated from the brains of adult rats and mice.

1.4.1.4 Modulating microglial activation

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram negative bacteria and is one of the most potent inducers of microbial inflammation (Bals, 2005; Perez-Perez et al, 1995). LPS is a prototypical PAMP that binds to PRRs and mediates it’s effects through TLR4 leading to distinct patterns of gene expression and appropriate adaptive immune response (Qin et al, 2005). It is a powerful activator of microglia leading to an upregulation of costimulatory molecules and cell surface molecules like CD40 and MHC II (Perez-Perez et al, 1995; Ribes et al, 2009; Kalmar et al, 2001) but it fails to induce CD45 expression by acutely isolated adult microglia. Therefore, LPS appears to exert differential effects on primary microglia or on microglia that are isolated acutely from the adult brain (Carson et al, 1998). LPS stimulates production of inflammatory cytokines like IL-6, IL-1β and TNF-α, chemokines, prostaglandin and nitric oxide (Nakamura, 2002; Laurenzi et al, 2001; Lindberg et al, 2005) and activates the NFκB signalling pathway (Qin et al, 2005). Peripheral
administration of LPS models the systemic infection that can occur in ageing or AD and evidence exists that systemic infection in an aged individual or AD patient can have a profound impact inducing exaggerated activation of the immune system resulting in an inappropriate and detrimental inflammatory response (Henry et al, 2009; Teeling and Perry, 2009).

Since microglia play an important role in the pathogenesis of AD and Aβ is the key component of senile plaques in the AD brain, it is important to understand the impact of Aβ on microglial activation (Nakamura, 2002). Aβ peptides have been shown to be potent activators of microglia and macrophages (Szczepanik et al, 2001) and in response to Aβ, microglia can induce complement proteins, inflammatory cytokines such as IL-1β and TNF-α, and the chemokine MCP-1. Microglia can also produce neurotoxic substances such as NO and ROS following exposure to Aβ and fibrillar Aβ can induce phagocytosis by microglia both in vitro and in vivo (Szczepanik et al, 2001; Weldon et al, 1998; Casal et al, 2002). Aβ stimulation of microglia is mediated by ligation of CD40 and CD40L which induces production of TNF-α via the MAP kinase pathway and expression of inducible NO synthase through the NFκB pathway (Nakamura, 2002).

The CNS is vulnerable to oxidative damage because it has a high energy requirement and a high oxygen consumption rate (Smith et al, 2000). Oxidative damage is caused by reactive oxygen species (ROS) which are free radicals and the most common ones include the superoxide anion (O₂⁻), the hydroxyl radical (OH), singlet oxygen, and the free radical-producing hydrogen peroxide (H₂O₂). ROS are highly reactive and oxidize lipids, proteins, and DNA, leading to tissue damage and cell death (Shen et al, 2008). H₂O₂ is a by-product of many in vivo reactions and can be converted to the highly damaging hydroxyl radical. Free radicals have been implicated in the etiology of neurodegenerative diseases.
including AD, and Parkinson's disease (Shen et al, 2008) and increased levels of ROS are also observed with age (Radak et al, 2004).

Microglial activation can be regulated by the oxidative state of the cell (Shih et al, 2006) and free radicals are both released from microglia and exert an effect on microglia (Takeda et al, 1998). Min and colleagues (2006) showed that induction of antioxidant gene expression in microglia by soluble astrocyte-derived factor can modulate microglial activation; this interaction may help to modulate brain inflammation in vivo. Evidence exists that supports the role played by H$_2$O$_2$ in both enhancing and inhibiting phagocytosis. Oosting and colleagues (1990) found that H$_2$O$_2$ inhibited phagocytic activity of alveolar macrophages in an irreversible, concentration- and time-dependent manner that was independent of the Fc receptor and of intracellular calcium concentration. They also showed that H$_2$O$_2$ caused an irreversible decrease in cellular ATP and phagocytosis and that a similar time course was involved in both processes (Oosting et al, 1990). Bejarano and colleagues (2006) reported that H$_2$O$_2$ enhanced phagocytic activity of human neutrophils in vitro by calcium mobilisation and promoted morphological changes in cultured amoeboid microglia that were indicative of increased phagocytic activity (Takeda et al, 1998).

In the brains of AD patients, elevated levels of ROS are found in senile plaques and in neurons that contain neurofibrillary tangles (NFT) and Aβ neurotoxicity correlates with free radical generation (Abramov and Duchen, 2005). While Aβ has been shown to increase production of ROS and accumulation of H$_2$O$_2$, oxidative stress has been reported to enhance Aβ levels and promote Aβ accumulation (Shen et al, 2008); this suggests that a self-perpetuating cycle exists which may drive AD pathogenesis. Consistent with a
role for ROS in AD, experiments utilising the transgenic mouse model of AD, Tg2576, showed that treatment with the anti-oxidant vitamin E reduced plaque formation and $\beta$ levels in the brain (Sung et al, 2004). However, this intervention was only effective when administered to young transgenic mice and did not slow progression of pathogenesis in aged mice. These results indicate that antioxidant therapy may be beneficial only if given at this stage of the disease process.

The ingestion phase of phagocytosis is an energy-dependent process and therefore, sodium fluoride (NaF) which is an inhibitor of glycolysis (Michl et al, 1976; Mazur and Williamson, 1977) inhibits phagocytosis. Michl and colleagues (1976) reported that 2-deoxyglucose (2-dG) caused a greater reduction in cellular ATP levels than NaF but that, unlike NaF, 2-dG did not inhibit opsonin-independent phagocytosis. These findings demonstrate that reduced cellular ATP is not a sufficient explanation for the inhibitory effects of NaF on phagocytosis.

Cytochalasin B is a cell-permeable fungal toxin which causes the disruption of actin filaments and inhibition of actin polymerization (Cooper, 1987) and as a consequence, inhibits phagocytosis, as well as glycolysis and respiration (Plagemann and Estensen, 1972). Axline and Reaven (1974) reported that the inhibition of phagocytosis produced by cytochalasin B could not be dissociated from it’s inhibitory effects on plasma membrane movement although it inhibited ATP synthesis.

1.5 The Ageing Brain.

In ageing, a shift towards a proinflammatory microenvironment occurs which makes the brain more susceptible to disruptive effects of disease, infection or stress (Sparkman and Johnson, 2008) and probably contributes to the
development of age-related neurodegenerative diseases (Blasko et al, 2004). The ageing process is defined by a slow deterioration of homeostatic functions and increased numbers of activated microglia accompany neuroinflammation; these microglia have been described as "primed" or "reactive" and therefore may be more responsive to a peripheral immune challenge (Godbout and Johnson, 2006; Perry et al, 2003). A similar state of heightened reactivity is also seen in neurodegenerative diseases such as multiple sclerosis, AD and prion disease and in parallel, the aged brain also exhibits increased basal levels of inflammatory cytokines (Sparkman and Johnson, 2008; Teeling and Perry, 2009).

Although a more proinflammatory environment exists in the aged brain, a decline in immune function is a hallmark of ageing (Renshaw et al, 2002). Expression of TLRs by macrophages and TLR function has been shown to decline in the aged population which could impact on the ability of the immune system to mount an adequate and effective response to infection (Renshaw et al, 2002). An increase in permeability of the BBB is seen with ageing in healthy individuals and this can facilitate the entry of chemical mediators of neurotoxicity and cellular mediators of inflammation (Popescu et al, 2009).

1.6 Alzheimer's Disease

AD, the leading cause of dementia in the elderly, is a progressive, incurable, debilitating disease of the brain that leads to memory loss and dementia (Benveniste et al, 2001; Blennow et al, 2006). The incidence of AD rises exponentially with advancing age and because of the increase in the ageing population, a significant increase in the number of individuals with AD is anticipated in the next two decades. Therefore, it is crucial that research into
neurodegenerative disorders focuses on developing therapeutic strategies to combat AD.

1.6.1 Pathogenesis of AD

Definitive diagnosis for AD requires pathological examination of brain tissue post-mortem (Selkoe, 2001). AD is characterised by gross cortical atrophy, widespread cellular degeneration and neuronal loss. Selective and profound loss of neuronal mass is observed in the temporal and frontal cortical regions, hippocampus and entorhinal cortex. These changes are accompanied by reactive gliosis, diffuse synaptic loss and by formation of neurofibrillary tangles (NFT) and the presence of extracellular neuritic plaques (Szczechanik et al, 2001; Blasko et al, 2004; Benveniste et al, 2001; Masters et al, 2006).

NFT are most easily identified in the hippocampus and are intraneuronal bundles of paired helical filaments of abnormally hyperphosphorylated tau proteins (Husain et al, 2008). They are not specific to AD and are seen in a number of other neurodegenerative diseases including Parkinson’s disease (Sasaki et al, 1998). In the healthy brain, tau is a normal structural component of cellular microtubules but in AD, hyperphosphorylated tau aggregates forming NFT resulting in cytoskeleton degeneration, impaired axonal transport, compromised neuronal and synaptic function, and neuronal death (Masters et al, 2006; Blennow et al, 2006).

Amyloid plaques consist of extracellular aggregates of Aβ peptide which is produced by the proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. Soluble Aβ peptide exists as monomers and dimers that begin to aggregate and form protofibrils. These protofibrils wrap around each other to form fibrils in a crossed β-sheet formation. This β-sheet content of the Aβ fibrils
renders it prone to aggregation as large insoluble fibrils in plaques (Blennow et al., 2006). The Aβ core in senile plaques varies in length from 38 to 43 amino acids, however, Aβ1-42 occurs more frequently and forms fibrillar aggregates far more readily than the Aβ1-40 peptide (Szczepanik et al., 2001). Amyloid plaques may represent a reservoir for soluble oligomers; they may provide a protective function and act as a sequestered pool of soluble and precipitated Aβ, or they may just be the final product of the Aβ cascade (Masters et al., 2006). Plaques are generally associated with reactive astrocytes, activated microglia and dystrophic axons and dendrites (Masters and Beyreuther, 2005).

1.6.2 Amyloid Precursor Protein

The APP gene is localised to chromosome 21 and is a ubiquitously-expressed, glycosylated transmembrane protein with a large hydrophobic extracellular domain, a single hydrophobic transmembrane domain and a small carboxy-terminal cytoplasmic domain (Kang et al., 1987). There are three common isoforms: APP695, APP751, and APP770; APP695 is expressed exclusively in neurons, whereas APP751 and APP770 are present in both neural and non-neural cells (Kitaguchi et al., 1988; Haass et al., 1991). Mutations in the APP gene are associated with increased Aβ self-aggregation and Aβ production by sequential cleavage by β- and γ-secretases. The α- and β-secretase cleavage sites are located in the extracellular domain of APP (Dulubova et al., 2004). The hydrophobic C-terminal portion of the Aβ peptide is contained in the single transmembrane domain of APP. The cytoplasmic domain of APP is short and is released following γ-secretase cleavage and may be transported to the nucleus.

The N-terminus of Aβ is generated from APP by proteolytic cleavage by β-secretase, a process during which β-APP is also produced. C-terminal cleavage
by γ-secretase releases the Aβ peptide from the β-secretase cleavage fragment C99 (Masters et al, 2006). Cleavage by γ-secretase occurs within the transmembrane region of APP and yields mainly 40 and 42 amino acid peptides. APP can also undergo non-amyloidogenic processing by α-secretase resulting in the production of α-APP and C83. These fragments can then undergo processing by γ-secretase resulting in the release of p3, a most likely non-pathogenic form of Aβ.

The exact function of APP is unknown although it has been implicated in maintenance of cellular Cu\(^{3+}\) homeostasis. This is supported by evidence from White and colleagues (1999) that shows that APP knockout mice have elevated Cu\(^{3+}\) levels both in the brain and the liver. Further evidence supporting this theory is that Cu\(^{3+}\) appears to be able to modulate APP processing in vivo. Higher Cu\(^{3+}\) levels result in reduced Aβ production and an increase in the non-amyloidogenic p3 form of the peptide (Borchardt et al, 1999).

β-APP functions as a neuronal acute-phase, injury-response protein. Graham and colleagues (1996) reported that there is excessive expression of β-APP, accompanied by microglial activation and cytokine expression, after traumatic head injury. Aβ deposition is also associated with brain injury, both in experimental animals and in humans (Smith et al, 2003; Graham et al, 1996).

1.6.3 Amyloid-β

Aβ is produced constitutively during normal cell metabolism and Aβ soluble monomers circulate in cerebrospinal fluid and blood suggesting that Aβ is a natural rather than a pathogenic product (Weldon et al, 1998). Under normal conditions, Aβ is degraded by the peptidases insulin-degrading enzyme, neprilysin and endothelin-converting enzyme (Blennow et al, 2006). Aβ clearance from the
brain is also mediated by a balanced process of efflux and influx across the BBB. Efflux is mediated by low-density lipoprotein receptor-related protein and influx is mediated by RAGE (Wang et al, 2006).

Aβ is markedly increased in AD patients where it accumulates as insoluble, fibrillar plaques (Benveniste et al, 2001). Neuronal toxicity to Aβ can occur via several different mechanisms; free radical induced damage appears to be one such mechanism. Aβ can trigger the production of ROS, nitrogen intermediates and inflammatory cytokines IL-1β, IL-6 and TNF-α from microglia (Meda et al, 2001; Johnstone et al, 1999). Aβ also increases the accumulation of H$_2$O$_2$ in a Cu$^{2+}$/Zn$^{2+}$-dependent manner resulting in free radical-induced lipid peroxidation and cell death (Bush and Tanzi, 2008). Aβ$_{1-42}$ is shown to induce apoptosis in cultured cortical neurons in vitro, possibly through alterations of cellular calcium homeostasis (Pereira et al, 2004) and it can also enhance glutamate release ultimately resulting in excitotoxicity, synaptic degeneration and neuronal death (Nakamura 2002).

1.6.4 Amyloid cascade hypothesis

The amyloid cascade hypothesis is the theory used most commonly to explain the molecular mechanisms leading to AD and states that an imbalance between production and removal of Aβ leads to progressive accumulation of the peptide, resulting in synaptic dysfunction, microgliosis and neuronal loss (Masters et al, 2006; Selkoe, 2001); it is supported by compelling genetic data (Hardy and Selkoe, 2002).

Four genes have been implicated in the pathophysiology of AD and all of these genes are associated with Aβ production or deposition. These genes include the APP gene on chromosome 21 and the presenilin 1 and presenilin 2 genes on
chromosome 14 and chromosome 1, and the ApoE4 gene (Masters et al, 2006). The main impact of mutations in these genes relates to their involvement in the APP processing pathway, which leads to increased production and elevated plasma levels of Aβ, especially Aβ\textsubscript{1-42} (Scheuner et al, 1996).

The presenilin (PS) proteins play a role in modulating γ-secretase activity and early onset familial AD cases are linked to mutations in the PS genes. Selkoe (2001) reported that mutations in both PS genes resulted in a selective increase in the production of Aβ\textsubscript{1-42} in cultured cells and in the brains of transgenic mice. PS 1 and 2 are ubiquitously expressed in the brain, primarily on neurons. The exact function of PS protein has not yet been elucidated but PS1 appears to play a role in normal neurogenesis and the formation of the axial skeleton, as well as γ-secretase activity. De Strooper and colleagues (1998) showed that PS1 is crucial for generation of Aβ.

1.6.5 Inflammation and immune response in Alzheimer’s Disease

The contributions of microglia and innate inflammation to the pathogenesis of AD are still unclear (Ransohoff and Perry, 2009). A wide variety of inflammatory mediators are released by microglia and histological evaluation of brain tissue from AD mouse models has shown Aβ plaques surrounded by reactive astrocytes, activated IL-1β immunoreactive microglia and degenerating neurons (Jimenez et al, 2008; Howlett et al, 2004). Neurons degenerate as a consequence of microglial activation and stimulate release of proinflammatory cytokines from microglia; it is proposed that this, in turn, results in further neuronal degredation thereby perpetuating the detrimental effects of inflammation in the AD brain (Blasko et al, 2004). Therefore, it is possible that inflammation is not simply a consequence of AD but that it is a key process involved in disease
development and progression (Szczepanik et al, 2001). The proinflammatory microenvironment of the aged brain assists development of age-related neurodegenerative diseases (Blasko et al, 2004). Several immunological systems such as the complement system, acute phase proteins, activated microglia and pro-inflammatory cytokines are engaged during AD development establishing that inflammation plays a major part in AD pathology (Ransohoff and Perry, 2009; Meda et al, 2001; Wyss-Coray and Mucke, 2002). The fundamental event in the development of AD appears to be the inappropriate metabolism of APP which results in an overproduction of Aβ leading to the development of neuritic plaques and subsequent inflammatory response and neuronal degeneration. The extent to which peripheral macrophages/monocytes contribute to this amyloid-associated microgliosis and its significance for AD remains unclear (Wyss-Coray, 2006).

Debate still exists regarding the role played by microglial phagocytic activity in Aβ metabolism and clearance from the brain. Hickman and colleagues (2008) reported that as AD progresses, phenotypic changes in microglia occur and cells become more pro-inflammatory losing their Aβ-clearing capabilities, resulting in reduced Aβ uptake and degradation, and increased Aβ accumulation. Wisniewski and colleagues (1991) argued that microglia could phagocytose Aβ fibrils in culture but not in vivo based on ultrastructural analysis which revealed the intimate relationship of amyloid plaques with microglia; these cells were never found to contain Aβ fibrils within their lysosomal compartments in the brains of AD patients. In contrast, Frackowiak and colleagues (1992) described phagocytosed Aβ fibrils in macrophages of elderly patients who suffered from fatal stroke (Wisniewski et al, 1991; Frackowiak et al, 1992). Further information comes from a study in which adult mouse microglia prepared from APPswe/PS1dE9 mice and from non-transgenic littermates were assessed for gene
expression of Aβ-binding receptors and Aβ-degrading enzymes (Borchelt et al, 1997; Jankowsky et al, 2001). As APPswe/PS1dE9 mice aged, their microglia became dysfunctional and exhibited a reduction in expression of their Aβ-binding receptors and Aβ-degrading enzymes, but continued to produce pro-inflammatory cytokines (Borchelt et al, 1997; Jankowsky et al, 2001). It has been suggested that these cytokines may act in an autocrine fashion and further reduce expression of Aβ-binding receptors and Aβ-degrading enzymes leading to decreased Aβ clearance and increased accumulation (Hickmann et al, 2008). The plaque load observed in mouse models of AD, and in the brains of AD patients post-mortem, have lead to the conclusion that microglia are inefficient phagocytes when it comes to removal of Aβ plaques (Ransohoff and Perry, 2009). Elucidating the interaction between the inflammatory cascade, microglia and the cells susceptible to neurodegenerative damage may lead to the development of new therapeutic strategies for AD and other neurodegenerative diseases.

1.7 Models of Alzheimer’s Disease

1.7.1 Non-transgenic animal models of AD

Initially, models of AD were developed to replicate the symptoms rather than pathology. Smith (1988) developed a model utilising pharmacological blockade of the cholinergic system to mimick the pre-synaptic cholinergic deficit seen in AD. However, these pharmacological models of AD did not increase Aβ plaques or NFT in the brain and therefore did not provide a platform on which to elucidate the mechanisms of disease progression. Investigation of the relationship between extracellular Aβ deposits and the pathological progression of AD was hindered by the lack of an in vivo model of AD where amyloid deposits could be induced (Frautschy et al, 1991) and attempts to develop a non-transgenic model of
AD focussed on reproducing the increased Aβ load that is observed in the brains of AD patients. The vast majority of these models involved intracerebral or intracerebroventricular (icv) infusion of synthetic Aβ peptides (Stephan and Phillips, 2005).

Frautschy and colleagues (1992) reported that the ability of Aβ infusion to replicate the inflammatory response and neuronal death seen in AD depended on a number of factors including the species and dose of Aβ used, the aggregation state of the peptide, the location of infusion and the animal strain. They injected SDS-isolated amyloid cores into rat hippocampus and cortex and found that there was considerable neuronal loss in the hippocampal layers in the immediate vicinity of the injected material. The same level of neuronal loss was not observed in the cortex. Infusion of Aβ1-40 resulted in a reduction of nicotine-stimulated acetylcholine (ACh) release and infusion of Aβ1-42 resulted a decrease in choline acetyltransferase activity which also led to decreased levels of ACh (Yamaguchi et al., 2006; Harkanay et al., 1999). Despite the fact that infusion of Aβ1-40 and Aβ1-42 had the same effect of ACh release, Games and colleagues (1992) failed to detect neuronal death or microglial activation following infusion of Aβ1-40 while Ryu and colleagues (2004) showed that intrahippocampal injection of Aβ1-42 induced significant neuronal loss and glial cell activation in rats.

Infusion of Aβ has been shown to exert negative effects on cognition and memory and the evidence suggests that the soluble Aβ oligomers, rather than the insoluble Aβ fibrils, exert more profound effects on learning and memory in AD. Yamaguchi and colleagues (2006) showed that icv infusion of soluble Aβ1-40 caused impairments in spatial reference and short-term memory in the Morris water maze task and in retention in passive-avoidance learning. Similarly, synaptic deficits in transgenic mouse models that overexpress mutant or wild-type
APP correlate with levels of soluble Aβ rather than amyloid deposits (Mucke et al., 2000). Fibrillar Aβ has been shown to induce impairments in learning and memory that are long lasting (Nakamura et al., 2001).

It is possible that the different forms of Aβ could impact on different mechanism of memory formation, perhaps playing an inhibitory role at different stages in the process. Maurice and colleagues (1996) showed that administration of Aβ25-35 did not affect storage or retention of a task that had already been learned whereas all species of Aβ, Aβ25-35, Aβ1-40 and Aβ1-42 appeared to induce deficits in short-term memory (Flood et al., 1991; Maurice et al., 1996; Nakamura et al., 2001).

1.7.2 Transgenic animal models of AD

The development of genetically-modified animal models of AD followed identification of genes associated with the disease. Transgenic mouse models exist with mutations in APP and the presenilin genes leading to increased production and progressive aggregation of Aβ. These transgenic mouse models can reproduce the major features of AD including the plaque-associated neuronal and microglial damage (Masters et al., 2006) but do not exhibit NFT. Behavioural testing of transgenic animal models of AD has revealed that they exhibit cognitive deficits (Eriksen and Janus, 2007). Although the transgenic mouse models that exist are extremely useful and have allowed significant progress to be made into the understanding of AD, no mouse model exists that accurately captures the complexity of AD and that models all of the pathological and cognitive symptoms associated with the disease.
1.7.2.1 APP-based transgenic mouse models

The PDAPP mouse, which has ten-fold overexpression of APP compared with wild-type mice, was the first transgenic mouse model to successfully develop neuritic Aβ plaques (Games et al, 1995). This mouse model favoured the production of Aβ1-42 over Aβ1-40, as a result of overexpression of human APP containing the V717F mutation. PDAPP mice display a number of the hallmarks of AD including microgliosis, astrogliosis, neuritic plaque formation and dystrophic neuritis (Chen et al, 1998). Although PDAPP mice show plaque localisation in the hippocampus and entorhinal cortex in a similar manner to that seen in AD, there is a lack of accompanying neuronal loss in these areas (Reilly et al, 2003; Irizarry et al, 1997). Deficits in spatial memory have been observed in PDAPP mice (Chen et al, 2000; Dodart et al, 1999).

The Tg2576 transgenic mouse model of AD is perhaps the most widely used of all the APP transgenic mice (Hsiao et al, 1996). This mouse model overexpresses the APP695 isoform of the APP gene with the Swedish double-mutation under the control of the prion protein (PrP) promoter. Kawarabayashi and colleagues (2001) reported a rapid increase in Aβ levels from 6 months of age with plaque deposition occurring between 9 and 12 months of age. These mice show a similar pattern of Aβ expression, similar hallmarks of the disease and a similar lack of neuronal loss to the PDAPP mouse (Dong et al, 2007; Frautschy et al, 1998; Eriksen and Janus, 2007). Tg2576 mice, however, display cognitive impairments in spatial and working memory that are associated with increased Aβ deposition (Hsiao et al, 1996; Zhuo et al, 2008).

Other APP transgenic mouse models of AD exist including the APP23 mouse and the TgCRND8 mouse which showed early lethality and is of limited use (Chishti et al, 2001). Double and triple transgenic models have been
developed more recently in an attempt to better encapsulate the true intricacies of the disease.

1.7.2.2 APP/PS1 transgenic mouse models

Since the presenilin genes had also been identified as playing an important role in development of AD, a transgenic mouse model was developed that incorporated all the genetic factors that were known to contribute to AD. A link was established between the presenilin genes and development of familial AD and a transgenic mouse that overexpressed mutant PS1 was developed and found to increase the amount of Aβ1-42 in the brain (Duff et al, 1996). These PS1 mice were crossed with Tg2576 mice and produced progeny that had 3 to 5 times greater accumulation of Aβ1-40 and Aβ1-42 than single transgenic littermates. These mice showed accelerated plaque pathology with widespread plaque deposition by 6-8 months old and impaired learning and memory in the radial arm water maze (Holcomb et al, 1998; Gordon et al, 2001).

The double transgenic mouse model that was used in this study and that is becoming the model of choice for therapeutic studies on AD is the APPswe/PS1dE9 mouse (Jankowsky et al, 2001; Jankowsky et al, 2004). This mouse model was produced by co-injection of the APP695 isoform containing the Swedish mutation and PS1 with exon-9 deleted into pronuclei. Each gene was under the control of an independent PrP promoter (Jankowsky et al, 2001). This leads to co-localisation and co-segregation of the genes. Aβ deposition is observed in these mice as early as 4 months of age with substantial plaque formation in the cortex and hippocampus by 6 months of age (Garcia-Alloza et al, 2006). This model favours increased expression of Aβ1-42 with levels of Aβ1-40 being unchanged (Jankowsky et al, 2004).
APPswe/PS1dE9 mice exhibit deficits in spatial memory in the Morris water maze (Cao \textit{et al}, 2007; Jankowsky \textit{et al}, 2005) and show a more profound deficit in episodic-like memory when tested in the repeated reversal and radial arm water maze tasks (Savonenko \textit{et al}, 2005; Jankowsky \textit{et al}, 2005). These behavioural deficits can become apparent from as early as 8 months-old (Cao \textit{et al}, 2005) with 6 month-old mice showing no impairment (Savonenko \textit{et al}, 2005). Reiserer and colleagues (2007) suggested that episodic-like memory is the first type of memory to be affected in APPswe/PS1dE9 mice, as it requires successful suppression of old memories alongside formation of new memories.

\textbf{1.8 Therapeutic Strategies for AD}

Despite much investigation, to date, no therapy has been identified that can halt or reverse progression of AD; current treatments are limited to symptomatic palliative intervention (Barrow, 2002). Cholinergic transmission is impaired in AD and most treatments increase intrasynaptic ACh levels (Auld \textit{et al}, 2002). Approved acetylcholinesterase inhibitors for treatment of AD include donepezil, rivastigmine and galantamine (Masters \textit{et al}, 2006). The other main drug used in the treatment of AD is memantine, a non-competitive N-methyl-D-aspartate (NMDA) antagonist. NMDA is a mediator of glutamatergic transmission and acts by blocking excessive NMDA receptor activity thereby preventing some of the excitotoxic cell death observed in AD (Chen and Lipton, 1997; Blennow \textit{et al}, 2006).

A number of potential sites of therapeutic intervention in AD have been identified. Since AD is characterised by excessive A\textbeta production, a rational strategy would be to halt or slow down the production of A\textbeta. In BACE1 knockout mice, A\textbeta production is abolished without any clinical phenotype (Luo \textit{et al}, 2004).
al., 2003). For this reason, BACE1 inhibitors are an attractive therapeutic target. β-secretase inhibitors have been developed that reduce brain Aβ in AD transgenic mice (Blennow et al., 2006). Drugs that stimulate α-secretase or that inhibit β- and γ-secretase could provide useful intervention for the treatment of AD.

It has been reported that active immunisation of AD mice with fibrillar Aβ attenuated Aβ deposition (Schenk et al., 1999) and that passive immunisation with antibodies against Aβ could achieve similar results (Bard et al., 2000). The effect may be mediated by increased microglial clearance of Aβ or by increased Aβ efflux from the brain (Weldon et al., 2006).

Development of anti-tau drugs and inhibitors of Aβ aggregation have been proposed as potential treatments of AD, as has the use of non-steroidal anti-inflammatory drugs (NSAIDs). Several NSAIDs have been shown to reduce brain Aβ burden in AD transgenic mice, an effect that may be mediated by inhibition of cyclo-oxygenase (COX) or by a direct effect on γ-secretase (Eriksen et al., 2003).

Rosiglitazone is a peroxisome proliferator-activated receptor-γ (PPARγ) agonist. PPARγ agonists have inhibitory effects on TNF-α suggesting that these agents may possess anti-inflammatory properties (Jiang et al., 1998; Ricote et al., 1998). Early experiments showed that PPARγ expression is upregulated in activated macrophages and monocytes, and that expression of iNOS and release of pro-inflammatory cytokines such as TNF-α and IL-6 are attenuated by treatment with natural and synthetic PPARγ agonists (Jiang et al., 1998; Ricote et al., 1998). Further studies have shown the anti-inflammatory properties of PPARγ agonists in animal models of aging, Parkinson’s disease, multiple sclerosis and many more neurodegenerative conditions.
Evidence suggests that PPARγ agonists can inhibit inflammatory responses in glia through receptor-independent mechanisms (Drew et al, 2006). In primary astrocytes inhibition of TNF-α, IL-1β and IL-6 gene expression, were seen to be independent of PPARγ activation (Giri et al, 2004) and inhibition of the inflammatory response was mediated by inhibition of IκB kinase (IKK) activity, which inhibits the translocation of the NFκB subunit p65 to the nucleus and thus the activity of NFκB (Giri et al, 2004; Ward et al, 2002). Recent evidence from this lab showed that the anti-inflammatory action of rosiglitazone in the brain of aged rats was independent of PPARγ; a synthetic PPARγ antagonist GW9662 failed to abrogate the inhibitory effect of rosiglitazone (Loane et al, 2009). Further studies are necessary to determine the mechanisms by which PPARγ agonists modulate inflammatory responses in the CNS. In this study, the PPARγ agonist rosiglitazone was assessed in the APPswe/PS1dE9 mouse model of AD.

1.9 Study Aims

The aims of these studies were as follows:

- To develop an assay to assess phagocytic activity using QD.
- To assess and modulate microglial activation in vitro using Aβ1-42, H2O2, NaF and cytochalasin B.
- To investigate age-related changes in microglial activation and to modulate activity of cells isolated from the brains of young and aged rats using Aβ1-42 or H2O2.
- To assess the effect of rosiglitazone on behaviour, plaque deposition, expression of inflammatory mediators and microglial activation in the APPswe/PS1dE9 mouse model of AD.
Chapter 2

Methods
2. Methods

2.1 Culture of Primary Cells

2.1.1 Preparation of sterile coverslips

Three days prior to cell culture, 13 mm glass coverslips (Chance Propper, UK) were placed in a sterile 50 ml falcon tube (Sarstedt, Ireland) containing 70% alcohol. The falcon tube was placed in the laminar airflow unit (Advanced Biosafety Cabinet Class II; AGB Scientific Ltd., Ireland) overnight under ultraviolet light. The following day the coverslips were removed from the falcon tube within the laminar airflow unit and laid out individually on sterile tissue paper where they remained overnight to dry under ultraviolet light. The following day, the coverslips were coated in sterile poly-L-lysine (1 mg/ml; diluted in sterile phosphate buffered saline (PBS; Sigma, UK)). The coverslips were placed individually in the solution in a Petri dish which was left to incubate for 1 hour in a humidified incubator (37°C; 5% CO₂:95% air; Nuaire Flow CO₂ incubator, Jencons, UK). Following incubation, the coverslips were laid out on sterile tissue paper in the laminar airflow unit and allowed to dry. Once the coverslips were dry, they were either placed into a sterile 24-well plate or stored in a sterile falcon tube in the fridge until they were needed.

2.1.2 Preparation of mixed glial cell cultures

Primary mixed glial cell cultures were prepared from 1-day old Wistar rats (BioResources Unit, Trinity College, Dublin, Ireland). All instruments used for dissection were sterilised by thorough cleaning with disinfectant followed by overnight baking at 200°C (Sanyo-Gallenkamp Hotbox Oven, Model #OHG050, Loughborough, UK). Within the laminar airflow unit, neonatal rats were
decapitated and the brains removed in the following manner. The skin along the midline of the skull was cut and the skull was exposed. A small sharp scissors was inserted at the brain stem and incisions were made along both sides of the head ensuring that the point of the scissors was touching the skull at all times. The skull was peeled back using sterile forceps. Once the skull was removed, the brain was removed and placed in a sterile Petri dish. The cerebral cortices of the brain were dissected out and the meninges removed. Tissue was bi-directionally chopped using a sterile disposable scalpel (Schwann-Mann, UK) and placed in sterile 15 ml falcon tubes containing pre-warmed complete Dulbecco’s Modified Eagle’s Medium (cDMEM; 2 ml; Gibco, UK). cDMEM is DMEM supplemented with 10% heat-inactivated foetal calf serum (FCS; Sigma, UK) and 1% streptomycin/penicillin (Gibco, UK) or 1% ciprofloxacin hydrochloride (Mediatech Inc., USA). Tissue was incubated for 20 minutes (37°C; 5% CO₂:95% air). Each sample was triturated using a sterile plastic Pasteur pipette (Sarstedt, UK) and the suspension was filtered through a sterile nylon mesh filter (40 μm; BD Biosciences, USA). Samples were centrifuged (1,200 rpm; 5 minutes; 20°C; Sorvall Legend RT, USA) and the pellet was resuspended in pre-warmed cDMEM.

Cells were counted and seeded at 1.0 x 10^6 cells/ml. For confocal analysis, 65 μl of the cell suspension was pipetted onto each poly-L-lysine coated coverslip in a sterile 24-well plate and incubated for 2 hours before addition of cDMEM (400 μl/well). For FACS analysis, 200 μl of the cell suspension was pipetted onto each well of a sterile 6-well plate and incubated for 2 hours before addition of cDMEM (1.5 ml/well). Cells were grown at 37°C in a humidified 5% CO₂:95% air environment and media was changed every 3 days for 12-14 days.
until cells reached greater than 70% confluence. Mixed glia were treated as outlined in section 2.3: ‘In vitro Treatment Protocols’. Following treatment, supernatants were removed and stored at -80°C for later analysis.

2.1.3 Maintenance of the L929 cell line and generation of M-CSF

Frozen L929 cells were thawed rapidly using 1 ml of FCS. The cell suspension was added to a T175 flask containing pre-warmed Roswell Park Memorial Institute medium (RPMI; 50 ml; Biosera, UK) supplemented with 10% heat-inactivated FCS and 1% ciprofloxacin hydrochloride. Cells were grown at 37°C in a humidified 5% CO₂:95% air environment and once confluent, cells were grown for 7 days. The supernatant was removed, cells were washed with PBS and trypsin was added to the flask (5 ml; 3-5 minutes; 37°C) to remove adherent cells. RPMI was added to each flask to neutralize the trypsin and the cell suspension was centrifuged (1,200 rpm; 5 minutes; 20°C). RPMI (50 ml) was added to T175 flasks. The cell pellet was resuspended in RPMI (1 ml) and 500 μl of the cell suspension was added to each T175 flask. Cells were allowed to become confluent and were grown for 7 days before undergoing the process again.

The supernatant from the mouse L929 cell line was used as a source of macrophage colony stimulating factor (M-CSF). On day 7, the supernatant was gently pipetted off and centrifuged (1,200 rpm, 5 minutes, 20°C) to remove any cells. The supernatant containing M-CSF was filter-sterilised and 20 ml aliquots were frozen at -20°C until required.
2.1.4 Preparation of Bone Marrow Derived Macrophages (BMDM)

Macrophages were generated in vitro from rat bone marrow cells (BMC). Cells in bone marrow are naïve and can be influenced to become the desired cell type. M-CSF is required for proliferation and differentiation of rat BMC into typical BMDM.

Young male Wistar rats (50 g) were sacrificed by cervical dislocation. The femurs and tibiae were removed from both hind legs and placed in a falcon tube containing DMEM (25 ml). The bone marrow was flushed into a sterile Petri dish using a sterile syringe needle and cDMEM. Repeated gentle aspiration of the marrow plugs through a sterile syringe needle (19 G) enabled cell aggregates to be dispersed and a single cell suspension to be created. The cell suspension was centrifuged (1,800 rpm; 5 minutes; 20°C), cells were resuspended in red blood cell lysis buffer (Sigma, UK) to assist erythrocyte lysis (5 ml; 2 minutes; room temperature (RT)) and the cell suspension was centrifuged again (1,800 rpm; 5 minutes; 20°C). This process was repeated until the supernatant was clear. Cells were washed and resuspended in 2-10 ml of cDMEM, depending on the size of the pellet. Cells from each rat were placed in a T175 flask containing cDMEM (50 ml) supplemented with 30% M-CSF and were incubated at 37°C in a humidified 5% CO₂:95% air environment for 24 hours. Non-adherent cells were harvested and added to a fresh T175 flask containing cDMEM (50 ml) supplemented with M-CSF. Adherent cells continued to grow in the original T175 flask.

On day 8-10, the supernatant was removed from the T175 flasks and discarded. Cells were washed with PBS, trypsin was added to the flask (5 ml; 3-5 minutes; 37°C) and cells were gently scraped. cDMEM (6 ml) was added to each
flask and the cell suspension was removed and centrifuged (1,800 rpm; 5 minutes; 20°C). For confocal analysis, cells were seeded at 1 x 10^5 cells/ml (1 ml/well) on poly-L-lysine coated coverslips in sterile 24-well plates. For FACS analysis, cells were seeded at 1 x 10^5 cells/ml (2 ml/well) in 6-well plates. Cells were incubated (37°C; 5% CO2:95%) for 1-2 days before treatment. BMDM were treated as outlined in section 2.3: 'In vitro Treatment Protocols'.

2.1.5 Cell Counting

Cells were resuspended in a known volume of media and viable cells were identified using trypan blue (Sigma, UK). Samples (10 µl) of the diluted cell suspension (1 in 10 dilution) were counted using a disposable haemocytometer (Hycor Biomedical, UK) and a light microscope. Trypan blue gives an indication of cell number, as well as cell viability since cells with a compromised cell membrane appear blue. Only viable cells, which did not stain and appear light under a light microscope, were counted. The number of cells/ml was determined using the formula: Cell count x dilution factor x 10^4.

2.2 Preparation of Amyloid-beta

2.2.1 Aggregation of Aβ1-42

Lyophilised amyloid β1-42 peptide (Aβ1-42; Biosource, Belgium) was aggregated according to manufacturer’s instructions. A stock solution (6 mg/ml) was prepared by dissolving in sterile dH2O. This was diluted to 1 mg/ml using calcium-free sterile PBS and allowed to aggregate (48 hours; 37°C). Aggregated Aβ1-42 was aliquoted and stored at -20°C until required.
2.2.2 Confirmation of the fibrillar nature of Aβ₁₋₄₂

The presence of Aβ fibrils in the aggregated samples was investigated using a thioflavin T (ThT) fluorescent assay. The binding of ThT to fibrillar Aβ was monitored by an increase in ThT fluorescence. Samples (5 μl), collected after reconstitution (0 hours) and after aggregation (24 hours and 48 hours), were applied in triplicate to a black fluorescent plate (Labsystems, Finland) and incubated with ThT (10 μl; 100 μM; Sigma, UK) in the presence of glycine (185 μl; 42.5 mM, pH 8.5; Sigma, UK). Fluorescence was read immediately (excitation 435 nm; emission 485 nm; Spectramax Gemini, Molecular Devices, UK). Fluorescence at 0, 24 and 48 hours was compared to the PBS control values.

2.3 In vitro Treatment Protocols

All compounds used to treat cells were diluted to the required concentration in the appropriate pre-warmed supplemented media and all solutions were filtered through a syringe with a cellulose acetate membrane filter (0.2μm Supor membrane; Acridisc syringe filters; Pall Corporation, UK). Details are listed below in Table 2.1.

Lipopolysaccharide (LPS) from Escherichia coli (1 mg/ml; Alexis, Switzerland) was diluted to a final concentration of 100 ng/ml in cDMEM and cells were treated for 24 hours. Recombinant rat interleukin-1 beta (IL-1β) (10 μg/ml; R&D Systems, UK) was diluted to a final concentration of 10 ng/ml in cDMEM and cells were treated for 24 hours. Aβ₁₋₄₂ peptide (200 μM; Biosource, Belgium) was diluted to a final concentration of 2 μM, 4 μM or 8 μM in cDMEM and cultured primary cells were treated with Aβ₁₋₄₂ for 24 hours. In other
experiments, mixed glial cells isolated from the brains of young and aged rats, or from the brains of wildtype and APPswe/PS1dE9 mice, were treated with Aβ_{1-42} diluted to a final concentration of 8 μM in cDMEM for 30 minutes. A stock concentration of hydrogen peroxide (H_{2}O_{2}; 10 mM) was prepared in distilled water (dH_{2}O) and stored at 4°C. It was diluted to a final concentration of 100 μM in cDMEM and cells were treated with H_{2}O_{2} for 30 minutes. In other experiments, mixed glial cells isolated from the brains of young and aged rats, or from the brains of wildtype and APPswe/PS1dE9 mice, were treated with H_{2}O_{2} (100 μM) for 15 minutes. A stock concentration of sodium fluoride (NaF; 1 M; Sigma, UK) was prepared in dH_{2}O and stored at +4°C. It was diluted to a final concentration of 10 mM in cDMEM and cells were treated with NaF for 1 hour. Granulocyte macrophage-colony stimulating factor (GM-CSF; 25 μg/ml; R&D Systems, UK) was diluted to a final concentration of 10 μg/ml in cDMEM and cells were treated for 1 hour. Cytochalasin B (CB; Sigma, UK) was reconstituted in dimethyl sulphoxide (DMSO) to prepare a stock concentration of 5 mg/ml. It was diluted to a final concentration of 10 μg/ml or 5 μg/ml in cDMEM and cells were treated with CB for 1 hour. The final concentration of DMSO was 0.1%. Quantum dots (QD) (Yurii Gunko, Trinity College, Dublin, Ireland) were diluted to a final concentration of 1 x 10^{-6} M in cDMEM and primary cells were incubated in the presence or absence of QD for 2 hours. Cells isolated from brains of adult rats or mice were incubated in the presence of QD for 15 minutes. In other experiments, commercially available fluorescent latex particles (L3030, L3280, L5155; Sigma, USA) were diluted 1:150 in cDMEM and mixed glial cells were incubated in the presence of these particles for 2 hours.
Table 2.1: In vitro treatment protocols.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stock Concentration</th>
<th>Neonatal Rat cells</th>
<th>Glial cells from young and aged rats.</th>
<th>Glial cells from wildtype and APPswe/PS1dE9 mice.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>1 mg/ml</td>
<td>100 ng/ml</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>10 μg/ml</td>
<td>10 ng/ml</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaF</td>
<td>1 M</td>
<td>10 mM</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>25 μg/ml</td>
<td>10 μg/ml</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>5 mg/ml</td>
<td>5/10 μg/ml</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Latex particles</td>
<td></td>
<td>1 in 150 diln</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ1-42</td>
<td>200 μM</td>
<td>2/4/8 μM</td>
<td>8 μM</td>
<td>8 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
<td>30 minutes</td>
<td>30 minutes</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>10 mM</td>
<td>100 μM</td>
<td>100 μM</td>
<td>100 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 minutes</td>
<td>15 minutes</td>
<td>15 minutes</td>
</tr>
<tr>
<td>QD</td>
<td>1 x 10⁻⁶ M</td>
<td>1 x 10⁻⁶ M</td>
<td>1 x 10⁻⁶ M</td>
<td>1 x 10⁻⁶ M</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 hours</td>
<td>15 minutes</td>
<td>15 minutes</td>
</tr>
</tbody>
</table>
2.4 Animals

Neonatal Wistar rats (0/1 day old) were used for preparation of mixed glial cultures. Male Wistar rats (4 weeks old) weighing approximately 50 g were used as a source of bone marrow for generation of bone marrow-derived macrophages. Young (3 months; 300-400 g) and aged (18-22 months; 600-700 g) male Wistar rats were used for isolation of mixed glial cells and for tissue analysis. Female wildtype and APPswe/PS1dE9 mice (7-8 months) were used in the behavioural study, for isolation of mixed glial cells from adult brains and for tissue analysis. Rats were housed in groups of 4 and mice were housed in groups of 5. Rats were obtained from Harlan, UK and mice were obtained from Jackson Laboratories, USA. All animals were maintained by the BioResources Unit, Trinity College, Dublin 2, Ireland on a 12 hour light/dark schedule with ambient room temperature controlled between 21 and 23°C. All animals were maintained under veterinary supervision.

These experiments were performed under a license issued by the Department of Health and Children (Ireland) with the approval of the local Ethics Committee and in compliance with the Cruelty to Animals Act, 1876 and the European Community Directive, 86/609/EC, and every effort was made to minimise stress to the animals.

2.5 Preparation of glial cells from the brains of adult rats

Young (3 months) and aged (18-22 months) male Wistar rats were sacrificed by cervical dislocation and decapitation. Brains were removed and the hippocampus and cortex were dissected free. Portions of the left hemisphere of
the brain were placed in liquid nitrogen for mRNA analysis, or in buffer containing CaCl₂ (0.2%) and DMSO (10%) for analysis by ELISA.

Tissue from the right hippocampus and cortex was bi-directionally chopped and incubated (37°C; 5% CO₂:95%) for 30 minutes in a 15 ml falcon tube containing 500 μl of collagenase D (1 mg/ml; Roche Applied Science, Germany) and 50 μl of DNase I (10 μg/ml, Sigma, UK) in 4.45 ml of PBS. The cell suspension was filtered through a sterile nylon mesh filter (40 μm) using the plunger of a 1 ml syringe and cDMEM supplemented with 0.5 M sucrose (Sigma, UK) and 10% w/v polyethylene glycol-1,000 (PEG-1,000; Fluka, Ireland). Samples were centrifuged (1,200rpm; 5 minutes; 20°C) and the pellet was resuspended in cDMEM supplemented with 0.5 M sucrose and 10% w/v PEG-1,000. Samples (500 μl) of the cell suspension were added to each well of a 24-well plate and cells were treated as outlined in section 2.3: 'In vitro Treatment Protocols'. Following treatment, cells were incubated with fluorescently-conjugated antibodies for analysis by flow cytometry and supernatants were removed and stored at -80°C for analysis.

2.6 Preparation of mononuclear cells

Female wildtype and APP/PS1dE9 mice (8 months; Jackson Laboratories, USA) were anaesthetised with sodium pentobarbital (Euthatal; 40 μl; Merial Animal Health Ltd, UK), perfused intracardially with ice cold PBS (20 ml) and their brains were removed. A portion of the right hemisphere of the brain, along the midline, was mounted in optimum cutting temperature compound (OCT; VWR International Ltd, Ireland) on a circular cork and frozen in ice-cold isopentane on dry ice for later preparation of cryostat sections and
immunohistochemical analysis. The cerebellum was placed in liquid nitrogen for later analysis. The remainder of the brain was placed in sterile PBS containing 3% FCS (PBS/FCS). Tissue was dissociated through a sterile nylon mesh filter (40 μm), washed with PBS/FCS, centrifuged (170 x g; 10 minutes; 20°C) and enzymatically digested in collagenase D (1 mg/ml) and DNase I (10 μg/ml) for 1 hour at 37°C. Cells were washed in PBS/FCS and resuspended in 1.088 g/ml Percoll (9 ml; Sigma, UK). This was underlayed with 1.122 g/ml Percoll (5 ml) and sequentially overlayed with 1.072 g/ml Percoll (9 ml), 1.030 g/ml Percoll (9 ml) and PBS (9 ml). Samples were centrifuged (1,250 x g; 45 minutes; 20°C) and mononuclear cells were removed from the 1.088:1.072 and 1.072:1.030 g/ml interfaces and washed twice in PBS/FCS. Cells were treated as outlined in section 2.3: ‘In vitro Treatment Protocols’. Following treatment, cells were incubated with fluorescent antibodies for analysis by flow cytometry and supernatants were removed and stored at -80°C for later analysis.

2.7 Flow Cytometry

2.7.1 Analysis of cell surface markers of activation

Expression of cell surface markers of microglial activation (CD11b, CD80, CD86, OX-6, CD68, MHC II, IA/IE) was assessed in glial cells from the brains of young and aged rats, in mononuclear cells from the brains of wildtype and APPswe/PS1dE9 mice, in primary mixed glial cells and in bone marrow-derived macrophages by flow cytometry using a DAKO CyAN ADP flow cyometer calibrated using Flow-Check Fluorospheres (Beckman Coulter, Ireland).

Mixed glial cells and BMDM were harvested following incubation with trypsin-ethylenediaminetetra acetic acid (trypsin-EDTA; 100 μl/well; 37°C; 5
minutes; Sigma, UK). Glial cells and mononuclear cells from the brains of adult rats and mice were resuspended in fluorescence activated cell sorting (FACS) buffer (100 μl). Cells were transferred to FACS tubes, centrifuged (1,200 rpm; 5 minutes; 20°C) and resuspended in FACS buffer (300 μl). Cells were washed twice in FACS buffer (300 μl) and the low-affinity IgG receptors (FcyRIII) were blocked by incubating with FACS block (1 ml/tube) for 15 minutes in the dark at 4°C or by incubating with CD16/CD32 FcyRIII block (1: 100 dilution; BD Pharmingen, USA) for 10 minutes at room temperature. Cells were washed in FACS buffer (1 ml/tube), resuspended in FACS buffer (100 μl) and incubated with the appropriate FACS antibody or isotype control antibody for 30 minutes in the dark at 4°C (See Table 2.2). Excess antibody was removed by washing each sample twice in FACS buffer (1 ml/tube). Samples were centrifuged (1,200 rpm; 5 minutes; 20°C) between washes. Immunofluorescence analysis was performed using Summit software and analysed using Summit software and Graphpad Prism.

2.7.2 Development of the phagocytic assay

An assay using quantum dots (QD) was developed to analyse the phagocytic activity of primary mixed glial cells, BMDM, mixed glial cells isolated from the brains of adult rats and mononuclear cells isolated from the brains of wildtype and APPswe/PS1dE9 mice. Initial experiments were carried out with a range of QD with emission wavelengths ranging from 511nm – 678 nm. Mixed glial cells were pre-treated with or without LPS (100 ng/ml; 24 hours), were incubated in the presence of a variety of QD preparations (1 x 10^{-7} M; 2 hours) and flow cytometry was used for analysis. A suitable QD preparation
was identified for use in later experiments based on uptake and emission wavelength.

2.7.3 Analysis of phagocytic activity

Cells were incubated in the presence or absence of QD (1 x 10^{-6} M) and were analysed by flow cytometry. Before acquiring data on any given day, the channels of the DAKO CyANA_{DP} flow cytometer were compensated using compensation beads (BD Pharmingen, UK) and the fluorescent antibodies in use on that day. During acquisition of the data, cells were chosen from the FS/SS (Forward Scanner/Side Scanner) dotplot based on size and granularity and these cells were displayed in a FS/CD11b dotplot. This allowed cells that were positive for CD11b to be isolated and displayed on other dotplots to investigate the percentage of CD11b-positive cells that were positive for QD and that were expressing cell surface markers of activation.

2.7.4 Optimising antibodies

To ensure that expression of cell surface markers of activation was accurate, a series of experiments were carried out to optimise the assay. Mixed glial cells were treated in duplicate with at least two concentrations of each antibody. Immunofluorescence analysis was performed and the results were analysed using Summit software and Graphpad Prism (GraphPad Software, USA).
**Table 2.2: Fluorochrome labeled antibodies used in flow cytometry studies.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fluorescent Label</th>
<th>Concentration (mg/ml)</th>
<th>Diln factor</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>APC (rat anti-mouse)</td>
<td>0.1</td>
<td>1/400, 1/100</td>
<td>BD Pharmingen UK</td>
</tr>
<tr>
<td></td>
<td>(M1/70) FITC (mouse anti-rat)</td>
<td>0.5</td>
<td>1/100, 1/50</td>
<td>BD Pharmingen UK</td>
</tr>
<tr>
<td></td>
<td>(ED8) PE (mouse anti-rat)</td>
<td>0.5</td>
<td>1/100, 1/50</td>
<td>BD Pharmingen UK</td>
</tr>
<tr>
<td>I-A/I-E</td>
<td>FITC (rat anti-mouse)</td>
<td>0.5</td>
<td>1/500</td>
<td>BD Pharmingen UK</td>
</tr>
<tr>
<td></td>
<td>(2G9) PE (hamster anti-mouse)</td>
<td>0.2</td>
<td>1/200, 1/100, 1/100, 1/50</td>
<td>eBiosciences UK</td>
</tr>
<tr>
<td></td>
<td>(3H5) FITC (mouse anti-rat)</td>
<td>0.5</td>
<td>1/50, 1/20</td>
<td>BD Pharmingen UK</td>
</tr>
<tr>
<td>CD80</td>
<td>PE</td>
<td>0.5</td>
<td>1/100, 1/50, 1/20</td>
<td>BD Pharmingen UK</td>
</tr>
<tr>
<td></td>
<td>(16-10A1) APC (anti-rat)</td>
<td>0.18</td>
<td>1/300, 1/30</td>
<td>eBiosciences, UK</td>
</tr>
<tr>
<td></td>
<td>(GL1) APC (anti-rat)</td>
<td>0.18</td>
<td>1/300, 1/30</td>
<td>eBiosciences, UK</td>
</tr>
<tr>
<td>MHC II</td>
<td>(HIS19) FITC (anti-rat)</td>
<td>0.25</td>
<td>1/100, 1/50</td>
<td>BD Pharmingen UK</td>
</tr>
<tr>
<td>OX6(RT1B)</td>
<td>FITC (anti-rat)</td>
<td>0.25</td>
<td>1/100, 1/50</td>
<td>BD Pharmingen UK</td>
</tr>
<tr>
<td>CD45</td>
<td>PeCy7 (rat anti-mouse)</td>
<td>0.2</td>
<td>1/100</td>
<td>BD Pharmingen UK</td>
</tr>
<tr>
<td></td>
<td>(30-F11) FITC (mouse anti-rat)</td>
<td>0.1</td>
<td>1/25</td>
<td>AbD Serotec, UK</td>
</tr>
</tbody>
</table>
2.8 Oral Administration of Rosiglitazone

Wild-type (n=10) and APPswe/PS1dE9 (n=10) mice (8 months; Jackson Laboratories, USA) were divided randomly into four groups (n=5). Each mouse received maple syrup (50 μl; Newforge, Canada) with or without added rosiglitazone (Rosiglitazone Maleate; 6 mg/kg/day; Alpha Technologies, Ireland) by oral administration for 28 days. The administration period began 2 weeks prior to behavioural testing and continued for the duration of testing. Body weight and general health was monitored throughout the duration of the study. The timeline of the study is shown in Figure 2.1.

2.9 Genotyping of APPswe/PS1dE9 mice

2.9.1 Isolation of genomic DNA from tail snips

Tail snips were obtained from wild-type and APPswe/PS1dE9 mice and stored overnight at -20°C. The following day, samples were thawed and were incubated overnight at 55°C in 500 μl of digestion buffer (97.8 ml SSC, 1 mM EDTA, 20 mM Tris-HCl pH 7-8, 10% SDS, 1% Proteinase K (20mg/ml; Sigma, UK)). DNA was extracted from the tail snips by adding phenol:chloroform:isoamyl alcohol (25:24:1; 500 μl; Sigma, UK) to each sample and samples were mixed by inversion for 5 minutes and centrifuged (15,000 rpm; 5 minutes; 20°C). The aqueous layer was removed to a fresh Eppendorf tube and the DNA was precipitated out by addition of isopropanol (500 μl; Sigma, UK) and inversion of the sample for 1 minute. Samples were centrifuged (8,000 rpm; 1 minute; 20°C) and the pellet washed with 70% ethanol (500 μl). The majority of the ethanol was removed by pipetting and samples were centrifuged (8,000 rpm; 1 minute; 20°C) and 10 mM Tris-HCl was added (50 μl; pH 8.2; Promega, USA).
Samples were heated at 65°C until all of the ethanol was evaporated. DNA concentration was determined spectrophotometrically at 260 nm using a NanoDrop Spectrophotometer (ND-1,000 v3.5, NanoDrop Technologies Inc, USA). Samples were stored overnight at 4°C.

### 2.9.2 Polymerase Chain Reaction for APPswe and PS1dE9 genes

The presence of the APPswe and PS1dE9 mutations in the mice was assessed using PCR. Samples (2 μl) were added to RT-PCR mastermix (23 μl). The RT-PCR mastermix was prepared by addition of 17.3 μl RNase-free H2O, 10X PCR reaction buffer (2.5 μl; Promega, USA), magnesium chloride (MgCl2; 1.5 μl; 25 mM; Promega, USA), nucleotide mix (0.2 μl; 25 mM; Bioline, UK), Jumpstart™ Taq DNA Polymerase (0.5 μl; Sigma, UK) and sense and antisense primers (0.5 μl; 100 μM; MWG Biotech, Germany) to a sterile tube (See Table 2.3 for primer sequences). The RT-PCR mastermix for the PS1dE9 gene also contained sense and anti-sense primers for the PrP gene as an internal control. The tubes containing the sample and the mastermix were placed in a thermocycler (MJ Research Peltier Thermal Cycler-200; Biosciences, Ireland). The amplification process consisted of an initial denaturing step of 94°C for 3 minutes, followed by 35 cycles comprising of a denaturing step of 94°C for 30 seconds, an annealing step of 67°C for 1 minute, and an extension step of 72°C for 1 minute. Upon completion of 35 cycles of amplification, a final extension step (72°C for 10 minutes) ensured complete extension of PCR products. Equal volumes of PCR products from each sample (10 μl) and a 100 base pair ladder (Promega, USA) were mixed with loading buffer (2 μl; Promega, USA) and were loaded onto a 1% (w/v) agarose gel containing ethidium bromide (0.5 μg/ml). Samples were
separated by application of 90 V for 120 minutes. PCR products were visualised under an ultraviolet light and photographed using an ultraviolet transiluminator (Labworks, Ultra Violet Products, Bioimaging Systems, USA).

**Table 2.3: Primers used for DNA amplification**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrP (control)</td>
<td>Fwd 5'-CCTCTTTGTGACTATGTGGACTGATGTCGG-3'</td>
<td>750</td>
</tr>
<tr>
<td>APPswe</td>
<td>Fwd 5'-GACTGACCACACTCGACCAGGTTCGCTG-3'</td>
<td>350</td>
</tr>
<tr>
<td>APPswe</td>
<td>Rev 5'-CTTGTAAGTGATTCTCATATCGC-3'</td>
<td></td>
</tr>
<tr>
<td>PS1dE9</td>
<td>Fwd 5'-AATAGAGAAGCGCCAGGAGCA-3'</td>
<td>608</td>
</tr>
<tr>
<td>PS1dE9</td>
<td>Rev 5'-GCCATGAGGGCACTAATCAT-3'</td>
<td></td>
</tr>
</tbody>
</table>

**2.10 Behaviour**

![Timeline of study involving wildtype and APPswe/PS1dE9 mice.](image)

*Figure 2.1: Timeline of study involving wildtype and APPswe/PS1dE9 mice.*
2.10.1 Motor Tests: Hangwire and inverted screen

Figure 2.2: Hangwire apparatus

The hangwire test assesses forelimb muscular strength and co-ordination. It consisted of a metal bar (26 cm long; 0.2 cm diameter) supported by a column at each end (19.5 cm high) (Figure 2.2). Each mouse was held by the tail, placed with its front paws at the central point of the bar, and released. A time score (maximum 60) was assigned as follows: if the mouse fell off the bar within 60 seconds, the number of seconds the mouse held onto the bar was assigned as a score; if the mouse held on for 60 seconds or reached a supporting column within 60 seconds, it received a maximum score of 60.

The inverted screen test assesses muscular strength for all four limbs. It consisted of a wooden frame (43 x 43 cm) covered with a wire mesh (12 x 12 mm squares; 1 mm diameter wire). The mouse was placed on the screen which was then slowly inverted. The time taken for the mouse to fall off the inverted screen was measured and assigned to each mouse as a time score. If the mouse had not fallen from the screen after 60 seconds, it was removed and assigned a maximum
score of 60. Padding was provided to cushion any mice that fell off either apparatus.

2.10.2 Footprint Analysis

Figure 2.3: Footprint Analysis Apparatus

Footprint analysis was used to assess motor coordination and balance in wildtype and APPswe/PS1dE9 mice. The apparatus included an open-top runway (50 cm long; 10 cm wide; 10 cm high) with an enclosed goal box (20 cm square with a 4 x 5 cm entrance hole) at one end. A3 sized white paper was laid underneath the runway and goal box. The forepaws and the hindpaws of the mouse were coated with different colours of non-toxic poster paint (Reads, Ireland) and the mouse was placed inside the runway on the sheet of white paper, at the end opposite the goal box. Each mouse was allowed 60 seconds to run over the white paper to the goal box. Data were not collected until performance was stable and the mouse ran rapidly and voluntarily to the goal box. The mouse was returned to it’s home cage and the apparatus was cleaned thoroughly with 70%
ethanol before beginning the next trial. Stride length (cm), hind-base width (cm) and front-base width (cm) were assessed.

2.10.3 Morris Water Maze Apparatus

The Morris water maze was set up in a sound-proof room with fluorescent lighting. The water maze consisted of a white circular tank 120 cm in diameter and 60 cm in height. It was filled with water at 20 - 22°C. The water was made opaque by the addition of non-toxic powder paint (Reeves Tempera Powder Paint; Crafty Devils, UK). A perspex platform, 15 cm in diameter and 23 cm in height, was submerged 1.5 cm below the water surface and 13 cm from the edge of the pool wall in the southeast quadrant. The surface of the platform was coarse to ensure that the mice could grip onto the platform once it was found. Two white walls and two white screens (110 cm in height by 120 cm in width; Figure 2.4) surrounded the water maze at an equal distance of 30 cm and each bore two distinct visual cue cards (30 cm x 30 cm; Figure 2.5) at differing heights. The visual cues were cut from coloured art paper and included a blue circle, a square box bearing a white and black diagonal striped pattern, a blue square and a green triangle among other shapes. A camera was fixed to the ceiling above the water maze and connected to the computer-based tracking programme Water 2020 (HVS Image, UK). The pool was divided into four quadrants namely, north, south, east and west, by the aid of an imaginary line using the Water 2020 software. The parameters recorded were latency to the platform (s), path length (cm), swim speed (cm/s), thigmotaxis (% time) and time spent in quadrant-containing platform (% time).
Figure 2.4: Morris Water Maze Apparatus
Figure 2.5: Visual Cues used in Morris Water Maze
2.10.4 Morris water maze training

Prior to recording, mice were subjected to a single habituation session in which the platform was visible above the water level and marked with a red flag. No visual cues were used during the habituation session. Each mouse was placed randomly into the pool facing the pool wall and was guided to the platform by the experimenter using a plastic rod. All mice found the platform within 2 minutes and then remained on it for one minute to become familiar with the trial objective and the surrounding area.

Training commenced the following day for 5 consecutive days. During the training sessions the platform was submerged 1.5 cm below the water and the visual cues were put in place. Each training day consisted of four 1 minute trials with an inter-trial interval of 3 minutes during which the mouse was returned to a holding cage. The holding cage was heated to 25°C using a hot water bottle placed underneath the cage. Each mouse was gently lowered into the pool facing the pool wall. During each trial, mice were released from random start points (i.e. north, south, east or west) and these start points were randomized daily by means of a random sequence generator programme (www.random.org). Each trial consisted of a fixed time period of 1 minute in which the mouse had to locate the platform; failure of the mouse to find the platform within this time period resulted in it being placed on the platform by the experimenter. Following each trial, regardless of success or failure to locate the position of the platform, each mouse was placed on the platform for 20 seconds to become familiar with the location of the platform relative to the proximal visual cues.

The probe trial was carried out 24 hours after training ended and consisted of a single 1 minute trial in which the platform was removed from the water maze.
Chapter 2 Methods

The probe trial is a measure of an animal's spatial learning ability and ability to retain and access previously learned information. The percentage of time each mouse spent swimming in the southeast quadrant (i.e. the quadrant in which the platform was previously located) was measured.

2.10.5 Morris water maze reversal training

Reversal training commenced 24 hours later and continued for 6 consecutive days. The location of the platform was reversed from it's location during the initial training phase and the platform was relocated to the northwest quadrant. All other training protocols remained the same. Reversal training gives an indication of plasticity of learning since it assesses the ability of the mouse to unlearn the initial location of the platform and to learn a new location. Following reversal training, another probe trial was conducted 24 hours later. The platform was removed from the maze and the percentage time each mouse spent swimming in the northwest quadrant was measured.

2.10.6 Morris water maze statistical analyses

Differences in latency, path length, swim speed, thigmotaxis and time spent in the quadrant-containing platform were assessed by two-way ANOVA analysis for repeated measures, using SPSS version 14.0, with latency, path length, swim speed, thigmotaxis and time spent in the quadrant-containing platform as within-subject factors and treatment with rosiglitazone as the between-subject factor. Post-hoc pair comparisons, using the Tukey's HSD test, were calculated where appropriate.
2.11 Cytokine Analysis

2.11.1 Analysis of IL-1β concentration

The enzyme-linked immunosorbent assay (ELISA) method was used to determine the concentration of IL-1β in supernatant from cultured primary mixed glia or BMDM. Cytokine concentration of IL-1β was measured using a commercially-available ELISA kit (R&D Systems, UK).

In brief, 96-well plates (Nunc Immuno, Denmark) were coated with capture antibody (50 µl/well; 0.8 µg/ml; goat anti-rat IL-1β) in PBS and incubated overnight at 4°C. Plates were washed several times in wash buffer (PBS containing 0.05% Tween-20) and non-specific binding sites were blocked with assay diluent (100 µl; 1 hour; RT) (PBS, pH 7.3, with 1% bovine serum albumin (BSA) for IL-1β. Plates were incubated with IL-1β standard recombinant protein (0 – 2,000 pg/ml; 50 µl/well; 2 hours; RT) and samples (50 µl/well; 2 hours; RT). Plates were washed three times in wash buffer, incubated with detection antibody (biotinylated goat anti-rat IL-1β; 50 µl/well; 350 ng/ml in assay diluent with 200 µl of normal goat serum (Vector Laboratories, UK); 2 hours; RT). Plates were washed three times in wash buffer and incubated with horseradish peroxidase (HRP)-conjugated streptavidin (50 µl/well; 1:200 in assay diluent for IL-1β ELISA; 20 – 30 minutes; RT) in the dark. Plates were washed three times with wash buffer and substrate solution (50 µl/well; R&D systems, UK) or tetramethylbenzidine (TMB; 50 µl/well; Sigma, UK) was added to each well for 20 – 30 minutes in the dark. The enzyme reaction was stopped by addition of 25 µl of 1 M H₂SO₄. Absorbance was read at 450 nm within 30 minutes (Labsystems Multiskan RC). A standard curve was constructed using the reference values of the standard recombinant proteins, and cytokine
concentrations contained in the test samples were evaluated with reference to this
standard curve. Data were expressed in pg/ml supernatant (GraphPad Prism v4.0;
GraphPad Software, USA).

2.12 Immunocytochemistry

2.12.1 Confocal analysis of CD11b, OX-6 and GFAP expression, and QD
uptake, by mixed glial cells.

Mixed glial cells were grown for 10 – 14 days on coverslips in 24-well
plates and were treated with Aβ1-42 (2 µM or 8 µM) for 30 minutes or 24 hours and
incubated in the presence or absence of QD (1 x 10^-6 M) for 2 hours. Cells were
washed with PBS, fixed with alcohol for 5 minutes and washed again with PBS.
Cells were blocked with Normal Goat Serum (NGS; 10% in PBS; 2 hours; RT;
Vector, UK), washed with PBS and incubated overnight at 4°C with the
appropriate 1° antibody (mouse anti-rat CD11b, 0.25 µg/ml; mouse anti-rat OX-6,
10 µg/ml; rabbit anti-rat GFAP, 1.2 µg/ml). Antibodies were diluted in PBS with
5% NGS. Isotype controls (normal mouse IgG; normal rabbit IgG) were also
added to the mixed glial cells at the same concentration as the corresponding 1°
antibody. The following day, cells were washed thoroughly with PBS and were
incubated in the presence of Hoechst reagent (1 in 1,000 dilution factor) and the
correct 2° antibody conjugated to a fluorophore (CD11b/OX-6 2° antibody: goat
anti-mouse Alexa 488 nm; 0.5 µg/ml; GFAP 2° antibody: goat anti-rabbit Alexa
488 nm; 0.5 µg/ml; 2 hours; RT; in the dark). The 2° antibodies were the same
for the isotype controls. Cells were washed with PBS every 5 minutes for 1 hour
in the dark. Coverslips were mounted using vectashield on glass slides and sealed
with clear nail polish. Slides were allowed to dry overnight in the dark and were
then ready to visualise on the confocal microscope. Slides were stored at 4°C in the dark until required for analysis.

2.13 Immunohistochemistry

2.13.1 Preparation of tissue sections for immunohistochemistry

Slices for immunohistochemistry were prepared from tissue isolated from the brains of female wild-type and APPswe/PS1dE9 mice either treated or untreated with rosiglitazone. For cryostat sectioning, a portion of the right hemisphere of the brain, along the midline, was mounted in OCT on a circular cork and frozen in ice-cold isopentane that was chilled on dry ice. Brain sections were stored at -80°C until required for sectioning. Frosted pre-subbed microscope slides (Fischer Scientific Ltd, Ireland) were used for mounting as they provided a suitable surface to which the section could adhere. On the day of sectioning the portion of brain was allowed to equilibrate to -20°C for 2 hours. Saggital sections (10 μm) were cut, and periodically stained with a 1% toluidine blue solution until the hippocampus was visible on the section. Toluidine blue was added for 30 seconds, washed off with running water and sections were viewed by light microscopy (Nikon Labophot, Nikon Instech Co, Japan). When the hippocampus became visible, 60 sections (10 μm) were cut from each animal onto 20 pre-subbed slides (3 sections per slide), making sure that one section from the outside, middle and inside of the brain portion was added to each slide. Sections were allowed to dry for 20 minutes and stored at -20°C until required.
2.13.2 Immunohistochemical staining for Aβ plaques using Congo red

Brain sections were stained for Aβ plaques using the Congo red staining method. Sections were allowed to equilibrate to RT for 30 minutes, were fixed with ice-cold ethanol (5 minutes) and were washed twice (5 minutes) in PBS. During the washes, sodium hydroxide solution (NaOH; 1 M; 2 ml) was added to saturated sodium chloride (NaCl; 200 ml) to produce an alkaline solution. Sections were incubated in alkaline-saturated NaCl for 20 minutes at RT. During this incubation, congo red solution (200 ml; Sigma, UK) was filtered through a syringe using a cellulose acetate membrane filter (0.2 μm) and mixed with NaOH (1 M; 2 ml) to generate an alkaline solution. Sections were incubated in alkaline congo red solution for 30 minutes at RT and were rinsed briefly (2 dips) in dH₂O to remove excess stain. Sections were incubated in a 1% (w/v) methyl green solution (Sigma, UK) for 30 seconds to counterstain the tissue, washed in running dH₂O for 1 minute to remove excess stain and dehydrated by dipping 6 times each in 95% ethanol, 100% ethanol and 100% ethanol. Sections were dried briefly to remove excess alcohol and incubated for 5 minutes each in 3 xylene dishes. Coverslips were mounted onto the slides using the xylene-based mountant, depex polystyrene (DPX; Electron Microscopy Sciences, USA), and allowed to dry in a fume hood overnight.

2.13.3 Quantification of cerebral amyloid load

Congo red-positive Aβ plaques were counted under the same light and magnification settings in 6 representative sections from each animal and results were expressed as the number of plaques per section.
2.13.4 Fluorescent immunohistochemical staining for Aβ plaques

Sections were allowed to equilibrate to RT for 30 minutes. Individual brain sections were surrounded with a hydrophobic well using a cytometion pen (Dako, UK). Sections were fixed in ice cold ethanol (5 minutes) and washed three times with PHEM buffer (25 mM HEPES, 10 mM EGTA, 60 mM PIPES, 2 mM MgCl₂, pH 6.9). Sections were permeabilised with Triton X-100 (5 minutes) and washed three times with PHEM buffer. Non-specific interactions were blocked using 250 µl of blocking solution per slice (10% NGS in 4% BSA in PHEM buffer) for 2 hours at RT. Blocking solution was removed and sections were incubated overnight with 1° antibody, rabbit anti-human anti-pan β amyloid15-30 (0.834 µg/ml in 2% BSA in PHEM with 5% NGS). Negative controls were incubated in 2% BSA in PHEM with 5% NGS alone. Sections were washed three times with PHEM buffer and incubated in the dark with 2° antibody (Alexa 488-conjugated goat anti-rabbit IgG; 0.25 µg/ml in 2% BSA in PHEM with 5% NGS; 1.5 hours; RT). Sections were washed with PHEM buffer for 1.5 hours while covered to avoid bleaching the fluorescent label. The buffer was changed 15 times to remove as much of the fluorescent background as possible. Coverslips were mounted with a DAPI nucleic counter-stain enhanced mountant (Vector, UK), sealed with clear nail polish and stored at 4°C until analysis.
2.13.5 Double immunofluorescent labelling: Analysis of microglial activation and Aβ plaque deposition.

Sections were prepared as described in section 2.13.4 ‘Fluorescent immunohistochemical staining for Aβ plaques’. Prior to mounting of coverslips, sections were blocked again for 2 hours at RT (250 µl blocking solution/slice; 10% NGS in 4% BSA in PHEM buffer). Blocking solution was removed and sections were incubated overnight with a second 1° antibody rat anti-mouse CD11b (20 µg/ml or 4 µg/ml in 2% BSA in PHEM with 5% NGS). Negative controls were incubated in 2% BSA in PHEM with 5% NGS alone. Sections were washed three times with PHEM buffer and incubated in the dark with a second 2° antibody, Alexa 633-conjugated goat anti-rat IgG (0.25 µg/ml in 2% BSA in PHEM with 5% NGS; 1.5 hours; RT). Sections were washed with PHEM buffer for 1.5 hours in the dark and the buffer was changed 15 times to remove as much of the fluorescent background as possible. Coverslips were mounted with a DAPI nucleic counter-stain enhanced mountant, sealed with clear nail polish and stored at 4°C until analysis.

2.13.6 Microscopy

Fluorescently stained cryostat sections were viewed with a Zeiss 510 Meta confocal laser microscope (Zeiss Ltd, UK) consisting of an Axiovert 200M inverted microscope. Images were acquired and optimised using the LSM 510 computer program at 20X, 40X and 60X magnification.
## Table 2.4: Antibodies used in immunohistochemical staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Host Species</th>
<th>Reactivity</th>
<th>Conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b (OX-42)</td>
<td>AbD Serotec</td>
<td>Mouse</td>
<td>Rat</td>
<td>0.25</td>
</tr>
<tr>
<td>MHC II (OX-6)</td>
<td>AbD Serotec</td>
<td>Mouse</td>
<td>Rat</td>
<td>10</td>
</tr>
<tr>
<td>CD68 (ED1)</td>
<td>AbD Serotec</td>
<td>Mouse</td>
<td>Rat</td>
<td>2.5</td>
</tr>
<tr>
<td>GFAP</td>
<td>AbD Serotec</td>
<td>Rabbit</td>
<td>Rat</td>
<td>1.2</td>
</tr>
<tr>
<td>Pan β-amyloid_{15.30}</td>
<td>Merck</td>
<td>Rabbit</td>
<td>Human</td>
<td>0.834</td>
</tr>
<tr>
<td>CD11b (M1/70.15)</td>
<td>AbD Serotec</td>
<td>Rat</td>
<td>Mouse</td>
<td>20, 4</td>
</tr>
<tr>
<td>MHC II (IBL-3/5)</td>
<td>AbD Serotec</td>
<td>Rat</td>
<td>Mouse</td>
<td>50, 5, 1</td>
</tr>
<tr>
<td>CD68 (FA-11)</td>
<td>AbD Serotec</td>
<td>Rat</td>
<td>Mouse</td>
<td>50, 5, 1</td>
</tr>
<tr>
<td>2° antibody</td>
<td>Biosciences, UK</td>
<td>Goat</td>
<td>Mouse</td>
<td>0.5</td>
</tr>
<tr>
<td>Alexa 488nm</td>
<td>Ireland</td>
<td>Goat</td>
<td>Rat</td>
<td>0.25</td>
</tr>
<tr>
<td>2° antibody Alexa</td>
<td>Biosciences, UK</td>
<td>Goat</td>
<td>Rat</td>
<td>0.25</td>
</tr>
<tr>
<td>546/633nm</td>
<td>Ireland</td>
<td>Goat</td>
<td>Rabbit</td>
<td>0.25</td>
</tr>
<tr>
<td>2° antibody</td>
<td>Biosciences, USA</td>
<td>Goat</td>
<td>Rabbit</td>
<td>0.25</td>
</tr>
<tr>
<td>Alexa 488nm</td>
<td>Ireland</td>
<td>Goat</td>
<td>Rabbit</td>
<td>0.25</td>
</tr>
<tr>
<td>Control mouse IgG</td>
<td>Santa Cruz Biotechnology, USA</td>
<td>Mouse</td>
<td>Non-specific</td>
<td>Same as 1° antibody</td>
</tr>
<tr>
<td>Control rabbit IgG</td>
<td>Santa Cruz Biotechnology, USA</td>
<td>Rabbit</td>
<td>Non-specific</td>
<td>Same as 1° antibody</td>
</tr>
</tbody>
</table>
2.14 Live Cell Imaging of QD uptake by mixed glial cells

Mixed glial cells were cultured in glass bottom dishes (MatTek Corporation, USA) at $1 \times 10^5$ cells/ml for 10-14 days and treated with Aβ$_{1-42}$ (2 μM; 24 hours). Cells were incubated in the presence of the nuclear stain Hoechst (1 in 10,000 dilution; 5 minutes), the culture dish was placed on the heated stage of the confocal microscope and the mixed glial cells were visualised. The confocal microscope was programmed to acquire one image every 2 minutes for 30 minutes. Once a single image had been acquired, the QD were added to the culture dish ($2.6 \times 10^8$ M). During recording intervals, the field of view could be adjusted on the confocal microscope until an ‘active’ cell was identified. Once the cell of interest was identified, it was recorded at 60X magnification for the remainder of the 30 minute period.

2.15 Analysis of Cell Viability in vitro

2.15.1 Propidium Iodide Assay

Mixed glial cells were incubated in the presence of Aβ$_{1-42}$ (8 μM; 24 hours), NaF (10 mM; 1 hour), H$_2$O$_2$ (100 μM; 15 minutes) or QD (1 x 10$^{-6}$ M; 2 hours), washed with PBS (5 ml), harvested by gentle scraping, centrifuged (1,200 rpm; 5 minutes; 20°C) and washed with ice-cold PBS (5 ml). The pellet was resuspended in PBS (200 μl), ice-cold ethanol (70%; 2 ml) was added and cells were fixed on ice (1 hour). Cells were centrifuged (1,200 rpm; 5 minutes; 20°C) and the ethanol was removed. The pellet was resuspended in PBS (400 μl), RNAse A (20 μl; 10 mg/ml) and Propidium iodide (PI; 75 μl; 1 mg/ml), and incubated at 37°C for 30 minutes in the dark. Cell cycle analysis was performed using Cellquest software package.
2.15.2 MTS Assay

CellTiter 96® AQquious One Solution Cell Proliferation Assay (Promega, Ireland) is a colorimetric method for determining the number of viable cells in culture. It contains MTS, a tetrazolium compound, (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron-coupling reagent (phenazine ethosulfate). Following treatment of mixed glial cells with H$_2$O$_2$ (100 μM; 15 minutes), MTS (20 μl/100 μl cDMEM) was added and incubated for 4 hours at 37°C. Absorbance was read at 490 nm. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

2.16 Real-time Polymerase Chain Reaction (PCR)

2.16.1 Preparation of tissue for RNA isolation

Tissue from in vivo studies was homogenised in 350 μl of cell lysis mastermix (Nucleospin RNA II, Macherey-Nagel) for extraction of RNA. Lysate was filtered using NucleoSpin Filter, collected in an Eppendorf tube and centrifuged (11,000 x g; 1 minute; RT). Ethanol (70%; 350 μl) was added to the filtrate, mixed and loaded onto NucleoSpin RNA II columns. Tubes were centrifuged (8,000 x g; 30 seconds; RT) and the RNA bound to the column. The silica membrane was desalted by adding membrane desalting buffer (350 μl) and centrifuged (11,000 x g; 1 minute; RT) to dry the membrane. To digest the DNA, DNase reaction mixture (95 μl) was added to the column and incubated at RT for 15 minutes. The silica membrane was washed and dried. RNA was eluted by adding RNase-free H$_2$O, and by centrifugation (11,000 x g; 1 minute; RT). RNA concentration was measured using a NanoDrop Spectrophotometer.
2.16.2 Reverse transcription for cDNA synthesis

Total mRNA (1 μg/ml) was reverse transcribed into cDNA using high-capacity cDNA archive kit (Applied Biosystems, Germany) according to the protocol provided by the manufacturer. Briefly, RNA (1 μg) was added to fresh tubes containing the appropriate volume of nuclease-free H₂O to make a 25 μl volume. A 2X mastermix was prepared containing the appropriate volumes of 10X RT buffer, 25X dNTPs and 10X random primer multiscribe reverse transcriptase (50 U/μl). The mastermix (25 μl) was added to the RNA and nuclease-free H₂O. Tubes were incubated for 10 minutes at 25°C followed by 2 hours at 37°C on a thermocycler.

2.16.3 Real-time PCR

Real-time PCR primers and probes were delivered as “TaqMan® Gene Expression Assays” for the rat and mouse genes listed in Table 2.4 (Applied Biosystems, Germany). Real-time PCR was performed on Applied Biosystems ABI Prism 7300 Fast Track Sequence Detection System v1.3.1 in 96-well format with 25 μl reaction volume per well. cDNA (200 pg/well) was mixed with Taqman Universal PCR Fast Mastermix (Applied Biosystems, Germany) and the respective target gene assay. Either mouse β-actin RNA (Applied Biosystems, Germany) or rat β-actin RNA (Applied Biosystems, Germany) was used as a reference. Each sample was measured in duplicate in a single RT-PCR run. Forty cycles were run with the following conditions: 2 minutes at 50°C, 10 minutes at 95°C and, for each cycle, 15 seconds at 95 °C for denaturation and 1 minute at 60°C for transcription. The expression of each target gene was determined using the efficiency-corrected comparative CT method, which calculated target gene
expression relative to β-actin endogenous expression. These values were
normalised to the control sample and the relative differences in expression of the
target gene between samples were expressed as a ratio.

Table 2.5. Primers used in this study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene Description</th>
<th>Assay number</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
<td>Mm00434228_ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
<td>Mm00446191_ml</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor Alpha</td>
<td>Mm00443258_ml</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Toll-like Receptor 4</td>
<td>Mm00445273_ml</td>
</tr>
<tr>
<td>TLR-2</td>
<td>Toll-like Receptor 2</td>
<td>Mm00442346_ml</td>
</tr>
<tr>
<td>CD11b</td>
<td>Cluster of Differentiation 11b</td>
<td>Mm1271263_ml</td>
</tr>
<tr>
<td>CD68</td>
<td>Cluster of Differentiation 68</td>
<td>Mm03047343_ml</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major Histocompatibility Complex II</td>
<td>Rn01768597_ml</td>
</tr>
<tr>
<td>CD11b</td>
<td>Cluster of Differentiation 11b</td>
<td>Rn00709342_ml</td>
</tr>
<tr>
<td>CD68</td>
<td>Cluster of Differentiation 68</td>
<td>Rn01495631_g1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor Alpha</td>
<td>Rn99999017_ml</td>
</tr>
<tr>
<td>CD40</td>
<td>Cluster of Differentiation 40</td>
<td>Rn00584362_ml</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation</td>
<td>Rn00584249_ml</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
<td>Rn00566603_ml</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon Gamma</td>
<td>Rn00594078_ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
<td>Rn00561420_ml</td>
</tr>
</tbody>
</table>
2.17 Protein Assay

Protein concentrations in cerebellar tissue prepared from wild-type and APPswe/PS1dE9 mice were determined using a bicinchoninic acid protein assay kit (Pierce, The Netherlands). To make the working reagent, 500 µl of Reagent B was added to 25 ml of Reagent A. A standard curve was prepared (0 – 2,000 µg/ml) using BSA diluted in the same buffer as the samples to be tested. Aliquots (25 µl) of sample/standard were added in triplicate to a 96-well plate. Working reagent (200 µl) was added to each well and the plate was then incubated for 30 minutes at 37°C to allow the colour to develop. The optical density values were determined by measuring the absorbance at 590 nm using a microtitre plate reader (Multiskan RC, Labsystems, Finland) and protein concentrations in the samples were calculated with reference to the standard curve.

2.18 Determination of Aβ concentration in tissue samples

2.18.1 Extraction of soluble and insoluble Aβ from brain tissue

Soluble and insoluble Aβ were extracted from snap-frozen cerebellar tissue obtained from wild-type and APPswe/PS1dE9 mice treated or untreated with rosiglitazone. Tissue for Aβ extraction was homogenised in 5 volumes (w/v) of homogenising buffer (SDS/NaCl in dH₂O with added proteases, pH 10) and samples were centrifuged (15,000 rpm; 40 minutes; 4°C). Pellets were stored at -80°C for isolation of insoluble Aβ.

Supernatants were used to extract SDS-soluble Aβ. Protein concentrations in supernatants were equalised to 8 mg/ml with homogenisation buffer using a bicinchoninic acid protein assay (see Section 2.18). The protein concentration was maintained above 2 mg/ml to ensure that SDS was diluted to less than 0.1%
on the plate. Equalised supernatants were aliquoted and neutralised by addition of 10% (v/v) 0.5 M Tris-HCl (pH 6.8). Samples were stored at -20°C for later detection of soluble Aβ.

Pellets were used for the extraction of insoluble Aβ and were disrupted by ultrasonication (23 kHz; 2 x 30 seconds) in guanidine buffer (5 M guanidine-HCl in ddH2O; Sigma, UK), incubated on ice (4 hours), and centrifuged (15,000 rpm; 30 minutes; 4°C). Supernatants were equalised to 0.4 mg/ml with guanidine buffer, aliquoted, and stored at -20°C for later detection of insoluble Aβ.

2.18.2. Detection of SDS-soluble and insoluble Aβ by multi-spot ELISA

Concentrations of Aβ in SDS-solubilised and guanidine-treated samples were determined using “MSD® 96-well multi-spot 4G8 Aβ triple ultra-sensitive assay” kits (Meso Scale Discovery, USA). Multi-spot Aβ 3-plex plates were blocked by adding 150 μl of 1% Blocker A (Meso Scale Discovery, USA) for 1 hour at RT with shaking. Plates were washed 3 times in 1X Tris wash buffer (TWB). Detection antibody solution (25 μl; Meso Scale Discovery, USA) was added to each well. SDS-solubilised samples were diluted to 200 μg/ml in kit buffer and 25 μl of sample was added in duplicate to the appropriate wells of a 96-well plate (5 μg/well). Guanidine-treated samples were diluted to 1 μg/ml in TWB and 25 μl of sample was added in duplicate to the appropriate wells of the 96-well plate (25 ng/ml). Serially-diluted recombinant human Aβ1-38 (0 - 3,000 pg/ml), Aβ1-40 (0 - 10,000 pg/ml), and Aβ1-42 (0 - 3,000 pg/ml) in 1% Blocker A solution were added to appropriate wells of the 96-well plate, which was incubated for 2 hours at RT. Plates were washed in wash buffer, and 150 μl of 2X MSD read buffer (Meso Scale Discovery, USA) was added. The plate was read
immediately using a Sector Imager plate reader (Meso Scale Discovery, USA) and Aβ concentrations in test samples were evaluated with reference to the standard curve prepared using recombinant Aβ1-38, Aβ1-40, and Aβ41-42.

2.19 Statistical Analysis

Data were analysed (GraphPad Prism v.5) using either Student’s t-test for independent means or a one-way or two-way ANOVA followed by Newman-Keuls or Bonferoni post hoc tests to determine statistical differences. Morris Water Maze data was analysed using a Two-Way Repeated Measures ANOVA and the statistical programme SPSS v.14. Data are expressed as means with standard errors (SEM) and deemed statistically significant when \( p<0.05 \).
Chapter 3

Development of an assay to assess phagocytosis using quantum dots
3.1 Introduction

Phagocytosis is a key mechanism of innate defence against invading pathogens and is a function of macrophages, neutrophils and dendritic cells of the PNS and the microglia of the CNS. It is crucial in ageing for maintenance of CNS homeostasis and prevention of secondary inflammation by removal of cellular debris and in AD it has been proposed that decreased clearance of Aβ by phagocytosis contributes to the increased accumulation of Aβ (Witting et al, 2000; Takata et al, 2007; Floden and Combs, 2006).

Microglia are described as the macrophages of the CNS. Within the CNS, peripheral macrophages that infiltrate during inflammation and disease, display similar markers to resident activated microglia (Smith, 2001). The primary objective of this study was to develop a method for analysis of phagocytosis by both microglia and macrophages. The potential modulatory effects of IL-1β, LPS and Aβ1-42, as well as two known inhibitors of phagocytosis, NaF and H₂O₂, were investigated. NaF is an inhibitor of glycolysis that depresses ATP levels in cells and thereby inhibits phagocytosis (Mazur and Williamson, 1977). Increased levels of ROS are observed in the aged brain and H₂O₂, which can generate ROS, was used as a model of oxidative stress (Radak et al, 2004).

The most common phagocytic assay involves the use of commercially available latex bead (Fukasawa et al, 1997; Tsujimoto et al, 2008; Dumrese et al, 2009; Smith, 2001; Koenigsknecht-Talboo and Landreth, 2005; Takeda et al, 1998). Visualisation of the internalisation of these beads is generally facilitated by fluorescent labelling. Here, QD which are fluorescent semiconductor nanocrystals that are produced with characteristic emission wavelengths that are size-dependent, were used to assess phagocytosis. Thioglycolic acid (TGA)-
stabilised QD have previously been used as live cell imaging tools in the human THP-1 monocyte cell line where they were internalised within 10 minutes (Byrne et al., 2006). Here, QD were used to develop a phagocytic assay using flow cytometry to investigate primary microglial and BMDM cultures as well as microglia isolated from the brains of adult rats and mice.

The aims of the study were:

- To develop a phagocytic assay using flow cytometry and QD.
- To assess the potential modulators of phagocytosis IL-1β, LPS, Aβ1-42, NaF, H2O2 and GM-CSF.
- To assess the effect of these modulators of phagocytosis on cell viability.
- To determine the optimal time point for aggregation of Aβ.
- To assess internalisation of QD by mixed glia.
3.2 Results

3.2.1 Identifying the cell population of interest

Phagocytic activity of cultured mixed glia, BMDM and glia from brain tissue of young and aged rats was investigated by FACS. Cells identified by size and granularity appeared in a specific area of a forward versus side scanner dot-plot (R1; Figure 3.1). The \textit{in vitro} preparations of mixed glia and bone marrow-derived macrophages displayed similar characteristics in terms of size and granularity and appeared in the same region. In both of the \textit{ex vivo} preparations, the cells of interest also displayed similar characteristics to each other.

A standardised method for analysis of FACS data was established and representative dot-plot scatter analyses of cells are seen in Figure 3.2. The areas of interest in these dot-plots are highlighted using red boxes; A identifies unstained cells, B identifies CD11b\(^+\) cells where CD11b is conjugated to fluorescein isothiocyanate (FITC), C identifies cells that have taken up QD (Sb184) and D identifies CD11b\(^+\) cells that have taken up QD. The negative control, where no CD11b antibody was present, is shown in Figure 3.3 A and C.

3.2.2 Choosing a Quantum Dot

Preliminary experiments in mixed glia and BMDM with a variety of QD preparations (Table 3.1) identified a selection that was taken by cells. Uptake of QD varied with preparation (Table 3.2) and QD preparations that were not taken up either by mixed glia (Figure 3.4A) or by BMDM (Figure 3.5 C and D) were eliminated from the study. Positively charged QD were unable to be taken up by
BMDM, treated with or without LPS, and were eliminated from the study (Figure 3.7 B and C). The emission spectra of the QD preparations that were taken up by mixed glia and BMDM in vitro were compared with the emission spectra of the fluorescently-labelled antibodies intended for use in the study. QD, whose emission spectra did not overlap with the emission spectra of the fluorescently-labelled antibodies (Table 3.3), were chosen for use in the phagocytic assay.

### 3.2.3 Choosing antibodies for flow cytometry

Antibodies against the cell surface markers of activation listed in Table 3.4 were tested in vitro with mixed glia and BMDM and ex vivo with cells isolated from the brains of young and aged rats, to assess their suitability for use in the phagocytic assay. Following preliminary experiments, MHC II-APC, CD45-FITC and OX-6-FITC were rejected as useful antibodies due to bad staining. CD11b (−PE, −FITC, −APC), OX-6-PerCP, CD40-FITC, CD80-PE and CD86-FITC were identified as suitable antibodies for use in analysis of expression of cell surface markers of activation. In cases where a single antibody is listed with more than one stain, the stain that facilitated use of all the required markers was chosen for a given experiment.

### 3.2.4 Assessing potential modulators of phagocytosis

The effects of potential modulators of phagocytosis on the CD11b+ cell population were assessed to determine if the modulators were suitable for use in the phagocytic assay (Table 3.5). Treatment of mixed glial cells with LPS (100ng/ml) for 24 h was found to significantly reduce CD11b expression by mixed glia (*p<0.05; student’s t-test for independent means; n=20; Figure 3.8 A).
Suitability of LPS as a stimulus for the phagocytic assay was further investigated by assessing the effect of LPS on uptake of five different QD preparations by mixed glial cultures. LPS did not enhance QD uptake of four out of five preparations by mixed glia (Figure 3.9). For these reasons, LPS was deemed unsuitable for use as a stimulus in the phagocytic assay. There was no significant effect of IL-1β, Aβ_{1-42}, NaF, GM-CSF or H_{2}O_{2} on CD11b expression by mixed glia (n=18; Figure 3.8 B-F). These treatments were used for the phagocytic assay.

### 3.2.5 Assessment of Aβ_{1-42} aggregation state and effect on phagocytic activity of CD11b^{+} cells.

A thioflavin T assay was conducted to assess aggregation of Aβ_{1-42}. Following incubation, aggregation increased in a time-dependent manner and at 48 hours post-reconstitution, Aβ_{1-42} was significantly aggregated (**p<0.01; ANOVA; n=8-17; Figure 3.10). Analysis of the effect of Aβ_{1-42} on phagocytic activity of CD11b^{+} cells revealed no significant effect at 2 μM, 4 μM or 8 μM on QD uptake by CD11b^{+} cells (n=6; Figure 3.11).

### 3.2.6 Comparison of QD and fluorescent latex particles

Fluorescent latex particles are commercially available and analysis of their uptake by flow cytometry is an established phagocytic assay (Steinkamp et al, 1982). Experiments were conducted to compare QD uptake with the uptake of a range of sizes of fluorescent latex particles (30 nm, 500 nm, 2,000 nm). Analysis by flow cytometry revealed that pre-treatment with H_{2}O_{2} significantly inhibited uptake of both fluorescent particles and QD by mixed glia (*p<0.05, **p<0.01, ***p<0.001; ANOVA; n=5-7; Figure 3.12). Similarly, NaF significantly
inhibited the uptake of all particles (*p<0.05, **p<0.001; ANOVA; n=3-7; Figure 3.12) except the 500nm particle (Figure 3.12 B). Aβ1-42 had no significant effect on the uptake of QD or of 500 nm or 2,000 nm fluorescent latex particles (Figure 3.13) but significantly increased uptake of 30 nm fluorescent latex particle (Figure 3.13 C).

3.2.7 Assessment of cell viability following treatment

A propidium iodide assay was performed to assess cell viability following treatment of mixed glia with Aβ1-42, H2O2, NaF, QD or Aβ1-42 and QD. There was no effect of any individual treatment on the percentage of cells in the apoptotic phase of the cell cycle (Figure 3.14 A). However, there was an increased percentage of cells in the apoptotic phase of the cell cycle when cells were treated with Aβ1-42 and QD in combination. A MTS assay confirmed that there was no significant effect of H2O2 on cell viability (Figure 3.14 B).

3.2.8 Assessment of internalisation of QD

Immunocytochemistry and confocal microscopy were performed to visualise internalisation of QD in a mixed glial culture. The images indicate that QD were internalised by less than 50% of the CD11b+ microglia (Figure 3.15 A) and were localised to the cytoplasm within 2 hours of incubation at 37°C (Figure 3.15 B). Assessment of QD internalisation by GFAP+ astrocytes showed that a small proportion of astrocytes were capable of internalising QD (Figure 3.16 A). Analysis of QD internalisation by microglia and astrocytes in the same mixed glial preparation showed that a significantly greater proportion of microglia internalised QD compared with astrocytes (Figure 3.17). A live cell imaging time
series analysis was performed to confirm that QD were being phagocytosed by mixed glia and that they were not passively diffusing across the membranes of cells (Figure 3.18). Cells were observed to be highly motile and capable of finding QD in the culture dish. Cells ingested QD (Figure 3.18 F), digested them (as indicated by a colour change in the QD; Figure 3.18 D) and released the digested product into the extracellular space by exocytosis (Figure 3.18 L). It was also observed that these phagocytic cells interacted with each other (Figure 3.18 I).
Chapter 3 Development of an assay to assess phagocytosis using quantum dots

Figure 3.1: Cell populations were identified based on forward and side scatter characteristics.

Representative dot-plot scatter analyses of cells from four different preparations with cells of interest R1 identified; (A) mixed glia, (B) BMDM, (C) cell suspension from brain of young adult rat and (D) cell suspension from brain of aged adult rat. Cells of interest were identified by size and granularity, characteristic of the cell type. Mixed glia and BMDM displayed similar characteristics in terms of size and granularity, and cells prepared from brains of young and aged rats also displayed similar characteristics to each other.
Figure 3.2: Analysis of FACS dot-plots.

Representative dot-plot scatter analyses of cells are displayed. Areas of interest are highlighted in red boxes: A represents cells unstained for cell surface markers of activation and that do not contain QD. B represents cells that express a given cell surface marker of activation; in this case, the cell surface marker is CD11b and it is conjugated to fluorescein isothiocyanate (FITC). C represents cells that have taken up QD (Sb184). D represents cells that express the cell surface marker CD11b and that have taken up QD.
Figure 3.3: Expression of CD11b by mixed glia.

Cells identified by size and granularity from a population of mixed glia were assessed for CD11b expression. Analyses are displayed in histogram (A and B) and dot-plot (C and D) format. As a negative control, cells were incubated in the absence of CD11b antibody (A and C) and no CD11b^\textsuperscript{+} cells were observed (A; R40). Incubation of cells in the presence of CD11b antibody identified labelled cells (B; R40 and D; R2). These CD11b^\textsuperscript{+} gated cells were later analysed for expression of cell surface markers of activation and phagocytic activity.
### Table 3.1: Characteristics of QD preparations.

<table>
<thead>
<tr>
<th>Label</th>
<th>Name</th>
<th>Emission λ (nm)</th>
<th>Concentration Used (M)</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>TGA 8 (5)</td>
<td>522</td>
<td>1 x 10^{-7} M</td>
<td>Negative</td>
</tr>
<tr>
<td>b</td>
<td>154 (5)</td>
<td>538</td>
<td>1 x 10^{-7} M</td>
<td>Negative</td>
</tr>
<tr>
<td>c</td>
<td>TGA 11 (6)</td>
<td>540</td>
<td>1 x 10^{-7} M</td>
<td>Negative</td>
</tr>
<tr>
<td>d</td>
<td>TGA 50</td>
<td>606</td>
<td>1 x 10^{-7} M</td>
<td>Negative</td>
</tr>
<tr>
<td>e</td>
<td>TGA 10-2 (4)</td>
<td>610</td>
<td>1 x 10^{-7} M</td>
<td>Negative</td>
</tr>
<tr>
<td>f</td>
<td>TGA 13 (1)</td>
<td>660</td>
<td>1 x 10^{-7} M</td>
<td>Negative</td>
</tr>
<tr>
<td>g</td>
<td>118-4 (1)</td>
<td>545</td>
<td>1 x 10^{-7} M</td>
<td>Negative</td>
</tr>
<tr>
<td>h</td>
<td>TGA 4 (4)</td>
<td>531</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>j</td>
<td>172 (1)</td>
<td>551</td>
<td>1 x 10^{-6} M</td>
<td>Positive</td>
</tr>
<tr>
<td>k</td>
<td>SbTGA14(1)</td>
<td>600</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>l</td>
<td>SbTGA14(2)</td>
<td>582</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>m</td>
<td>SbGel</td>
<td>511</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>n</td>
<td>Sb171c(3)</td>
<td>678</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>p</td>
<td>Sb184(1)</td>
<td>623</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>q</td>
<td>Sb186(3)</td>
<td>600</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>r</td>
<td>Sb186(4)</td>
<td>585</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>s</td>
<td>Sb186(5)</td>
<td>576</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>t</td>
<td>Tc44B-6</td>
<td>620</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>u</td>
<td>Tc44B-7</td>
<td>618</td>
<td>1 x 10^{-6} M</td>
<td>Negative</td>
</tr>
<tr>
<td>Quantum Dot</td>
<td>Uptake by glia</td>
<td>Uptake by BMDM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>---------------</td>
<td>---------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a: TGA 8 (5)</td>
<td>Yes</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b: 154 (5)</td>
<td>No</td>
<td>Not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c: TGA 11 (6)</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d: TGA 50</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e: TGA 10-2 (4)</td>
<td>Yes</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f: TGA 13 (1)</td>
<td>Yes</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g: 118-4 (1)</td>
<td>No</td>
<td>Not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>h: TGA 4 (4)</td>
<td>Not tested</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>j: 172 (1)</td>
<td>Not tested</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k: SbTGA14(1)</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>l: SbTGA14(2)</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m: SbGel</td>
<td>No</td>
<td>Not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n: Sbl71c(3)</td>
<td>Yes</td>
<td>Not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p: Sb184(1)</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>q: Sb186(3)</td>
<td>Not tested</td>
<td>Not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r: Sb186(4)</td>
<td>Not tested</td>
<td>Not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>s: Sb186(5)</td>
<td>Not tested</td>
<td>Not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t: Tc44B-6</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>u: Tc44B-7</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2: Uptake of QD varies in mixed glia and BMDM.

Mixed glia and BMDM were pre-treated with LPS (100 ng/ml) for 24 hours and incubated in the presence of several preparations of QD (1 x 10^{-7} M) for 2 hours in an effort to identify the optimal preparation. Experiments were undertaken to determine which quantum dots were taken up by mixed glia and BMDM. Those chosen are identified in bold font.
Figure 3.4: QD uptake by mixed glia.

Mixed glia were pre-treated with LPS (100 ng/ml) for 24 hours and were incubated in the presence of different preparations of QD (1 x 10^{-7} M) for 2 hours. CD11b fluorescence intensity is displayed on the x-axis and QD fluorescence intensity is displayed on the y-axis. (A) Analysis by flow cytometry indicated that there was no evidence of uptake of QD preparation b by mixed glia (R31 and R32). (B) QD preparation e was taken up by CD11b^{+} cells only (R32) and QD preparations c and d were taken up by all cells in the mixed glial culture (R31 and R32; C and D respectively).
Chapter 3 Development of an assay to assess phagocytosis using quantum dots

Figure 3.5: QD uptake by BMDM.

BMDM were pre-treated with LPS (100 ng/ml) for 24 hours and were incubated in the presence of different preparations of QD (1 x 10^{-7} M) for 2 hours. CD11b fluorescence intensity is displayed on the x-axis and QD fluorescence intensity is displayed on the y-axis. Analysis by flow cytometry indicated that QD preparations c and d were taken up by CD11b+ BMDM (R24; A and B respectively) while there is no evidence of uptake of QD preparations e or j (R24; C and D respectively).
Figure 3.6: QD uptake varies with preparation.

Mixed glia and BMDM were incubated in the presence of QD (1 x 10^{-7} M) for 2 hours. There was no evidence of uptake of QD preparation b by mixed glia or of uptake of QD preparation e or f by BMDM (A and B respectively).
Figure 3.7: Uptake of QD varies with charge on the particle.

BMDM were pre-treated with (A and C) or without (B) LPS (100 ng/ml) for 24 hours and incubated in the presence of QD (1 x 10^{-7} M) for 2 hours. QD preparation h was negatively charged and was taken up by LPS-treated BMDM (A: R32). The positively charged QD preparation j was not taken up by cells that were untreated (B: R29) or treated with LPS (C: R35).
Chapter 3 Development of an assay to assess phagocytosis using quantum dots

Table 3.3: QD and fluorescent labels suitable for use in the phagocytic assay.

The characteristic emission spectra of the QD used for analysis of phagocytosis and the corresponding fluorescent labels conjugated to the antibodies of interest are given. The fluorescent labels that could be used in conjunction with the QD are highlighted in red.
### Table 3.4: Antibodies used in flow cytometry.

Antibodies, for the cell surface markers of activation analysed in this study, are given along with the corresponding stains and suppliers.
<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Concentration</th>
<th>Exposure Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>100 ng/ml</td>
<td>2 hours</td>
</tr>
<tr>
<td>LPS</td>
<td>100 ng/ml</td>
<td>24 hours</td>
</tr>
<tr>
<td>Aβ_{1-42}</td>
<td>2 μM</td>
<td>2 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td>Aβ_{1-42}</td>
<td>4 μM</td>
<td>2 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td>Aβ_{1-42}</td>
<td>8 μM</td>
<td>2 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td>IL-1β</td>
<td>10 ng/ml</td>
<td>24 hours</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>100 μM</td>
<td>30 minutes</td>
</tr>
<tr>
<td>NaF</td>
<td>10 mM</td>
<td>1 hours</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>1 μg/ml</td>
<td>1 hours</td>
</tr>
</tbody>
</table>

Table 3.5: Potential modulators of phagocytosis.

Concentrations and incubation times used to assess potential modulators of phagocytosis are listed.
Chapter 3 Development of an assay to assess phagocytosis using quantum dots

Figure 3.8: Effect of potential modulators of phagocytosis on the percentage of CD11b⁺ cells.

Mixed glia were pre-treated with or without LPS (100 ng/ml), IL-1β (10 ng/ml) or Aβ₁₋₄₂ (2 μM) for 24 hours, NaF (10 mM) or GM-CSF (10 μg/ml) for 1 hour or H₂O₂ (100 μM) for 30 minutes and were assessed using flow cytometry for cell surface expression of CD11b. LPS significantly reduced the percentage of cells that expressed CD11b on their surface (A; *p<0.05; student’s t-test for independent means; n=20). There was no significant effect of any other treatment on the percentage of cells that expressed CD11b on their surface (n=18). These data are representative of 4 series of experiments. Data are expressed as means ± SEM.
Chapter 3 Development of an assay to assess phagocytosis using quantum dots

A

% CD11b+ cells

10

0

Control

LPS

B

% CD11b+ cells

10

0

Control

IL-1β

C

% CD11b+ cells

10

0

Control

Alβ1-42

D

% CD11b+ cells

12.5

0

Control

H₂O₂

E

% CD11b+ cells

7.5

0

Control

NaF

F

% CD11b+ cells

7.5

0

Control

GM-CSF
Figure 3.9: Effect of LPS on QD uptake by mixed glia.

Mixed glia were pre-treated with or without LPS (100 ng/ml) for 24 hours and were incubated in the presence of 5 different preparations of QD ($1 \times 10^{-6}$ M) for 2 hours. QD uptake was assessed using flow cytometry. LPS stimulated uptake of QD preparation c but did not stimulate uptake of any other QD preparation.
Aggregation of Aβ_{1-42} (200 μM) increased in a time-dependent manner as assessed by thioflavin T assay. Aβ_{1-42} was significantly aggregated at 48 hours (***p<0.001; ANOVA; n=8-17). Aggregation was expressed as units of fluorescence intensity and data are expressed as means ± SEM.
Figure 3.11: Effect of Aβ_{1-42} on QD uptake by CD11b⁺ cells.

Mixed glia were pre-treated in the presence or absence of Aβ_{1-42} (2 μM, 4 μM or 8 μM) for 24 hours and were incubated in the presence of QD (1 x 10⁻⁶ M) for 2 hours. There was no significant effect of treatment on QD uptake (n=6). The percentage of cells which remained unstained was unchanged by treatment (data not shown). These data are representative of 3 separate series of experiments. Data are expressed as means ± SEM.
Mixed glia were pre-treated with control media, NaF (10 mM) for 1 hour or H$_2$O$_2$ (100 μM) for 30 minutes and were incubated in the presence of fluorescent latex particles (30 nm, 500 nm, 2,000 nm; 1 in 150 dilution) or QD (1 x 10$^{-6}$ M) for 2 hours. Analysis by flow cytometry revealed that pre-treatment with H$_2$O$_2$ significantly inhibited uptake of both fluorescent particles and QD by mixed glia (*p<0.05, **p<0.01, ***p<0.001; ANOVA; n=5-7). NaF significantly inhibited the uptake of all particles except the 500 nm particle (B) (*p<0.05, ***p<0.001; ANOVA; n=3-7). Data are expressed as mean ± SEM and are given as arbitrary values.
Figure 3.13: Effect of Aβ₁₋₄₂ on uptake of QD and fluorescent latex particles

Mixed glia were pre-treated with or without Aβ₁₋₄₂ (2 μM) for 24 hours and were incubated in the presence of fluorescent latex particles (30 nm, 500 nm, 2,000 nm; 1 in 150 dilution) or QD (1 x 10⁻⁶ M) for 2 hours. Aβ₁₋₄₂ had no effect on uptake of the 500 nm or 2,000 nm fluorescent particles and QD by mixed glia. Aβ₁₋₄₂ significantly increased uptake of the 30 nm fluorescent particle (**p<0.01; student’s t-test for independent means; n=2-7). Data are expressed as means ± SEM and are given as arbitrary values.
Chapter 3 Development of an assay to assess phagocytosis using quantum dots

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Events in M1</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>288</td>
<td>2.88</td>
</tr>
<tr>
<td>QD</td>
<td>329</td>
<td>6.42</td>
</tr>
<tr>
<td>Aβ&lt;sub&gt;1-42&lt;/sub&gt;</td>
<td>422</td>
<td>4.96</td>
</tr>
<tr>
<td>QD &amp; Aβ&lt;sub&gt;1-42&lt;/sub&gt;</td>
<td>1105</td>
<td>13.78</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>186</td>
<td>2.01</td>
</tr>
<tr>
<td>NaF</td>
<td>482</td>
<td>4.82</td>
</tr>
</tbody>
</table>

B

Figure 3.14: Assessment of cell viability following treatment

Mixed glia were treated with control media or Aβ<sub>1-42</sub> (2 μM) for 24 hours, H<sub>2</sub>O<sub>2</sub> (100 μM) for 30 minutes or NaF (10 mM) for 1 hour or were incubated in the presence of QD (1 x 10<sup>-6</sup> M) for 2 hours. A single sample was treated with Aβ<sub>1-42</sub> (2 μM) for 24 hours and was incubated in the presence of QD (1 x 10<sup>-6</sup> M) for 2 hours. Cells were prepared as described in section 2.16.1. (A) There was an increased percentage of cells in the apoptotic phase of the cell cycle when cells were treated with Aβ<sub>1-42</sub> and QD in combination. (B) There was no significant effect of H<sub>2</sub>O<sub>2</sub> on mixed glial cell viability as measured by MTS assay and spectrophotometry.
Figure 3.15: Assessment of QD internalisation by microglia.

Mixed glia were treated with Aβ_{1-42} (2 μM) for 24 hours and incubated in the presence of QD (1 x 10^{-6} M) for 2 hours. Cells were prepared as described in section 2.13.1. (A and B) CD11b^{+} (green) cells show intracellular localisation of QD (red) to the cytoplasm. (C) CD11b^{+} cells (green) in the same preparation exhibit no QD internalisation. Pictures were acquired on a confocal laser scanning microscope (Zeiss 510 META).
Figure 3.16: Assessment of QD internalisation by astrocytes.

Mixed glia were treated with control media or with Aβ_{1-42} (2 μM) for 24 hours and incubated in the presence of QD (1 x 10^{-6} M) for 2 hours. Cells were prepared as described in section 2.13.1. (A) GFAP^+ (green) astrocytes that were treated with control media did not internalise QD (red). (B) A single GFAP^+ (green) astrocyte pre-treated with Aβ_{1-42} shows internalisation of QD. Pictures were acquired on a confocal laser scanning microscope (Zeiss 510 META).
Figure 3.17: QD are internalised by microglia and astrocytes.

Mixed glia were treated with Aβ1-42 (2 μM) for 24 hours and incubated in the presence of QD (1 x 10⁻⁶ M) for 2 hours. Cells were prepared as described in section 2.13.1. A single GFAP⁺ (green) cells show intracellular localisation of QD (red) to the cytoplasm (white arrow). Nuclei (blue) of microglia are seen surrounded by QD (yellow arrows). Pictures were acquired on a confocal laser scanning microscope (Zeiss 510 META).
Figure 3.18: Live cell imaging time series analysis of QD uptake by mixed glia

Cells were incubated in the presence of the nuclear stain Hoescht (1 in 10,000 dilution; 5 minutes) and visualised with the confocal microscope. QD (2.6 × 10^{-8} M) were added to the culture dish and a single image was acquired every 2 minutes for 25 minutes. ‘Active’ cells were identified and recorded at 60X magnification. Images were acquired using an Olympus FluoView TM FV1,000 Confocal microscope. Cells were observed to actively seek out QD, ingest them, digest them and expel them by exocytosis (white arrows). These ‘active’ cells are then seen to communicate with each other.
3.3 Discussion

The main objective of this study was to develop an assay to assess phagocytosis using QD. Flow cytometry, ELISA, confocal microscopy and live cell fluorescent imaging were used to assess the usefulness of QD as phagocytic probes. The significant new finding of this study is that QD represent a practical option as a novel phagocytic probe.

Fluorescent latex particles and other probes are commercially available to assess the phagocytic activity of cells in vitro (Fukasawa et al, 1997; Tsujimoto et al, 2008; Dumrese et al, 2009; Smith, 2001; Koenigsknecht-Talboo and Landreth, 2005; Takeda et al, 1998). Aside from these commercially available methods, laboratories have established techniques using apoptotic cells and a wide variety of other substance including bacteria, IgG coated erythrocytes, zymosan, oxidised LDL and myelin vesicles that microglia and other phagocytic cells have been shown to ingest (Smith, 2001; Adayev et al, 1998; Witting et al, 2000; Magnus et al; 2001). The development of a technique using QD for assessing phagocytic activity of cells recognises that nanotechnology is a rapidly expanding area of scientific research that potentially offers many advantages over traditional techniques. QD are superior to conventional organic dyes that are available for diagnostic purposes and live cell imaging due to their high degree of photostability which results in a long-lived signal, their tuneable emission spectra and their broadband excitation over a wide range of wavelengths (Byrne et al, 2006). To date, the most successful application of QD is as biological tags, a strategy first demonstrated in 1998 (Bruchez et al, 1998; Chan and Nie, 1998). Due to their size, QD are also suitable for tagging to a range of biological targets.
that are of interest to researchers. For all these excellent reasons, it is important to elucidate all potential applications of QD.

As previously established, microglia and BMDM displayed similar characteristics in terms of size and granularity and consequently appeared in the same location on the forward scatter/side scatter dot plot. It was observed that QD uptake varied with preparation specifically in relation to surface coating, size and charge on the particle. These are characteristics of QD that have previously been identified as influencing their uptake (Zhang and Monteiro-Riviere, 2009; Choi and Maysinger, 2008). QD with an emission wavelength of between approximately 600 nm and 630 nm were most readily taken up by the microglia and BMDM. In this study it was demonstrated that positively charged QD were not taken up by either unstimulated or LPS-stimulated microglia or BMDM. Previous reports are contradictory and appear to be dependent on the type of QD in question and the synthesis procedures involved. In support of the present findings, Byrne and colleagues (2006) showed that positively-charged QD were not taken up by cells but tended to stick to the surface of the cell. In contrast, Wang and colleagues (2009) reported that positively-charged Tren-QD showed efficient cellular uptake by the BV-2 microglial cell line. However, our studies were conducted using primary cells which may behave differently to cell lines and QD which were prepared in manner that was different to the synthesis of the Tren-QD.

Following identification of QD that were suitable for use as phagocytic probes, it was necessary to establish which cellular markers could be used to identify the cells of interest. The fluorescent antibodies that emitted a strong fluorescent signal by flow cytometric analysis and which were identified for use
in the phagocytic assay included CD11b-FITC, CD11b-APC, OX-6-PerCP, CD80-PE, CD86-FITC and CD45-FITC.

LPS has previously been reported to enhance the phagocytic activity of microglia via TLR activation and filamentous actin reorganisation (Sun et al, 2008; Semple et al, 2007; Honstetter et al, 2004). TLR ligands have been shown not only to amplify the percentage of phagocytes taking up bacteria, but also to increase the number of bacteria phagocytosed by an individual macrophage (Doyle et al, 2004). However, contradictory evidence suggests that LPS can also suppress phagocytosis mediated by Fc receptors (Sundaram et al, 1993). In this study, LPS significantly reduced CD11b^+ cells in the mixed glial culture and had no significant effect on the percentage of CD11b^+ cells that took up QD. This contrasts with the findings of Roy et al (2008) who reported that LPS induced expression of CD11b by the BV-2 microglial cell line and by primary microglia. However, since LPS significantly reduced the percentage of cells expressing CD11b, the interpretation of any change in phagocytic activity was compromised and therefore, LPS was not pursued as a potential modulator of phagocytosis in this study. In contrast to LPS, the other potential modulators of phagocytosis that were assessed in this study, IL-1β, Aβ_{1-42}, H_2O_2, NaF and GM-CSF, had no significant effect on expression of CD11b by mixed glial cells.

Fibrillar Aβ is considered to be the most neurotoxic species of Aβ found in the AD brain. In this study, Aβ_{1-42} was aggregated to produce a solution containing fibrillar and soluble Aβ species. Aggregation of Aβ_{1-42} increased in a time-dependent manner with significant aggregation occurring at 48 hours. Aβ_{1-42} did not significantly affect QD uptake by CD11b^+ cells in a mixed glial culture. This contrasts with previous findings which indicated that fibrillar Aβ induced
phagocytosis and increased proinflammatory cytokine secretion by microglia (Weldon et al, 1998; Koenigsknecht-Talboo and Landreth, 2005; Casal et al, 2002). There are many possible explanations for this discrepancy. It is possible that in a mixed glial culture, the presence of astrocytes maintained the microglia in a resting state (Sparkman and Johnson, 2008), although it was observed that a basal level of phagocytic activity existed. It is also possible that addition of Aβ1-42 induced proinflammatory cytokine release that prevented phagocytosis and this is consistent with the findings of Koenigsknecht-Talboo and Landreth (2005) who reported that LPS treatment prior to stimulation with fibrillar Aβ suppressed phagocytic activity of primary microglia. Finally, it is possible that phagocytic activity is already increased in mixed glia isolated from the brains of neonatal rats and that the cells were unable to respond to the additional stimulus of Aβ1-42 (Smith, 2001).

To further investigate the potential of QD as phagocytic probes, their uptake was compared with the uptake of commercially-available fluorescent latex particles. The uptake of 30 nm, 500 nm and 2,000 nm fluorescent latex particles was assessed alongside the uptake of the QD. All of the fluorescent latex particles were taken up by CD11b⁺ cells in mixed glial cultures in agreement with previous studies (Adayev et al, 1998; Witting et al, 2000; Magnus et al; 2001), as were the QD. Having demonstrated that the fluorescent latex particles and the QD could be similarly taken up by CD11b⁺ cells, the impact of two known inhibitors of phagocytosis, NaF and H₂O₂, on the uptake of the fluorescent latex particles and the QD was assessed. Both H₂O₂ and NaF inhibited uptake of QD and fluorescent latex particles by CD11b⁺ cells in a mixed glial culture. These data suggest that QD are ideal for use as phagocytic probes.
Aβ₁₋₄₂ had no effect on uptake of the 500 nm or 2,000 nm fluorescent latex particles or on uptake of QD. However, Aβ₁₋₄₂ increased uptake of 30 nm fluorescent latex particle. Importantly, the data indicated that there was no effect of Aβ₁₋₄₂, H₂O₂ or NaF on cell viability. Incubation of mixed glial cells in the presence of QD alone also had no effect on cell viability. This is consistent with a study by Byrne et al (2006) which reported that TGA-stabilised CdTe QD maintained cell viability of THP-1 cells over extended incubation periods.

To confirm that QD were indeed suitable for use as phagocytic probes, it was necessary to confirm that they were internalised by the cells. Flow cytometry simply detects fluorescent signals and cannot provide any information regarding location of the QD and therefore, confocal imaging was undertaken. QD were found to be localised to the cytoplasm of CD₁₁b⁺ microglia and GFAP⁺ astrocytes in a mixed glial culture. These data are consistent with Byrne and colleagues (2006) who demonstrated that TGA-stabilised QD bound to the surface of THP-1 cells and were then internalised within 10 minutes. Although both CD₁₁b⁺ and GFAP⁺ cells took up QD, closer observation revealed that a greater number of CD₁₁b⁺ cells took up QD which is consistent with the observation that microglia are professional phagocytes in the CNS but that other cells are also capable of phagocytosis (Kullberg et al, 2001; Dong and Benveniste, 2001). With longer incubation times, Byrne and colleagues (2006) observed a diffuse cytoplasmic and enhanced nuclear membrane localisation of the QD. In this study, a diffuse cytoplasmic localisation of QD in both the CD₁₁b⁺ and GFAP⁺ cells was observed after 2 hours. Live cell imaging revealed that, over the course of 30 minutes, cells in the mixed glial culture actively moved towards QD; cells were motile and extended processes to interact with QD. One cell in particular,
following interaction with QD, underwent morphological changes from a classical ramified shape with extended processes to an amoeboid morphology with retracted processes and an enlarged cell soma. Another cell in the same culture expelled an ingested QD and these two cells then extended process to interact with each other. The live cell imaging data from this study confirm that QD uptake by mixed glial cells is an active process.

The findings of this study demonstrate that analysis of QD uptake by flow cytometry represents a feasible technique for assessing phagocytosis. The data illustrate that the role played by Aβ_{1-42} in stimulating microglial phagocytic activity is complex and still requires much work to establish why discrepancies exist.
Chapter 4

Analysis and modulation of activation states of mixed glia and bone marrow-derived macrophages.
4.1 Introduction

Microglia and macrophages are professional phagocytes, derived from the myeloid cell lineage, that can produce cytokines and express cell surface markers upon activation (Mueller et al, 2003; Kim et al, 2005). Peripheral macrophages can infiltrate the CNS in disease states and become indistinguishable from amoeboid microglia (Smith, 2001; Tanaka et al, 2003; Simard et al, 2006).

Assessment of the modulation of microglial and BMDM activation by chemical mediators that are upregulated in the brains of aged or AD subjects may provide a better understanding of the role played by these cells in these conditions and in this study, the focus was on investigation of mechanisms by which phagocytosis was altered. In particular, the role of Aβ in modulating phagocytic activity was a key area of interest due to reports that activated microglia are observed in the vicinity of amyloid plaques in vivo, and the theory that Aβ phagocytosis by microglia may represent a valid mechanism of Aβ clearance from the brain and a viable therapeutic target (Benveniste et al, 2001; Frackowiak et al, 1992).

In addition to assessment of phagocytosis by QD uptake, expression of CD68 was also investigated. CD68 is a member of the lysosomal/endosomal-associated membrane glycoprotein (LAMP) family and has been shown to be upregulated in normal ageing and in multiple sclerosis (Wong et al, 2005). CD68 expression is thought to be upregulated during phagocytosis and therefore, it is often used as a marker of phagocytic activity of cells.
The aims of this study were:

- To assess the phagocytic activity of microglia and BMDM.
- To investigate whether cells that were phagocytic also expressed cell surface markers of activation and produced cytokines.
- To investigate the effects of the potential modulators Aβ1-42, H₂O₂, NaF and cytochalasin B on phagocytosis, expression of cell surface markers and production of cytokines by CD11b⁺ microglia and BMDM.
- To compare the effects of Aβ1-42, H₂O₂, NaF and cytochalasin B on expression of CD68 and phagocytosis of fluorescent latex particles and QD.
4.2 Results

4.2.1 $\text{H}_2\text{O}_2$ and NaF significantly inhibited QD uptake by CD11b$^+$ BMDM.

Bone marrow cells (BMC) were harvested from the femur and tibiae of 50g male Wistar rats and were differentiated into BMDM by culturing in cDMEM with 30% M-CSF for 8-10 days. Cells were treated with medium or in the presence of IL-1β (10 ng/ml) or Aβ1-42 (2 μM) for 24 hours, NaF (10 mM) or GM-CSF (10 μg/ml) for 1 hour or $\text{H}_2\text{O}_2$ (100 μM) for 30 minutes and were incubated in the presence of QD (1 x $10^{-6}$ M) for 2 hours. The data show that QD uptake was significantly inhibited in CD11b$^+$ BMDM treated with $\text{H}_2\text{O}_2$ (***p<0.001; ANOVA; n=12; Figure 4.1 B) or NaF (***p<0.001; ANOVA; n=12; Figure 4.2 B).

4.2.2: $\text{H}_2\text{O}_2$ significantly reduced expression of OX-6 by BMDM.

Cells were treated as described in section 4.2.1 and incubated in the presence of fluorescently-labeled antibodies against CD80 and OX-6 as described in section 2.7.1. Expression of these cell surface markers of microglial activation was assessed by flow cytometry. $\text{H}_2\text{O}_2$ significantly reduced expression of OX-6 by CD11b$^+$ BMDM (**p<0.01; student's t-test for independent means; n=8; Figure 4.3 B). There was no effect of treatment on expression of the co-stimulatory molecule CD80 by CD11b$^+$ BMDM (n=8; Figure 4.3 A). Pre-treatment with IL-1β (10 ng/ml) or Aβ1-42 (2 μM) for 24 hours had no effect on expression of OX-6 or CD80 by CD11b$^+$ BMDM.
4.2.3: A\beta_{1-42}, H_2O_2 and NaF significantly increased IL-1\beta release by BMDM.

Cells were treated as described in section 4.2.1. Supernatants were assessed for IL-1\beta concentration by ELISA. A\beta_{1-42}, H_2O_2 and NaF significantly increased IL-1\beta release by BMDM (*p<0.05, **p<0.01, ***p<0.001; ANOVA; n=4; Figure 4.4 A and B). GM-CSF had no significant effect on IL-1\beta release by BMDM (n=4; Figure 4.4 B).

4.2.4: H_2O_2 and NaF significantly inhibited QD uptake by mixed glia.

Mixed glial cells were prepared from neonatal Wistar rats and cultured for 10-14 days in cDMEM. Cells were treated with control medium or were treated with IL-1\beta (10 ng/ml) or A\beta_{1-42} (2 \mu M) for 24 hours, NaF (10 mM) or GM-CSF (10 \mu g/ml) for 1 hour or H_2O_2 (100 \mu M) for 30 minutes and were incubated in the presence of QD (1 x 10^{-6} M) for 2 hours. Phagocytic activity was assessed by analysing QD uptake by flow cytometry. The data show that phagocytic activity was significantly inhibited in mixed glia treated with H_2O_2 (**p<0.01; ANOVA; n=18; Figure 4.5 B) or NaF (***p<0.001; ANOVA; n=18).
4.2.5: Effect of Aβ1-42 on expression of cell surface markers of activation and phagocytosis by CD11b⁺ microglia.

Aβ1-42 has previously been shown to increase CD68 immunofluorescence reactivity by microglial cells (Szaingurten-Solodkin et al, 2009) and to increase expression of OX-6 by microglia in vivo (Jantaratnotai et al, 2003).

Cells were treated with Aβ1-42 (2 μM, 4 μM, 8 μM) for 24 hours and incubated in the presence of QD (1 x 10⁻⁶ M) for 2 hours. Following this, cells were incubated in the presence of fluorescently-labeled antibodies against CD80, OX-6 and CD68 as described in section 2.7.1. Expression of cell surface markers of activation by CD11b⁺ microglia was assessed using flow cytometry. The data show that 2 μM Aβ1-42 significantly increased expression of CD68 by CD11b⁺ microglia (***p<0.001; student’s t-test for independent means; n=8-24; Figure 4.7 A). Aβ1-42 significantly increased expression of CD68 by CD11b⁺ microglia in a concentration-dependent manner (**p<0.01, ***p<0.001; student’s t-test for independent means; n=8-24; Figure 4.7 A).

The data show that Aβ1-42 significantly increased expression of CD80 by CD11b⁺ microglia at both 4 μM and 8 μM concentrations (**p<0.01; ANOVA; n=2-12; Figure 4.7 B) while expression of OX-6 by CD11b⁺ microglia was only significantly increased by 8 μM Aβ1-42 (***p<0.01; ANOVA; n=6-13; Figure 4.7 C).

The ability of CD11b⁺ microglia that were phagocytosing QD, to express cell surface markers of activation, was also assessed. The data show that between 1.2% and 3.3% of CD11b⁺ microglia were capable of phagocytosing QD and expressing either OX-6 or CD80 (Control; Figure 4.8 A.)
and B). Aβ_{1-42} had no significant effect on expression of cell surface markers or uptake of QD by CD11b^+ microglia (n=2-16; Figure 4.8 A and B). Confocal analysis revealed that in a mixed glial culture OX-6-expressing microglia were also capable of internalising QD (Figure 4.9).

4.2.6: Aβ_{1-42} and H_2O_2 significantly increase IL-1β release by mixed glia.

Cells were treated in medium or medium containing IL-1β (100 ng/ml) or Aβ_{1-42} (2 μM) for 24 hours or H_2O_2 (100 μM) for 30 minutes or NaF (10 mM) or GM-CSF (100 ng/ml) for 1 hour and were incubated in the presence of QD (1 x 10^{-6} M) for 2 hours. Supernatants were assessed for IL-1β and IL-6 concentrations by ELISA. Aβ_{1-42} and H_2O_2 significantly increased IL-1β release by mixed glial cells (*p<0.05; ANOVA; n=14; Figure 4.10 A). Treatment with NaF or GM-CSF had no significant effect on IL-1β release by mixed glia (n=14; Figure 4.10 B).

4.2.7 Comparison of mixed glial cell activity in the presence of commercially available fluorescent latex particles or in the presence of QD.

Cytochalasin B is a microfilament-disrupting agent that interferes with the interaction of actin and myosin thereby increasing cell deformability and inhibiting phagocytosis. It has been reported to decrease uptake of latex particles, which is the standard method of assessing phagocytosis (Tsujimoto et al, 2008; Dumrese et al, 2009; Houalla and Levine, 2003; Mitrasinovic et al, 2003), by macrophages in a concentration-dependent manner (Mazur and Williamson, 1977; Axline and Reaven, 1974).

Mixed glia were pre-treated with or without Aβ_{1-42} (2 μM, 4μM, 8 μM) for 24 hours, H_2O_2 (100 μM) for 30 minutes, NaF (10 mM), DMSO (0.1%) or
cytochalasin B (5 μg/ml or 10 μg/ml) for 1 hour and were incubated in the presence of fluorescent particles (30 nm, 500 nm, 2,000 nm; 1 in 150 dilution) or QD (1 x 10^{-6} M) for 2 hours. Expression of cell surface markers of activation and phagocytosis were assessed using flow cytometry.

The data show that 2 μM Aβ_{1-42} significantly increased the proportion of CD11b^{+} microglia that took up the 30 nm and 500 nm fluorescent particles or the QD and expressed CD68 (*p<0.05, **p<0.01; student's t-test for independent means; n=2-7; Figure 4.11 B, C and D). There was no significant effect of any other concentration of Aβ_{1-42} on the proportion of CD11b^{+} microglia that were phagocytosing the 30 nm particle or the QD, and, expressing CD68 (n=2-7; Figure 4.11 C and D). Aβ_{1-42} at 4 μM and 8 μM concentrations significantly increased the proportion of CD11b^{+} microglia that took up the 500 nm and 2,000 nm fluorescent particles and expressed CD68 (*p<0.05, **p<0.01, ***p<0.001; student's t-test for independent means; n=2-7; Figure 4.11 A and B).

Neither NaF nor H_{2}O_{2} had an effect on CD68 expression or phagocytosis of the 500 nm or 2,000 nm fluorescent particle by CD11b^{+} microglia (n=5-7; Figure 4.12 A and B). However, NaF significantly reduced the proportion of CD11b^{+} microglia that expressed CD68 and took up the 2,000 nm particle (*p<0.05; student's t-test for independent means; n=3-7; Figure 4.12 A). H_{2}O_{2} and NaF significantly reduced the proportion of CD11b^{+} microglia that expressed CD68 and took up either the 30 nm particle or QD (***p<0.001; ANOVA; n=3-7; Figure 4.12 C and D).

Cytochalasin B had no effect on uptake of either the fluorescent latex particles or QD by CD11b^{+} microglia (Figure 4.13). However, cytochalasin B significantly reduced the proportion of CD11b^{+} microglia that were expressed
CD68 and phagocytosed the 30 nm particle or QD (*p<0.05, **p<0.01; ANOVA; n=5-7; Figure 4.14 C and D).
BMDM were incubated in control media or pre-treated with IL-1β (10 ng/ml) or Aβ1-42 (2 μM) for 24 hours or H$_2$O$_2$ (100 μM) for 30 minutes and cells were incubated in the presence of QD (1 x 10$^{-6}$ M) for 2 hours. Phagocytic activity was assessed by flow cytometry analysis of QD uptake. (A) Representative dot-plots from each treatment group are shown. (B) QD uptake was significantly inhibited in BMDM pre-treated with H$_2$O$_2$ (***p<0.001; ANOVA; n=12). These data are representative of 3 separate series of experiments and are expressed as means ± SEM. The number of CD11b$^+$ cells was unchanged by treatment (data not shown).
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

A

Control media-treated

IL-1β-treated

Aβ-treated

H₂O₂-treated

B

% CD11b+QD+ cells

Control  IL-1β  Aβ₁-₄₂  H₂O₂

132
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

Figure 4.2: NaF significantly inhibited QD uptake by BMDM.

BMDM were incubated in control media or pre-treated with NaF (10 mM) or GM-CSF (10 μg/ml) for 1 hour and were incubated in the presence of QD (1 x 10⁻⁶M) for 2 hours. (A) Representative dot-plots from each treatment group are shown. (B) Phagocytic activity was significantly inhibited in BMDM pre-treated with NaF (***p<0.001; ANOVA; n=12). These data are representative of 3 separate series of experiments and are expressed as means ± SEM. The number of CD11b⁺ was unchanged by treatment (data not shown).
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

A

Control media-treated

NaF-treated

GM-CSF-treated

B

% CD11b+QD+ cells

Control  NaF  GM-CSF

134
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

Figure 4.3: H$_2$O$_2$ significantly reduced OX-6 expression by BMDM.

BMDM were incubated in control media or pre-treated with IL-1β (10 ng/ml) or Aβ$_{1-42}$ (2 μM) for 24 hours or H$_2$O$_2$ (100 μM) for 30 minutes and were incubated in the presence of QD (1 x 10$^{-6}$ M) for 2 hours. (A) Treatment had no effect on CD80 expression by CD11b+ BMDM. (B) H$_2$O$_2$ significantly reduced OX-6 expression by CD11b+ BMDM (**p<0.01; student’s t-test for independent means; n=8). These data are representative of 2 separate series of experiments and are expressed as means ± SEM.
Figure 4.4: Aβ_{1-42}, H_2O_2 and NaF significantly increased IL-1β release.

BMDM were incubated in control media or pre-treated with Aβ_{1-42} (2 μM) for 24 hours, H_2O_2 (100 μM) for 30 minutes or NaF (10 mM) or GM-CSF (100 ng/ml) for 1 hour and incubated in the presence of QD (1 x 10^{-6} M) for 2 hours. Aβ_{1-42}, H_2O_2 and NaF significantly increased IL-1β release (*p<0.05, **p<0.01, ***p<0.001; ANOVA; n=4). Data are expressed as means ± SEM.
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

Figure 4.5: H$_2$O$_2$ significantly inhibited QD uptake by mixed glia.

Mixed glia were incubated in control media or pre-treated with IL-1β (10 ng/ml) or Aβ$_{1-42}$ (2 μM) for 24 hours or H$_2$O$_2$ (100 μM) for 30 minutes and incubated in the presence of QD (1 x 10$^{-6}$ M) for 2 hours. (A) Representative dot-plots from each treatment group are shown. (B) Phagocytic activity was significantly inhibited in mixed glia pre-treated with H$_2$O$_2$ (**p<0.01; ANOVA; n=18). These data are representative of 4 separate series of experiments and are expressed as means ± SEM. The number of CD11b$^+$ cells was unchanged by treatment (data not shown).
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

A

Control media-treated

IL-1β-treated

Aβ-treated

H₂O₂-treated

CD11b

B

% CD11b+QD+ cells

Control  IL-1β  Aβ₁₋₄₂  H₂O₂

138
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

Figure 4.6: NaF significantly inhibited QD uptake by mixed glia.

Mixed glia were incubated in control media or pre-treated with NaF (10 mM) or GM-CSF (10 μg/ml) for 1 hour and incubated in the presence of QD (1 x 10^-6 M) for 2 hours. (A) Representative dot-plots from each treatment group are shown. (B) Phagocytic activity was significantly inhibited in mixed glia treated with NaF (***p<0.001; ANOVA; n=18). These data are representative of 4 separate series of experiments and are expressed as means ± SEM. The number of CD11b^+ cells was unchanged by treatment.
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

A

Control media-treated  NaF-treated

GM-CSF-treated

CD11b

B

% CD11b+QD+ cells

Control  NaF  GM-CSF

140
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

Figure 4.7: Aβ1-42 increased expression of cell surface markers of activation in a concentration-dependent manner.

Mixed glia were incubated in control media or pre-treated with Aβ1-42 (2 μM, 4 μM, 8 μM) for 24 hours and were incubated in the presence of QD (1 x 10^{-6} M) for 2 hours. Aβ1-42 increased expression of CD68 (A: **p<0.01, ***p<0.001; student’s t-test for independent means; n=8-24), CD80 and OX-6 (B and C: **p<0.01; ANOVA; n=2-12, n=6-13 respectively) by CD11b+ mixed glia in a concentration dependent manner. Data are expressed as means ± SEM and are given as arbitrary values.

141
Mixed glia were incubated in control media or pre-treated with Aβ1-42 (2 µM, 4 µM, 8 µM) for 24 hours and were incubated in the presence of QD (1 x 10^{-6} M) for 2 hours. Expression of cell surface markers of activation and QD uptake were assessed using flow cytometry. Aβ1-42 had no effect, at any concentration, on the percentage of CD11b^+ cells that were expressing OX-6 or CD80 and taking up QD (n=2-16). Data are expressed as means ± SEM.
Figure 4.9: Co-localisation of OX-6 expression and QD fluorescence.

Mixed glia were incubated in the presence of QD (1 x 10^{-6} M) for 2 hours and cells were prepared as described in section 2.13.1. OX-6 expression (A) and QD fluorescence (B) associated with an individual cell from a mixed glia preparation. (C) Co-localisation of OX-6 fluorescence and QD fluorescence in an individual cell from a mixed glia preparation. Pictures were acquired on a confocal laser scanning microscope (Zeiss 510 META).
Figure 4.10: \(\text{A\beta}_{1-42}\) and \(\text{H}_2\text{O}_2\) significantly increased IL-1\(\beta\) release by mixed glia.

Mixed glia were incubated in control media or pre-treated with \(\text{A\beta}_{1-42}\) (2 M) for 24 hours, \(\text{H}_2\text{O}_2\) (100 \(\mu\)M) for 30 minutes or NaF (10 mM) or GM-CSF (100 ng/ml) for 1 hour and incubated in the presence of QD (1 x 10\(^{-6}\) M) for 2 hours. (A) \(\text{A\beta}_{1-42}\) and \(\text{H}_2\text{O}_2\) significantly increased IL-1\(\beta\) production by mixed glia (*\(p<0.05\); ANOVA; \(n=14\)). Data are expressed as means ± SEM.
Figure 4.11: Aβ_{1-42} significantly increased expression of CD68 and phagocytosis by CD11b^+ mixed glia

Mixed glia were incubated in the presence or absence of Aβ_{1-42} (2 μM, 4μM, 8 μM) for 24 hours and were incubated in the presence of fluorescent latex particles (30 nm, 500 nm, 2,000 nm; 1 in 150 dilution) or QD (1 x 10^6 M) for 2 hours. Aβ_{1-42} significantly increased the proportion of CD11b^+ mixed glia that took up fluorescent particles and QD and expressed CD68 (*p<0.05, **p<0.01, ***p<0.001; student’s t-test for independent means; n=2-7). Data are expressed as means ± SEM and are given as arbitrary values.
Figure 4.12: H$_2$O$_2$ and NaF significantly reduced expression of CD68 and phagocytosis by CD11b$^+$ mixed glia

Mixed glia were incubated in the presence or absence of H$_2$O$_2$ (100 μM) for 30 minutes or NaF (10 mM) for 1 hour and were incubated in the presence of fluorescent latex particles (30 nm, 500 nm, 2,000 nm; 1 in 150 dilution) or QD (1 x 10$^{-6}$ M) for 2 hours. H$_2$O$_2$ significantly reduced the proportion of CD11b$^+$ mixed glia that expressed CD68 and took up either QD or 30 nm fluorescent latex particles (C and D; ***$p<0.001$; ANOVA; n=5-7). NaF significantly reduced the proportion of CD11b$^+$ mixed glia that expressed CD68 and took up the 2,000 nm fluorescent latex particle (A: *$p<0.05$; student's t-test for independent means; n=3-7), the 30 nm fluorescent latex particle or the QD (C and D: ***$p<0.001$; ANOVA; n=3-7). Data are expressed as means ± SEM and are given as arbitrary values.
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

A

![Graph A](image)

Control  H$_2$O$_2$  NaF

B

![Graph B](image)

Control  H$_2$O$_2$  NaF

C

![Graph C](image)

Control  H$_2$O$_2$  NaF

D

![Graph D](image)

Control  H$_2$O$_2$  NaF
Figure 4.13: Cytochalasin B did not significantly reduce phagocytosis by mixed glia.

Mixed glia were incubated in control media or pre-treated with DMSO (0.1%) or cytochalasin B (5 μg/ml or 10 μg/ml) for 1 hour and incubated in the presence of fluorescent latex particles (30nm, 500 nm, 2,000 nm; 1 in 150 dilution) or QD (1 x 10^{-6} M) for 2 hours. There was no significant effect of treatment on QD or fluorescent latex particle uptake by CD11b^+ mixed glia (n=2-7). Data are expressed as means ± SEM and are given arbitrary values.
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

Figure 4.14: Cytochalasin B significantly reduced expression of CD68 and phagocytosis by CD11b+ mixed glia

Mixed glia were incubated in control media or pre-treated with DMSO (0.1%) or cytochalasin B (5 µg/ml or 10 µg/ml) for 1 hour and incubated in the presence of fluorescent latex particles (30nm, 500 nm, 2,000 nm; 1 in 150 dilution) or QD (1 x 10-6 M) for 2 hours. Cytochalasin B significantly reduced the proportion of CD11b+ mixed glia that expressed CD68 and took up either QD or 30 nm fluorescent latex particles (C and D; *p<0.05, **p<0.01; ANOVA; n=5-7). Data are expressed as means ± SEM and are given as arbitrary values.
4.3 Discussion

The main objective of this study was to assess the effects of modulators on phagocytic activity, cytokine production and expression of cell surface activation markers by microglia and BMDM using uptake of QD as the analytical method. The most significant findings of this study are that H$_2$O$_2$ and NaF decreased phagocytic activity and expression of cell surface activation markers, and increased cytokine secretion by microglia and BMDM. NaF, H$_2$O$_2$ and cytochalasin B decreased expression of CD68 by phagocytic microglia while A$\beta_{1,42}$ increased CD68 expression by phagocytic microglia.

In this study, H$_2$O$_2$ and NaF inhibited QD uptake by both BMDM and microglia consistent with previous reports and supporting the conclusion in the previous chapter that QD uptake was a suitable method for analysis of phagocytic activity. Previous studies provide evidence supporting the role of H$_2$O$_2$ as an inhibitor of phagocytosis. Oosting et al (1990) found that phagocytic activity of alveolar macrophages was impaired in a time- and concentration-dependent manner by H$_2$O$_2$. However, H$_2$O$_2$ has also been implicated in the enhancement of phagocytic activity of microglia (Takeda et al, 1998) and studies also report that either H$_2$O$_2$ or superoxide can induce autophagy (Chen et al, 2009; Byun et al, 2009). NaF has also previously been shown to inhibit phagocytosis via inhibition of ATP synthesis (Michl et al, 1976; Mazur and Williamson, 1977) and the present findings which concur with this suggest that QD uptake occurs by the active process of phagocytosis and that this phagocytosis can be modulated by H$_2$O$_2$. IL-1$\beta$, A$\beta_{1,42}$ and GM-CSF had no effect on QD uptake by either BMDM or microglia.
It was observed that both Aβ1-42 and H2O2 increased IL-1β secretion by BMDM and mixed glia. This is consistent with the data of previous studies which have shown that Aβ can activate microglia and macrophages and stimulate secretion of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α (Weldon et al, 1998; Koenigsknecht-Talboo and Landreth, 2005). H2O2 readily induces free radical generation promoting a proinflammatory environment and stimulating secretion of proinflammatory cytokines and specifically, H2O2 has been shown to stimulate the NFκB signaling pathway resulting in synthesis of TNF-α (Dimayuga et al, 2001). Similarly, Aβ1-42 also stimulates the NFκB signaling pathway to induce cytokine production (Sastre et al, 2006). The present data demonstrated that NaF increased IL-1β secretion by BMDM, but not by microglia. There were no reports that NaF exerted differential effects in different cell types but Loftenius et al (1999) reported a differential effect of NaF on cytokine production in human blood lymphocytes where IFN-γ was induced but IL-6 was not.

Considering the data acquired in BMDM, it appears that when the phagocytic activity of a cell is decreased, cytokine production by the cell is increased. The same effect is also seen in mixed glia following treatment with H2O2. IL-6 secretion was not significantly altered by any of the modulators in either BMDM or mixed glia (data not shown) suggesting that IL-6 does not play a role in the complex response initiated by incubation of BMDM and mixed glia in the presence of QD. The fact that none of the modulators stimulated an increase in IL-6 suggests that when these cells are involved in phagocytosis and production of IL-1β, they are not capable of increasing secretion of IL-6.

In parallel with it’s effect on QD uptake, H2O2 decreased OX-6 expression by BMDM. To my knowledge, no previous data exists that describes a
relationship between exogenous H$_2$O$_2$ and OX-6 expression by BMDM. This is very interesting result since OX-6 is an epitope of MHC II which plays a crucial role in antigen presentation (McMenamin et al, 1992; Tadokoro et al, 2008) and previous studies have shown that antigen presentation is impaired by H$_2$O$_2$ as a consequence of modification of intracellular events involved in the generation of the MHC II-peptide complexes (Preynat-Seauve et al, 2003). H$_2$O$_2$ and other pro-oxidant species have also been shown to decrease the capacity of T lymphocytes to be activated (Flescher et al, 1994, 1998; Tatla et al, 1999). OX-6 expression by BMDM was unaffected by IL-1β, and Aβ$_{1-42}$ had no effect on OX-6 expression by either BMDM or microglia. Similarly, expression of CD80 which is a costimulatory molecule that is expressed on the surface of APC and must be activated for T cell immune response to occur (Vasu et al, 2003), was unaffected by any of the treatments used.

The effect of H$_2$O$_2$ on the activation of microglia and BMDM is important as H$_2$O$_2$ is implicated in AD by several studies that demonstrated that brains of AD patients showed evidence of oxidative stress (Choi et al, 2005; Hensley et al, 1995; Palmer and Burns, 1994; Mecocci et al, 1993). Moreover, free radicals have been implicated in the etiology of Alzheimer's disease as well as Parkinson's disease in which oxidative stress is implicated in Aβ production (Shen et al, 2008). Collectively, these data suggest that oxidative stress modulates microglial activation which concurs with the proposal that it contributes to the neurodegeneration observed in AD (Zekry et al, 2003).

Data from Chapter 3 revealed that there was no effect of Aβ$_{1-42}$ on QD uptake at any of the concentrations used. In this study, an investigation into the effect of increasing concentration of Aβ$_{1-42}$ on expression of cell surface
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

activation markers revealed that Aβ1-42 increased expression of cell surface markers by mixed glia in a concentration-dependent manner. With the highest concentration of Aβ1-42 (8 µM) used here, an increase in expression of CD68, CD80 and OX-6 by CD11b+ microglia was observed. This is consistent with previous studies that have reported that Aβ1-42 is a potent activator of microglia. The majority of published studies describe Aβ-induced microglial activation in terms of cytokine production and information regarding expression of cell surface markers is not as readily available (Smith, 2001). However, analysis of post-mortem tissue from brains of AD patients revealed elevated levels of glial-derived proteins involved in the immune response, such as MHC II (Szczepanik et al, 2001). Here, expression of OX-6 by mixed glia that had taken up QD was confirmed by immunohistochemistry and confocal microscopy analysis. However, Aβ1-42 did not affect the percentage of CD11b+ cells that expressed the cell surface activation markers CD80 and OX-6 and engulfed QD; this finding reflects the lack of effect that Aβ1-42 exerted on the phagocytic activity of BMDM and microglia. In contrast, Aβ1-42 increased the number of CD11b+ cells that expressed CD68 and took up QD or the fluorescent latex particles. These data suggest that Aβ1-42 is capable of inducing microglial activation by more than one mechanism simultaneously, i.e. stimulating expression of cell surface markers and phagocytosis. H2O2 and NaF decreased the number of CD11b+ cells that expressed CD68 and that took up either QD or the 30 nm fluorescent latex particles confirming the earlier data presented here. Parallel changes in CD68 expression and QD uptake by mixed glia confirms the proposal that CD68 is an indicator of phagocytic activity.
Chapter 4 Analysis and modulation of activation states of mixed glia and BMDM

The evidence indicates that uptake of the 500 nm and 2,000 nm particles was different to the uptake of the 30 nm particle suggesting that the mechanism of phagocytic uptake of a foreign pathogen is dependent on the size of the pathogen (Groves et al, 2008). Consistent with this, Harashima et al (1994) reported that opsonins bound to liposomes in a manner that was dependent on their size and that this impacted on phagocytosis of liposomes since uptake was correlated with extent of opsonisation. Therefore, size of the liposome directly influenced it’s phagocytosis.

Cytochalasin B is reported to inhibit phagocytosis by preventing actin polymerisation essentially paralysing reorganization of the cytoskeletal elements required for phagocytosis. However, in this study, cytochalasin B did not significantly affect QD uptake or uptake of fluorescent latex particles by CD11b+ cells but it decreased the number of CD11b+ cells that expressed CD68 and took up either QD or the 30 nm particle. This mirrors the effects of the other inhibitors, H2O2 and NaF, which also significantly inhibited expression of CD68 and uptake of QD and the 30 nm particle. In the same fashion as NaF and H2O2, cytochalasin B had no effect on the number of CD11b+ cells that expressed CD68 and took up the 500 or 2,000 nm particle.

The findings of this study demonstrate the BMDM and microglia have similar activation profiles and that the impact of modulators on their activity is also similar. The data suggest that there are different activation states of BMDM and microglia and that phagocytosis, cytokine production and expression of cell surface activation markers can be decoupled. Previous studies have suggested that several activation states exist (Perry, 2004) rather than the traditionally-described two states of microglial activation; activated non phagocytic microglia
and reactive phagocytic microglia (Minagar et al, 2002). \( \text{H}_2\text{O}_2 \) profoundly inhibited of phagocytosis and expression of cell surface activation markers while it increased secretion of pro-inflammatory cytokines and this profile of activation was also observed with NaF; this may describe the activated non-phagocytic state of microglia proposed by Minagar et al (2002). Another significant finding of this study was the change in CD68 expression that was observed on CD11b\(^+\) cells that phagocytosed QD or fluorescent latex particles. That this expression could be modulated by A\( \beta_{1-42} \), NaF, \( \text{H}_2\text{O}_2 \) and cytochalasin B provided evidence that CD68 is an indicator of phagocytosis at least \emph{in vitro}. It is important to recognise that \emph{in vivo} the response of microglia to pathological events is context-dependent and varies as the microenvironment changes (Hanisch and Kettenmann, 2007) and that this microenvironment is much more complex than the controlled \emph{in vitro} environment.
Chapter 5

Assessment and modulation of microglial activation in ageing


5.1 Introduction

A fundamental difference in the activation state of microglia in the aged, but otherwise healthy, brain exists and thus it has been suggested that ageing itself acts as a priming stimulus leading to increased microglial activation (Dilger and Johnson, 2008). In the aged brain, secretion of proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α, is up-regulated paralleling the altered microglial activation (Campuzano et al., 2009). There is also an age-related increase in expression of cell surface activation markers such as CD45, MHC II, CD40 and CD68 (Stolzing and Grune, 2003; Henry et al, 2009). These molecules play a role in antigen presentation to, and activation of, T-cells.

Brain aging is associated with a progressive imbalance between antioxidant defences and production of ROS (Droge and Schipper, 2007). It has been suggested that microglia in the aged brain may be dysfunctional and that this can result in production of increased levels of ROS from older mitochondria in the microglia (Nakanishi and Wu, 2009). Since, age is the most significant risk factor for neurodegenerative diseases such as AD, it is likely that the inflammation and skewed microenvironment significantly contribute to this risk. Shen and colleagues (2008) reported that Aβ accumulates in the brain with aging and it may also play a pivotal role in modulating the altered microglia activation observed in the brains of aged and AD subjects since Aβ has been shown to be a potent inducer of microglial activation (Tan et al, 1999; Bamberger and Landreth, 2001; Floden and Combs, 2006). Increased oxidative stress observed in the brain of aged subjects contributes to the proinflammatory environment by enhancing binding of the redox-sensitive

The aims of this study were:

- To investigate the effect of ageing on the phagocytic activity of CD11b\(^+\) cells.
- To investigate the effect of ageing on expression of cell surface markers of activation by CD11b\(^+\) cells.
- To determine the effects of Aβ\(_{1-42}\) or H\(_2\)O\(_2\) on the activity of CD11b\(^+\) cells isolated from the brains of young and aged rats.
- To investigate the effect of aging on expression of cell surface markers of activation and cytokines at the mRNA level.
Chapter 5 Assessment and modulation of microglial activation in ageing

5.2 Results

5.2.1: Ageing increased phagocytic activity of CD11b+ cells.

Ageing is one of the principal risk factors for AD and it is thought that microglial activation is not simply a consequence of AD but that in fact it may also be a cause (Blasko et al, 2004). It has previously been reported that during the ageing process, microglia can be activated either chronically or pathologically (Nakanishi and Wu, 2009) and for this reason, the effect of aging on phagocytic activity, expression of cell surface markers and expression of pro-inflammatory cytokines in microglia was assessed.

Cells were isolated from cortical and hippocampal tissue obtained from the brains of young and aged adult male Wistar rats and incubated in the presence of QD (1 x 10^{-6} M) for 15 minutes. The data show that QD uptake by CD11b+ cells isolated from the brains of aged rats was significantly increased compared with QD uptake by CD11b+ cells isolated from the brains of young rats (*p<0.05; student’s t-test for independent means; n=26; Figure 5.1 B).

5.2.2: Ageing increased OX-6 expression by CD11b+ cells.

It has previously been reported that with ageing the inflammatory profile of the brain moves in a more pro-inflammatory direction (Lynch et al, 2007; Blasko et al, 2004) and this has been demonstrated in terms of increased expression of cell surface markers of activation, for example MHC II and CD68 (Perry et al, 2003). OX-6 expression by CD11b+ cells isolated from the brains of aged rats was significantly increased compared with young rats (*p<0.05; t-test; n=14; Figure 159)
5.2 B). There was no significant effect of ageing on the expression of CD86 by CD11b$^+$ cells (Figure 5.3 B).

5.2.3: Ageing increased the proportion of CD11b$^+$ cells that were expressed OX-6 and took up QD.

The proportion of CD11b$^+$ cells isolated from the brains of aged rats that expressed OX-6 and that took up QD was significantly increased compared with activity of CD11b$^+$ cells isolated from the brains of young rats (**p<0.01; student’s t-test for independent means; n=13; Figure 5.4 A). There was no significant effect of ageing on CD11b$^+$, CD86$^+$ cells that were phagocytic (Figure 5.4 B).

5.2.4: Aβ$_{1-42}$ increased QD uptake by CD11b$^+$ cells isolated from the brains of young rats.

It has been suggested that phagocytic clearance undertaken by microglia following brain injury may play a role in repair (Neumann et al, 2009). Aβ peptides have been shown to be potent activators of microglia and macrophages (Szczepanik et al, 2001) which are already reported to exist in a more activated state in the ageing brain. In the AD brain, microglia are found surrounding Aβ plaques and it is possible that dysfunction of these microglia results in a lack of clearance of Aβ from the ageing brain and thus a lack of regeneration (Neumann et al, 2009). In this study, the effect of Aβ$_{1-42}$ on phagocytic activity of cells prepared from the brains of young and aged animals was assessed. Aβ$_{1-42}$ significantly increased QD uptake by CD11b$^+$ cells isolated from the brains of young rats (*p<0.05; student’s t-test for independent
means; n=27; Figure 5.5). In contrast, Aβ1-42 did not have an effect on QD uptake by CD11b⁺ cells isolated from the brains of aged rats.

5.2.5: H₂O₂ increased QD uptake by CD11b⁺ cells isolated from the brains of young, but not aged, rats.

Cells were pre-treated in the presence or absence of H₂O₂ (100 μM) for 15 minutes and incubated in the presence of QD (1 x 10⁻⁶ M) for 15 minutes. H₂O₂ significantly increased QD uptake by CD11b⁺ cells isolated from the brains of young rats (*p<0.05; student’s t-test for independent means; n=27; Figure 5.6) while H₂O₂ significantly decreased QD uptake in CD11b⁺ cells isolated from the brains of aged rats (#p<0.05; student’s t-test for independent means; n=27; Figure 5.6).

5.2.6: Aβ1-42 or H₂O₂ had no effect on expression of cell surface markers of activation by CD11b⁺ cells.

Cells were pre-treated in the presence or absence of Aβ1-42 (8 μM) or H₂O₂ (100 μM) for 15 minutes and in the presence of QD (1 x 10⁻⁶ M) for 15 minutes. Neither Aβ1-42 nor H₂O₂ affected the expression of OX-6 or CD86 by CD11b⁺ cells isolated from the brains of young or aged rats (Figures 5.7 and 5.8). Similarly, neither Aβ1-42 nor H₂O₂ had any effect on the proportion of CD11b⁺OX-6⁺ or CD11b⁺CD86⁺ cells isolated from the brains of young or aged rats that took up QD (Figures 5.9 and 5.10).
5.2.7: Expression of cell surface markers of activation and cytokines is increased at the mRNA level in tissue isolated from the brains of aged rats.

Snap frozen cortical and hippocampal tissue isolated from the brains of the young and aged rats was analysed for CD11b, CD68, MHC II, CD40, RAGE, GFAP, IL-1β and TNF-α mRNA expression by real-time PCR. Expression of CD11b, CD68 and GFAP mRNA was significantly increased in cortical and hippocampal tissue isolated from the brains of aged, compared with young, rats (*p<0.05, **p<0.01, ***p<0.001; student’s t-test for independent means; n=14; Figure 5.11 A, B, C and D; n=7; Figure 5.12 E and F). MHC II mRNA expression was increased in cortical tissue, but not hippocampal tissue, isolated from the brains of aged, compared with young rats (**p<0.01; student’s t-test for independent means; n=14; Figure 5.11 E). CD40 mRNA expression was significantly increased in hippocampal tissue isolated from the brains of aged, compared with young, rats (**p<0.001; student’s t-test for independent means; n=7; Figure 5.12 B). RAGE mRNA expression was significantly decreased in cortical tissue isolated from the brains of aged, compared with young, rats (*p<0.05; student’s t-test for independent means; n=7; Figure 5.12 C). IL-1β mRNA expression was significantly decreased in hippocampal and cortical tissue isolated from the brains of aged, compared with young, rats (*p<0.05; student’s t-test for independent means; n=7; Figure 5.13 A and B). TNF-α mRNA expression was unchanged in either cortical or hippocampal tissue isolated from the brains of young and aged rats (Figure 5.13 C and D).
Figure 5.1: QD uptake was increased in CD11b^ cells prepared from the brains of aged rats.

CD11b^ cells isolated from the brains of young and aged rats were incubated in the presence of QD (1 x 10^{-6} M) for 15 minutes and phagocytic activity was assessed using flow cytometry. (A) Representative dot plots from each treatment group are shown. (B) QD uptake was significantly increased in CD11b^ cells isolated from the brains of aged, compared with young, rats (*p<0.05; student's t-test for independent means; n=7). Data are representative of 7 separate series of experiments and are expressed as means ± SEM given as arbitrary values.
CD11b+ cells isolated from the brains of young and aged rats were incubated in the presence of QD (1 x 10^{-6} M) for 15 minutes and expression of cell surface markers of activation was assessed by flow cytometry. (A) Representative dot plots from each treatment group are shown. (B) OX-6 expression was significantly increased on CD11b+ cells isolated from the brains of aged, compared with young, rats (*p<0.05; student’s t-test for independent means; n=7). Data are representative of 7 separate series of experiments and are expressed as means ± SEM given as arbitrary values.
CD11b⁺ cells isolated from the brains of young and aged rats were incubated in the presence of QD (1 x 10⁻⁶ M) for 15 minutes. (A) Representative dot plots from each treatment group are shown. (B) CD86 expression was unchanged on CD11b⁺ cells isolated from the brains of aged, compared with young, rats. Data are representative of 7 separate series of experiments and are expressed as means ± SEM given as arbitrary values.

Figure 5.3: CD86 expression was unchanged on CD11b⁺ cells prepared from the brains of aged rats.
Figure 5.4: The number of CD11b+ cells that expressed OX-6 and took up QD was increased in the brains of aged rats.

Cells isolated from the brains of young and aged rats were incubated in the presence of QD (1 x 10^{-6} M) for 15 minutes. (A) The number of CD11b+ cells from the brains of aged rats that expressed OX-6 and took up QD was significantly increased compared with CD11b+ cells from the brains of young rats (**p<0.01; student’s t-test for independent means; n=13). (B) There was no effect of ageing on the number of CD11b+ cells that expressed CD86 and took up QD. These data are representative of 7 separate series of experiments and are expressed as means ± SEM given as arbitrary values.
Figure 5.5: Aβ₁₋₄₂ increased QD uptake by CD11b⁺ cells isolated from the brains of young rats.

CD11b⁺ cells isolated from the brains of young and aged rats were pre-treated in the presence or absence of Aβ₁₋₄₂ (8 μM) for 15 minutes and in the presence of QD (1 x 10⁻⁶ M) for 15 minutes. Aβ₁₋₄₂ significantly increased QD uptake by CD11b⁺ cells isolated from the brains of young rats (*p<0.05; student’s t-test for independent means; n=7). In contrast, Aβ₁₋₄₂ did not further effect QD uptake by CD11b⁺ cells isolated from the brains of aged rats. These data are representative of 7 separate series of experiments and are expressed as means ± SEM given as arbitrary values.
Figure 5.6: \( \text{H}_2\text{O}_2 \) increased QD uptake by CD11b\(^+\) cells isolated from the brains of young rats but reduced QD uptake by CD11b\(^+\) cells isolated from the brains of aged rats.

CD11b\(^+\) cells isolated from the brains of young and aged rats were pre-treated in the presence or absence of \( \text{H}_2\text{O}_2 \) (100 \( \mu \text{M} \)) for 15 minutes and were incubated in the presence of QD (1 \( \times 10^{-6} \text{ M} \)) for 15 minutes. \( \text{H}_2\text{O}_2 \) significantly increased QD uptake by CD11b\(^+\) cells isolated from the brains of young rats (*\(p<0.05\); student’s t-test for independent means; \(n=27\)) while in contrast, \( \text{H}_2\text{O}_2 \) significantly decreased QD uptake in CD11b\(^+\) cells isolated from the brains of aged rats (#\(p<0.05\); student’s t-test for independent means; \(n=27\)). These data are representative of 7 separate series of experiments and are expressed as means ± SEM given as arbitrary values.
Figure 5.7: Aβ₁₄₂ or H₂O₂ had no effect on expression of OX-6 by CD₁₁b⁺ cells isolated from the brains of young and aged rats.

CD₁₁b⁺ cells isolated from the brains of young and aged rats were pre-treated in the presence or absence of: (A) Aβ₁₄₂ (8 μM) or (B) H₂O₂ (100 μM) for 15 minutes, and in the presence of QD (1 x 10⁻⁶ M) for 15 minutes. There was no effect of Aβ₁₄₂ or H₂O₂ on expression of OX-6 by CD₁₁b⁺ cells isolated from the brains of young or aged rats. These data are representative of 7 separate series of experiments and are expressed as means ± SEM given as arbitrary values.
Chapter 5 Assessment and modulation of microglial activation in ageing

**Figure 5.8:** Aβ_{1-42} or H_{2}O_{2} had no effect on expression of CD86 by CD11b<sup>+</sup> cells isolated from the brains of young and aged rats.

CD11b<sup>+</sup> cells isolated from the brains of young and aged rats were pre-treated in the presence or absence of: (A) Aβ_{1-42} (8 μM) or (B) H_{2}O_{2} (100 μM) for 15 minutes, and in the presence of QD (1 x 10^{-6} M) for 15 minutes. There was no effect of Aβ_{1-42} or H_{2}O_{2} on expression of CD86 by CD11b<sup>+</sup> cells isolated from the brains of young or aged rats. These data are representative of 7 separate series of experiments and are expressed as means ± SEM given as arbitrary values.
Figure 5.9: Aβ<sub>1-42</sub> or H<sub>2</sub>O<sub>2</sub> had no effect on the number of CD11b<sup>+</sup> cells that expressed OX-6 and took up QD.

CD11b<sup>+</sup> cells isolated from the brains of young and aged rats were pre-treated in the presence or absence of: (A) Aβ<sub>1-42</sub> (8 μM) or (B) H<sub>2</sub>O<sub>2</sub> (100 μM) for 15 minutes, and in the presence of QD (1 x 10<sup>-6</sup> M) for 15 minutes. There was no effect of Aβ<sub>1-42</sub> or H<sub>2</sub>O<sub>2</sub> on the number of CD11b<sup>+</sup> cells isolated from the brains of young or aged rats that expressed OX-6 and took up QD. These data are representative of 7 separate series of experiments and are expressed as means ± SEM given as arbitrary values.
Figure 5.10: Aβ₁-₄₂ or H₂O₂ had no effect on the number of CD11b⁺ cells that expressed CD86 and took up QD.

CD11b⁺ cells isolated from the brains of young and aged rats were pre-treated in the presence or absence of: (A) Aβ₁-₄₂ (8 μM) or (B) H₂O₂ (100 μM) for 15 minutes, and in the presence of QD (1 x 10⁻⁶ M) for 15 minutes. There was no effect of Aβ₁-₄₂ or H₂O₂ on the number of CD11b⁺ cells isolated from the brains of young or aged rats that expressed CD86 and took up QD. These data are representative of 7 separate series of experiments and are expressed as means ± SEM given as arbitrary values.
Figure 5.11: Expression of cell surface markers of activation was increased at the mRNA level in tissue isolated from the brains of aged rats.

Snap frozen cortical (A, C and E) and hippocampal (B, D and F) tissue isolated from the brains of young and aged rats was analysed for CD11b, CD68 and MHC II mRNA expression by real-time PCR. Expression of CD11b and CD68 mRNA was significantly increased in cortical and hippocampal tissue isolated from the brains of aged, compared with young, rats (A, B, C and D; \(^{*}p<0.05\), \(^{**}p<0.01\), \(^{***}p<0.001\); student’s t-test for independent means; n=14). Expression of MHC II mRNA was increased in cortical, but not hippocampal, tissue isolated from the brains of aged, compared with young, rats (E; \(^{**}p<0.01\); student’s t-test for independent means; n=14). Target gene expression was calculated relative to β-actin endogenous expression. Data are expressed as means ± SEM and are given as arbitrary values.
Chapter 5 Assessment and modulation of microglial activation in ageing

Cortex

A

\[
\begin{array}{c}
\text{CD11b mRNA (RQ)} \\
\text{Young} & \text{Aged} \\
\end{array}
\]

B

\[
\begin{array}{c}
\text{CD11b mRNA (RQ)} \\
\text{Young} & \text{Aged} \\
\end{array}
\]

Hippocampus

C

\[
\begin{array}{c}
\text{CD98 mRNA (RQ)} \\
\text{Young} & \text{Aged} \\
\end{array}
\]

D

\[
\begin{array}{c}
\text{CD98 mRNA (RQ)} \\
\text{Young} & \text{Aged} \\
\end{array}
\]

E

\[
\begin{array}{c}
\text{MHC II mRNA (RQ)} \\
\text{Young} & \text{Aged} \\
\end{array}
\]

F

\[
\begin{array}{c}
\text{MHC II mRNA (RQ)} \\
\text{Young} & \text{Aged} \\
\end{array}
\]
Figure 5.12: Expression of cell surface markers of activation was increased at the mRNA level in tissue isolated from the brains of aged rats.

Snap frozen cortical (A, C and E) and hippocampal (B, D and F) tissue isolated from the brains of young and aged rats was analysed for CD40, RAGE and GFAP mRNA expression by real-time PCR. Expression of CD40 mRNA was significantly increased in hippocampal tissue isolated from the brains of aged, compared with young, rats (B; ***p<0.001; student’s t-test for independent means; n=7). Expression of RAGE mRNA was significantly decreased in cortical tissue isolated from the brains of aged, compared with young, rats (*p<0.05; student’s t-test for independent means; n=7). Expression of GFAP mRNA was significantly increased in hippocampal and cortical tissue isolated from the brains of aged, compared with young, rats (*p<0.05, **p<0.01; student’s t-test for independent means; n=7). Data are expressed as means ± SEM and are given as arbitrary values.
Chapter 5 Assessment and modulation of microglial activation in ageing

Cortex

A

CD40 mRNA (RQ)

0.6

0.3

0.0

Young

Aged

B

CD40 mRNA (RQ)

0.8

0.4

0.0

Young

Aged

C

RAE mRNA (RQ)

2.0

1.0

0.0

Young

Aged

D

RAE mRNA (RQ)

1.2

0.6

0.0

Young

Aged

E

GFAP mRNA (RQ)

2.0

1.0

0.0

Young

Aged

F

GFAP mRNA (RQ)

3.0

1.5

0.0

Young

Aged

176
Figure 5.13: Expression of IL-1β mRNA was decreased in tissue isolated from the brains of aged rats.

Snap frozen cortical (A and C) and hippocampal (B and D) tissue isolated from the brains of young and aged rats was analysed for expression of IL-1β and TNF-α mRNA by real-time PCR. Expression of IL-1β mRNA was significantly decreased in hippocampal and cortical tissue isolated from the brains of aged, compared with young, rats (A and B; *p<0.05; student’s t-test for independent means; n=7). Expression of TNF-α mRNA was unchanged. Target gene expression was calculated relative to β-actin endogenous expression. Data are expressed as means ± SEM and are given as arbitrary values.
Chapter 5 Assessment and modulation of microglial activation in ageing

5.3 Discussion

The main objective of this study was to assess endogenous microglial activation in ageing and to assess whether Aβ1-42 and H2O2 exerted differential effects in tissue prepared from young and aged animals. The significant findings of this study are that an age-related increase in phagocytosis and expression of OX-6 was observed. Differential effects of Aβ1-42 and H2O2 were observed with ageing. Aβ1-42 and H2O2 increased phagocytosis by cells isolated from the brains of young rats but Aβ1-42 had no effect on phagocytosis, and H2O2 decreased phagocytosis, by cells isolated from the brains of aged rats.

The phagocytic assay developed in chapter 3 revealed that there was an age-related increase in QD uptake by CD11b⁺ cells. Previously, Sheng and colleagues (1998) reported an age-associated increase in the number of phagocytic microglia present in the human brain as identified by immunohistochemical analysis of morphology. In addition, Hodkinson and colleagues (2006) reported an increase in monocyte phagocytic function, as determined by engulfment of E.coli, in cells prepared from male and female adults with age. In contrast with these findings, studies have reported that phagocytosis declines with ageing. Zhao and colleagues (2006) reported that ageing is associated with delayed recruitment of phagocytic cells and reduced clearance of myelin following toxin-induced lesion of the spinal cord. However, many of these studies relate to dendritic cells or macrophages or the Kupffer cells of the liver and to the authors knowledge little information on declining microglial phagocytosis exist. The majority of studies that report an age-related decrease in phagocytosis report a simultaneous increase
in secretion of pro-inflammatory cytokines (Neumann et al, 2009); cytokine release was not assessed in this study.

The data described here show that there was an age-related increase in OX-6 expression by CD11b\(^+\) cells. This is consistent with many reports which demonstrated that there is an increased expression of MHC II in the brain with normal healthy ageing (Henry et al, 2009; Nicolle et al, 2001; Ogura et al, 1994; Perry et al, 1993; Sheffield and Berman, 1998; Sloane et al, 1999). In this study, CD86 expression was unchanged with age. Griffin and colleagues (2006) have previously shown an age-related increase in CD86 in the rat hippocampus by western immunoblotting and immunohistochemistry. Similarly, CD86 has been shown by flow cytometry to be increased on microglia isolated from brains of 12 month old rats compared with expression by neonatal primary microglia (Stolzing and Grune, 2003). Interestingly, the number of CD11b\(^+\) cells that expressed OX-6 and took up QD was also increased in cells prepared from the brains of aged rats indicating that phagocytic cells also express OX-6. In contrast, there was no age-related change in the number of CD11b\(^+\) cells that expressed CD80 and took up QD.

The action of A\(\beta\)\(_{1-42}\) on phagocytic activity was assessed in CD11b\(^+\) cells isolated from the brains of young and aged rats. A\(\beta\)\(_{1-42}\) increased QD uptake by CD11b\(^+\) cells isolated from the brains of young animals and this contrasts with the lack of effect of A\(\beta\)\(_{1-42}\) on phagocytic activity \textit{in vitro} in mixed glia that was described in the previous chapter. It is possible that the primary mixed glial cells isolated from the brains of neonatal rats were not fully differentiated into a phenotype that could become phagocytic or that indeed the process of isolation caused the cells to become over-stimulated so that they could not respond to a

179
phagocytic insult. However, the present data concur with Weldon and colleagues (1998) who showed that injection of fibrillar Aβ into the rat striatum resulted in increased phagocytosis by microglia. Koenigsknecht-Talboo and Landreth (2005) reported that fibrillar Aβ could induce phagocytosis in vitro in the BV-2 microglia cell line. These data contrast with the results obtained in chapter 4 where Aβ1-42 was found to have no significant effect on phagocytic activity in a mixed glial culture or in BMDM. Overall, it must be concluded that data obtained in the in vitro system cannot necessarily be extrapolated into the in vivo situation.

In contrast to the Aβ1-42 related increase in phagocytosis observed in the CD11b+ cells isolated from the brains of young animals, Aβ1-42 had no effect on QD uptake by CD11b+ cells isolated from the brains of aged animals. This is an interesting finding and suggests that strategies which target phagocytic activity to increase clearance of Aβ may be useful in the treatment of AD. The data are consistent with the view that the build up of Aβ and the plaque deposition observed in the brains of AD patients is due to a malfunction in microglial phagocytic activity as previously suggested (Koenigsknecht-Talboo and Landreth, 2005; Bard et al, 2000; Schenk et al, 1999). Microglial cells isolated from the brains of the aged animals appear to exist in a hyperactivated state with increased phagocytic activity and increased expression of cell surface activation markers. It is possible that these cells have reached their maximal activation state and further activation is not possible. Interestingly, the lack of effect of Aβ1-42 on QD uptake in cells prepared from the brains of aged rats is similar to the results obtained in vitro. Smith (2001) suggested that primary microglial cultures are already
activated as a consequence of the culture procedure and therefore their activity cannot be enhanced.

Like Aβ_{1-42}, H_2O_2 significantly increased QD uptake by CD11b^+ cells isolated from the brains of young animals; this contrasts with the results obtained when the effect of H_2O_2 was assessed in mixed glial cultures. However, previous studies have reported that H_2O_2 significantly increases phagocytic activity, for example Bejarno and colleagues (2006) reported that H_2O_2 significantly increased the phagocytic activity of neutrophils isolated from the adult human. Interestingly, differential age-related effects of H_2O_2 on phagocytic activity were observed; whereas H_2O_2 increased QD uptake in cells prepared from the brains of young rats, it decreased QD uptake by CD11b^+ cells isolated from the brains of aged animals. It is known that ROS production is upregulated in the brains of aged rats (Murray and Lynch, 1998) and it has been reported that microglial activation can be regulated by the oxidative state of the cell (Shih et al, 2006). It is possible that treatment with H_2O_2 caused an overload of ROS in the CD11b^+ cells isolated from the brains of aged animals resulting in an inhibition of their function (Shih et al, 2006). When all of the data are considered together, a very interesting story regarding phagocytic activity emerges. Both Aβ and ROS are upregulated in the brains of AD patients (Sun et al, 2009; Shen et al, 2008). The data from this study indicate that, in contrast with cells from young animals, Aβ and H_2O_2 are unable to stimulate phagocytic activity in microglia from aged animals; indeed H_2O_2 actually reduces the microglial phagocytic activity occurring. Overall, microglia in the aged brain may be defective in their ability to clear Aβ and this is likely to contribute to the development of plaques in AD. Interestingly, expression of
RAGE, which is considered to play a role in clearance of Aβ (Yan et al, 2009) and has been shown to mediate uptake of Aβ and amyloid fibrils by microglia in vitro (Paresce et al, 1996; Yan et al, 1996), was decreased in tissue prepared from aged animals.

Neither Aβ1-42 nor H₂O₂ had any effect on expression of OX-6 or CD86 by CD11b⁺ cells isolated from the brains of young and aged rats and they had no effect on the number of CD11b⁺ cells isolated from the brains of young and aged rats that took up QD and expressed cell surface markers. In contrast, Aβ1-42 has been shown both by the author and by others to increase expression of OX-6 in vitro by primary mixed glia again highlighting the differences in cells that mature in vitro versus cells that mature in vivo (O’Reilly et al, 2009; Lyons et al, 2007).

Expression of cell surface markers of microglial activation was examined in hippocampal and cortical tissue prepared from the brains of young and aged rats. The data show that CD11b mRNA was increased in the cortex and hippocampus with age which supports previous data from this lab (Moore et al, 2005). It has been reported in several models of neuroinflammatory diseases that CD11b expression correlates with the extent of microglial activation and at least in one study it was suggested that this upregulation in CD11b was driven by NO (Roy et al, 2008). In parallel with the change in CD11b mRNA, CD68 mRNA expression was also increased in the cortex and hippocampus of aged, compared with young, rats. This supports previous data which reported that expression of CD68 was increased during normal brain ageing, but also in brains obtained from transgenic mouse models of AD (Wong et al, 2005). In this role, changes in CD68 indicate a change in phagocytosis or in clearance of cellular debris since the
findings in chapter 4 indicated a close correlation between QD uptake and CD68 expression.

An age-related increase in MHC II mRNA expression was observed in the cortex which correlates with the age-related increase in OX-6 expression at a protein level that was observed by flow cytometry. This result was anticipated since several papers previously reported an age-related increase in microglial expression of MHC II (Henry et al., 2009; Martin et al., 2009). Similarly, an age-related increase in CD40 mRNA expression was observed which concurs with earlier reports (Martin et al., 2009; Griffin et al., 2006). CD40-CD40L interactions are implicated in a variety of CNS diseases with increased expression of CD40 and CD40L detected in AD (Togo et al., 2000) and MS (Gerritse et al., 1996). Since depletion of CD40 and CD40L genes in mouse models of MS or AD renders the animals resistant to disease progression, a detrimental role has been attributed to these molecules in promoting inflammation in the CNS (Chen et al., 2006).

In addition to the age-related increase in activation of microglia, there was also an age-related increase in GFAP mRNA in the cortex and hippocampus of aged rats, which is indicative of astrogliosis. Astrocyte proliferation is enhanced in the aged brain (Dong and Benveniste, 2001) but it is unclear whether this is damaging or not since depending on the stimulus that provokes it, astrogliosis can be either beneficial by promoting neuronal survival or detrimental by the formation of glial scars. Both microglia and astrocytes synthesise and release inflammatory cytokines like IL-1β but in contrast with increased activation of both cells, IL-1β mRNA was decreased in the cortex and hippocampus of aged rats. In
contrast with this, IL-1β concentration has been shown to be increased in the brains of aged, compared with young, rats (Moore et al, 2005; Murray et al, 1999).

The data presented in this study indicate that microglial activation increases with age and support the concept proposed in chapter 4, that there is a direct relationship between phagocytosis and expression of cell surface markers of activation. The inability of cells prepared from the brains of aged rats to respond to Aβ1-42 and H2O2 in terms of phagocytic activity suggests that the microenvironment in the brains of aged individuals, in which ROS is increased, has a detrimental effect on phagocytic activity and this may, at least in part, explain the significant risk that age represents in terms of the development of AD. Manipulation of microglial activation may represent a potential therapeutic target for treatment of AD.
Chapter 6

Investigation into the effect of rosiglitazone on microglial activation, plaque deposition and behaviour in the APPswe/PS1dE9 mouse.
6.1 Introduction

The development of transgenic mouse models of AD has contributed greatly to our understanding of the disease process. The APPswe/PS1dE9 mouse is a double transgenic model of AD co-overexpressing APP with the Swedish mutation and exon-9-deleted PS1 (Jankowsky et al., 2001) which results in increased production of A\(_{\beta_{1-42}}\). These mice show plaque deposition as early as 4-6 months of age and an increased A\(_{\beta_{1-42}}\):A\(_{\beta_{1-40}}\) ratio has been described in brain tissue described from these mice (Garcia-Alloza et al., 2006; Jankowsky et al., 2004). It has been reported that spatial and episodic memory is impaired in 18 month-old APPswe/PS1dE9 mice (Savonenko et al., 2005).

In this study, spatial learning was tested during the acquisition phase of the Morris water maze and reference memory was assessed during the probe trial. Reversal training in the Morris water maze reveals whether or not animals can extinguish their initial learning of the platform’s position and acquire a direct path to the new platform location (Vorhees and Williams, 2006). It facilitates enhanced detection of impairments in spatial learning.

The APPswe/PS1dE9 mouse also provides a model of the inflammation that occurs in the brains of AD patients. A pro-inflammatory microenvironment exists around A\(_{\beta}\) plaques as they are surrounded by activated microglia and reactive astrocytes (Zhang et al., 2009; Ho et al., 2005). This glial cell-driven inflammation in AD contributes to the pathogenesis of the disease and, as a result, there is an increased focus on therapies designed to modulate inflammation.

PPAR\(_{\gamma}\) agonists have been shown to inhibit the expression of TNF\(_{\alpha}\) which is triggered by inflammatory stimuli like LPS, indicating that they
possess anti-inflammatory properties (Jiang et al., 1998; Ricote et al., 1998). Specifically, rosiglitazone, which is a synthetic agonist of PPARγ has been shown to attenuate LPS-induced increases in pro-inflammatory cytokine production by microglia and astrocytes in vitro (Storer et al., 2005) and LPS-induced IL-1β in the hippocampus in vivo (Loane et al., 2009). The mechanism by which this attenuation in microglial activation occurs is still unclear. Pedersen and colleagues (2006) showed that rosiglitazone could attenuate learning and memory deficits in the Tg2576 transgenic mouse model of AD. Taking these previous studies into consideration, it was proposed to assess the effects of rosiglitazone on performance in the MWM, microglial activation and plaque deposition in APPswe/PS1dE9 mice.

The aims of this study were:

- To assess the performance of APPswe/PS1dE9 mice in the MWM and to determine if oral administration of rosiglitazone would alter any deficits.
- To investigate the Aβ burden in the brains of APPswe/PS1dE9 mice and to assess if rosiglitazone could modulate this burden.
- To determine the effect of genotype on microglial activation and to assess if microglial activation could be modulated in vivo by rosiglitazone or ex vivo by Aβ1-42 or H2O2.
6.2 Results

6.2.1: APPswe/PS1dE9 mice were unimpaired in muscular strength and coordination.

Mice were genotyped prior to assignment of treatment group or behavioural testing. Figure 6.1 displays a representative picture with bands for both transgenes, PS1 (A) and APPswe (B), and a band displaying the internal control. Lanes 1 and 2 represent wild-type mice while lanes 3, 4 and 5 represent mice that contain both transgenes.

Transgenic mouse models have previously been shown to exhibit alterations in behaviour and motor coordination that are not necessarily linked to the mutation associated with the model (Crawley and Paylor, 1997). Double transgenic mice derived from a cross between Tg2576 mice and the mutant PS1 transgenic line 5.1 have shown impairments on the balance beam but were unimpaired on the hangwire test at 5-7 months of age (Arendash et al. 2001). The APPswe/PS1dE9 mice used in this study co-overexpress a chimeric mouse/human APP695 isoform containing the K595N/M596L Swedish mutations, and a mutant human PS1 with exon 9 deleted, under the control of independent mouse prion promoters (Jankowsky et al. 2001, Jankowsky et al. 2004). These mutations could lead to impairments in motor coordination and consequently altered performance in the behavioural paradigm, the Morris water maze. To ensure that any changes in behaviour observed in the Morris water maze were due cognitive deficits in the APPswe/PS1dE9 mice, wild-type and transgenic mice underwent testing in sensorimotor tasks prior to testing in the Morris water maze.
Muscular strength was assessed using the hang-wire and inverted screen tests. Latency to fall from either the hang-wire or the inverted screen was measured and there was no significant difference between the wild-type and APPswe/PS1dE9 mice in either task (Figure 6.2). To assess motor coordination and balance, gait was assessed using footprint analysis. There was no difference in either front stride length or hind stride length between the wild-type and APPswe/PS1dE9 mice (Figure 6.3). These results indicate that the APPswe/PS1dE9 mice were unimpaired in muscular strength and coordination.

6.2.2 Wild-type and APPswe/PS1dE9 mice showed significant learning during the acquisition phase of the Morris water maze.

Two weeks prior to testing in the Morris water maze, wild-type and APPswe/PS1dE9 mice received oral administration of maple syrup or maple syrup containing rosiglitazone on a daily basis. Administration of maple syrup either with or without rosiglitazone did not affect the body weights of the mice (Figure 6.4).

During the acquisition phase of the Morris water maze both wild-type and APPswe/PS1dE9 mice showed a significant decrease in pathlength (**p<0.01, ***p<0.001; ANOVA; n=5) and latency to find the platform (**p<0.01; ANOVA; n=5) on day five compared with day one of training (Figures 6.5 and 6.6). Oral administration of rosiglitazone had no significant effect on performance of wild-type or APPswe/PS1dE9 mice during the acquisition phase of the MWM (Figure 6.7). During the probe trial, there was no effect of genotype or treatment with rosiglitazone on the percentage time spent in the quadrant that
previously contained the platform (Figure 6.8). There were no genotype- or treatment-related differences in swim speed which shows that all groups had equal swimming abilities and were not differentially affected by exertion in the maze (data not shown). Our data are in agreement with previous studies that have shown that, at 6 months of age, there is no significant difference in spatial reference memory between APPswe/PS1dE9 mice and their wild-type counterparts (Savonenko et al. 2005).

6.2.3 APPswe/PS1dE9 mice had a significantly longer pathlength to the platform during reversal training.

During the reversal phase of the Morris water maze, the platform was relocated to the diagonally-opposite quadrant of the maze and mice were required to learn the new platform location as well as inhibit memories of the previous location. This task assesses plasticity of learning. There was a significant difference between wild-type and APPswe/PS1dE9 mice during the reversal phase of the Morris water maze. APPswe/PS1dE9 mice took a significantly longer pathlength to the platform (Day five: (F(3,16)=6.31) and six (F(3,16)=6.61); *p<0.05, ***p<0.001; ANOVA; n=5) and mean latency to find the platform was also significantly longer (**p<0.01; ANOVA; n=5) than for wild-type mice (Figures 6.9 and 6.10).

6.2.4 Oral administration of rosiglitazone significantly improved performance of APPswe/PS1dE9 mice during the reversal phase of the Morris water maze.

Oral administration of rosiglitazone had no effect on performance of the wild-type mice during the reversal phase of the Morris water maze. However, it
significantly reduced the pathlength taken by the APPswe/PS1dE9 mice to find the platform (**p<0.01; ANOVA; n=5; Figure 6.11 B). Oral administration of rosiglitazone also significantly reduced the time taken by APPswe/PS1dE9 mice to find the platform on day 5 of reversal training (**p<0.01; ANOVA; n=5; Figure 6.12 B).

### 6.2.5 Insoluble Aβ1-42 concentration was significantly greater in the brains of APPswe/PS1dE9 mice.

The transgenic mouse model used in this study provides a model of Alzheimer-like amyloidosis (Savonenko et al. 2005). The APP and PS1 mutations are linked to familial Alzheimer’s disease and cause increased levels of the Aβ peptide in the brain by altering metabolism of APP (Arendash et al. 2001). Analysis by multi-spot elisa found that there was no significant effect of either genotype or rosiglitazone treatment on the concentration of soluble Aβ1-38, Aβ1-40 or Aβ1-42 in cerebellar tissue (Figure 6.13). However, there was a significantly higher concentration of insoluble Aβ1-42 in cerebellar tissue isolated from APPswe/PS1dE9 mice than from wild-type mice (**p<0.01; ANOVA; n=5; Figure 6.14). Oral administration of rosiglitazone significantly attenuated the increased concentration of insoluble Aβ1-42 observed in cerebellar tissue isolated from APPswe/PS1dE9 mice (**p<0.01; ANOVA; n=5; Figure 6.14).

### 6.2.6 Oral administration of rosiglitazone significantly decreased the number of plaques found in the cortex of APPswe/PS1dE9 mice.

There was a significantly greater number of Congo red-positive Aβ plaques in the hippocampus and cortex of APPswe/PS1dE9 mice (**p<0.01,
***p<0.001; ANOVA; n=5; Figure 6.15 and 6.16). Oral administration of rosiglitazone had no significant effect on the number of plaques found in the hippocampus of APPswe/PS1dE9 mice but it caused a significant decrease in the number of plaques present in the cortex of APPswe/PS1dE9 mice (***p<0.01; ANOVA; n=5; Figure 6.16).

6.2.7 A pro-inflammatory environment existed in the brains of APPswe/PS1dE9 mice.

In the brains of patients with AD, and in some animal models of AD (Stadler et al, 1999; Koenigsknecht-Talboo et al, 2008) activated microglia and reactive astrocytes are found around amyloid plaques. In addition, pro-inflammatory cytokines have been reported to be up-regulated in the brains of AD patients and in transgenic animal models (Aarli 2003, Zhang et al. 2009). Expression of GFAP and CD68 mRNA were significantly increased in hippocampal tissue isolated from the brains of APPswe/PS1dE9 mice (*p<0.05, ***p<0.001; student’s t-test for independent means; n=5; Figure 6.18). There was no change in CD11b mRNA expression. IL-1β, TLR-2 and TLR-4 mRNA expression were also significantly increased in hippocampal tissue isolated from the brains of APPswe/PS1dE9 mice (*p<0.05, ***p<0.001; student’s t-test for independent means; n=5; Figure 6.19).

Analysis by flow cytometry revealed that, although there was no difference in QD uptake by cells isolated from the brains APPswe/PS1dE9 mice compared to wild-type mice, there was a significant increase in expression of the cell surface marker of activation, IA/IE, by CD11b+ cells isolated from the brains of APPswe/PS1dE9 mice (*p<0.05; student’s t-test for independent means; n=5;
Figure 6.20 B). Oral administration of rosiglitazone significantly increased expression of IA/IE by CD11b\(^+\) cells isolated from the brains of wild-type, but not APPswe/PS1dE9, mice (*p<0.05; student’s t-test for independent means; n=5; Figure 6.21).

### 6.2.8 \(\text{A}\beta_{1-42}\) significantly increased expression of IA/IE by CD11b\(^+\) cells isolated from the brains of wild-type and APPswe/PS1dE9 mice.

CD11b\(^+\) cells isolated from the brains of control- and rosiglitazone-treated wild-type and APPswe/PS1dE9 mice were incubated with \(\text{A}\beta_{1-42}\) (8 \(\mu\text{M}\)) for 15 minutes and QD uptake and expression of cell surface markers of activation was assessed. \(\text{A}\beta_{1-42}\) had no significant effect on QD uptake by CD11b\(^+\) cells (Figure 6.22 A). However, \(\text{A}\beta_{1-42}\) significantly increased expression of IA/IE by CD11b\(^+\) cells isolated from the brains of wild-type and APPswe/PS1dE9 mice (*p<0.05, **p<0.001; two-way ANOVA; n=5; Figure 6.22 B). \(\text{A}\beta_{1-42}\) significantly increased expression of CD80 by CD11b\(^+\) cells isolated from the brains of wild-type mice (*p<0.05; two-way ANOVA; n=5; Figure 6.22 C). There was no significant effect of \(\text{A}\beta_{1-42}\) on QD uptake or expression of cell surface markers of activation by CD11b\(^+\) cells isolated from the brains of wild-type or APPswe/PS1dE9 mice that received oral administration of rosiglitazone (Figure 6.22).

\(\text{H}_2\text{O}_2\) significantly reduced QD uptake by CD11b\(^+\) cells isolated from the brains of APPswe/PS1dE9 mice that had received oral administration of rosiglitazone (*p<0.05; two-way ANOVA; n=5; Figure 6.23 A). There was no significant effect of \(\text{H}_2\text{O}_2\) on expression of cell surface markers of activation by
CD11b+ cells isolated from the brains of control- or rosiglitazone-treated wild-type or APPswe/PS1dE9 mice (Figure 6.23).

6.2.9: Aβ plaques were visible in the hippocampus and cortex of control- and rosiglitazone-treated APPswe/PS1dE9 mice.

Aβ plaques were observed in the hippocampus, dentate gyrus and cortex of control- and rosiglitazone-treated APPswe/PS1dE9 mice (Figures 6.24 and 6.25). Magnification of Aβ plaques (60 X) in the hippocampus and the cortex of an APPswe/PS1dE9 mouse revealed the presence of CD11b+ cells in the area of Aβ plaques (red staining; Figure 6.26).
Figure 6.1: Genotyping of APPswe/PS1dE9 mice

Tail snips were taken from the wild-type and APPswe/PS1dE9 transgenic mice and were analysed by reverse transcriptase-PCR and gel electrophoresis for expression of the PS1dE9 (A) and the APPswe (B) transgenes. Mice 3, 4 and 5 contain both transgenes while mice 1 and 2 are wild-type. The lower band on gel (A) present in all lanes corresponds to the PrP control gene.
Figure 6.2: Front- and hind-paw strength were not impaired in APPswe/PS1dE9 mice.

Front- and hind-paw strength of wild-type and APPswe/PS1dE9 (APP Tg) mice were assessed prior to oral administration of rosiglitazone using the hangwire and inverted screen tests. (A) The hangwire test consisted of 4 one-minute trials during which time the mice were suspended from the wire by their front paws. (B) The inverted screen consisted of a single one-minute trial during which time the mice were suspended from the screen by all four paws. The duration the mice held onto the wire or screen was recorded. There was no difference in latency to fall from either apparatus between the wild-type and transgenic mice. Values are expressed as means ± SEM (n=10).
Figure 6.3: Motor co-ordination and balance was not impaired in APPswe/PS1dE9 mice.

The gait of wild-type and APPswe/PS1dE9 (APP Tg) mice was examined prior to oral administration of rosiglitazone using footprint analysis. There was no difference in front stride length (A) or hind stride length (B) in transgenic compared with wild-type mice. Values are expressed as means ± SEM (n=10).
Figure 6.4: Oral administration of maple syrup or maple syrup containing rosiglitazone did not significantly affect the body weights of the mice.

Mice were weighed 3 days prior to beginning oral administration of either maple syrup or maple syrup containing rosiglitazone and were then weighed at regular intervals over the 28-day administration period. Mean body weight over the 3 week period was similar in all treatment groups. Values are expressed as means ± SEM (n=5).
Chapter 6 APPswe/PS1dE9 study

A

Wt Control-treated  Tg Control-treated

Pathlength (cm)

Days

B

Wt Control-treated  Tg Control-treated

Pathlength (cm)

Day 1  Day 5

C

Wild-type  APPswe/PS1dE9  Wild-type  APPswe/PS1dE9

Day 1  Day 5
Figure 6.6: There was no significant difference in latency to the platform between wild-type and APPswe/PS1dE9 transgenic mice.

Wild-type (Wt) and APPswe/PS1dE9 (Tg) mice were tested for spatial memory in the Morris water maze. (A) There was no significant difference in time taken to find the platform between the wild-type and transgenic mice over 5 days of training. (B) Both the wild-type and APPswe/PS1dE9 mice showed a significant reduction in latency over the 5 days of training (***p<0.01; ANOVA; n=5). Values are expressed as means ± SEM (n=5).
Following oral administration of rosiglitazone for 2 weeks, mice underwent behavioural training in the Morris water maze and the effect of rosiglitazone on learning was assessed. Treatment of wild-type or APPswe/PS1dE9 transgenic mice with rosiglitazone had no significant effect on pathlength taken by mice to the platform (A) or the time taken to find the platform (B). All mice showed significant learning over the 5 days of training. Values are expressed as means ± SEM (n=5).
Figure 6.8: Performance in the probe trial of the Morris water maze was unaffected by genotype or rosiglitazone.

Following oral administration of rosiglitazone for 3 weeks and behavioural training in the Morris water maze, mice underwent a single 60 second probe trial during which the escape platform was removed from the water maze. (A) There was no significant difference in the percentage time wild-type or APPswe/PS1dE9 mice spent swimming in the quadrant that previously contained the platform. Oral administration of rosiglitazone had no significant effect on performance. (B) Representative traces of the swim-path of mice in each treatment group are displayed. Values are expressed as means ± SEM (n=5).
Chapter 6 APPswe/PS1dE9 study

A

![Graph showing path length over days for Wt Control-treated and Tg Control-treated groups.]

B

![Bar graph showing path length on Day 5 and Day 6 for Wt Control-treated and Tg Control-treated groups.]

C

![Images showing behavior of Wild-type and APPswe/PS1dE9 mice on Day 5 and Day 6.]
Figure 6.10: Wild-type mice had a significantly shorter latency to the platform than APPswe/PS1dE9 transgenic mice.

Wild-type (Wt) and APPswe/PS1dE9 (Tg) mice were tested for spatial memory in the Morris water maze. (A and B) There was a significant difference in the time taken to find the platform between the wild-type and transgenic mice on day 5 of reversal training (**p<0.01; ANOVA; n=5). Values are expressed as mean ± SEM (n=5).
Figure 6.11: Oral administration of rosiglitazone significantly reduced the length of the path taken to the platform by APPswe/PS1dE9 mice.

Following oral administration of rosiglitazone for 3 weeks, mice underwent reversal training in the Morris water maze and the effect of rosiglitazone on plasticity of learning was assessed. (A) Treatment of wild-type (Wt) mice with rosiglitazone had no significant effect on pathlength to the platform but (B) rosiglitazone significantly reduced the length of the path taken to the platform by transgenic (Tg) mice on day 5 and 6 of reversal training (**p<0.01; ANOVA; n=5). Values are expressed as means ± SEM (n=5).
Figure 6.12: Oral administration of rosiglitazone significantly reduced the latency to the platform by APPswe/PS1dE9 mice.

Following oral administration of rosiglitazone for 3 weeks, mice underwent reversal training in the Morris water maze and the effect of rosiglitazone on plasticity of learning was assessed. (A) Treatment of wild-type (Wt) mice with rosiglitazone had no significant effect on the time taken to reach the platform but (B) rosiglitazone significantly reduced the latency by transgenic (Tg) mice on day 5 of reversal training (*p<0.05; ANOVA; n=5). Values are expressed as means ± SEM (n=5).
Figure 6.13: There was no significant difference in soluble \(A\beta_{1-38}\), \(A\beta_{1-40}\) and \(A\beta_{1-42}\) concentrations in cerebellar tissue isolated from wild-type or APPswe/PS1dE9 mice.

Soluble \(A\beta_{1-38}\), \(A\beta_{1-40}\) and \(A\beta_{1-42}\) concentrations in the cerebellum of wild-type and APPswe/PS1dE9 mice, that had or had not received oral administration of rosiglitazone, were determined by multi-spot ELISA. There was no significant difference in the levels of soluble \(A\beta\) among treatment groups. Values are expressed as means ± SEM (n=5)

209
Figure 6.14: There was significantly greater insoluble Aβ_{1-42} in cerebellar tissue isolated from APPswe/PS1dE9 mice.

There was a significantly greater concentration of insoluble Aβ_{1-42} in the cerebellum of the APPswe/PS1dE9, compared with wild-type, mice (***p<0.01; ANOVA; n=5). Oral administration of rosiglitazone significantly reduced the insoluble Aβ_{1-42} concentration in the cerebellum of the APPswe/PS1dE9 mice (***p<0.01; ANOVA; n=5). Values are expressed as means ± SEM (n=5).
Figure 6.15: There was a significantly greater number of Congo red-positive Aβ plaques in the hippocampus of APPswe/PS1dE9 mice.

Cryostat sections prepared from the brains of control or rosiglitazone-treated wild-type or APPswe/PS1dE9 mice were stained with Congo red for detection of Aβ plaques in the brain. Sections were counter-stained with 1% w/v methyl green solution. (A) Average counts of plaque number in the hippocampus of standardised sections of tissue from each group. (B and C) Representative images of Congo red stained sections from each group; (B) 10 x magnification, (C) 20 x magnification. Scale bars are 100 μm. (**p<0.01; ANOVA; n=6-20). Values are expressed as means ± SEM (n=6-20).
Figure 6.16: Oral administration of rosiglitazone significantly decreased the number of Congo red-positive Aβ plaques in the cortex of APPswe/PS1dE9 mice.

Cryostat sections prepared from the brains of control or rosiglitazone-treated wild-type or APPswe/PS1dE9 mice were stained with Congo red for detection of Aβ plaques in the brain. Sections were counter-stained with 1% w/v methyl green solution. (A) Average counts of plaque number in the cortex of standardised sections of tissue from each group. (B and C) Representative images of Congo red stained sections from each group; (B) 10 x magnification, (C) 20 x magnification. Scale bars are 100 μm. (**p<0.01, ***p<0.001; ANOVA; n=6-20). Values are expressed as means ± SEM (n=6-20).
Chapter 6 APPswe/PS1dE9 study

Wild-type Control-treated

Wild-type Rosiglitazone-treated

APPswe/PS1dE9 Control-treated

APPswe/PS1dE9 Rosiglitazone-treated
Figure 6.17: Representative image of a Congo red-positive Aβ plaque in a section of the brain of an APPswe/PS1dE9 mouse.

This cryostat section was prepared and stained with Congo red for detection of Aβ plaques and counter-stained with 1% w/v methyl green solution to identify nearby nuclei. This image was acquired at 40 x magnification on a light microscope. The scale bar is 100 μm.
Figure 6.18: Expression of GFAP and CD68 mRNA significantly increased in the hippocampus of APPswe/PS1dE9 transgenic mice.

Snap-frozen hippocampal tissue from wild-type and APPswe/PS1dE9 mice was analysed for CD11b, GFAP and CD68 mRNA expression by real-time PCR. Mean expression of GFAP and CD68 mRNA was significantly increased in tissue prepared from APPswe/PS1dE9, compared with wild-type, mice (*p<0.05, ***p<0.001; student’s t-test for independent means; n=5). Values are expressed as means ± SEM (n=5).
Figure 6.19: IL-1β, TRL-2 and TLR-4 mRNA expression was significantly increased in the hippocampus of APPswe/PS1dE9 transgenic mice.

Snap-frozen hippocampal tissue from wild-type and APPswe/PS1dE9 mice was analysed for IL-1β, TLR-2 and TLR-4 mRNA expression by real-time PCR. Mean expression of each marker was significantly increased in tissue prepared from APPswe/PS1dE9, compared with wild-type, mice (*p<0.05, ***p<0.001; student’s t-test for independent means; n=5). Values are expressed as means ± SEM (n=5).
CD11b$^+$ cells isolated from the brains of wild-type and APPswe/PS1dE9 mice were incubated in the presence of QD (1 x $10^{-6}$ M) for 15 minutes and QD uptake and expression of cell surface markers of activation were assessed using flow cytometry. The number of CD11b$^+$QD$^+$ (A) cells and CD11b$^+$CD80$^+$ (C) cells was similar in wild-type and APPswe/PS1dE9 mice whereas the number of CD11b$^+$IA/IE$^+$ (B) cells was increased in APPswe/PS1dE9, compared with wild-type, mice (*$p<0.05$; Student’s t-test for independent means; $n=5$). Data are expressed as means ± SEM and are given as arbitrary values.
Chapter 6 APPswe/PS1dE9 study

A

Wild-type  APPswe/PS1dE9

B

Wild-type  APPswe/PS1dE9

C

Wild-type  APPswe/PS1dE9
Figure 6.21: Rosiglitazone significantly increased expression of IA/IE on CD11b+ cells from the brains of wild-type mice.

CD11b+ cells isolated from the brains of wild-type and APPswe/PS1dE9 mice were incubated in the presence of QD (1 x $10^{-6}$ M) for 15 minutes and QD uptake and expression of cell surface markers of activation were assessed. There was no effect of genotype or rosiglitazone on the number of CD11b+QD+ (A) or CD11b+CD80+ (C) cells in the brains of young or aged rats. (B) Expression of IA/IE was significantly increased on CD11b+ cells isolated from the brains of APPswe/PS1dE9 mice (*p<0.05; Student’s t-test for independent means; n=5). Data are expressed as means ± SEM and are given as arbitrary values.
Figure 6.22: Aβ_{1-42} significantly increased expression of IA/IE by CD11b^ cells isolated from the brains of wild-type and APPswe/PS1dE9 mice.

CD11b^ cells isolated from the brains of control- and rosiglitazone-treated wild-type and APPswe/PS1dE9 mice were pre-treated with or without Aβ_{1-42} (8 μM) for 15 minutes and incubated in the presence of QD (1 x 10^{-6} M) for 15 minutes. QD uptake and expression of cell surface markers of activation were assessed using flow cytometry. (A) There was no significant effect of Aβ_{1-42} on QD uptake by cells. (B) Aβ_{1-42} significantly increased expression of IA/IE on CD11b^ cells isolated from the brains of wild-type and APPswe/PS1dE9 mice (*p<0.05, ***p<0.001; Two-way ANOVA; n=5). (C) Aβ_{1-42} significantly increased expression of CD80 on CD11b^ cells isolated from the brains of wild-type mice (*p<0.05; Two-way ANOVA; n=5). Values are expressed as means ± SEM and are given as arbitrary values.
Chapter 6 APPswe/PS1dE9 study

**Figure 6A**

- Control-treated
- $\text{A}_\beta_{1-42}$-treated

**Figure 6B**

- Wild-type
- APPswe/PS1dE9

**Figure 6C**

- Wild-type
- APPswe/PS1dE9
CD11b+ cells isolated from the brains of control- and rosiglitazone-treated wild-type and APPswe/PS1dE9 mice were pre-treated with or without H$_2$O$_2$ (100 μM) for 15 minutes and incubated in the presence of QD (1 x 10$^{-6}$ M) for 15 minutes. QD uptake and expression of cell surface markers of activation were assessed using flow cytometry. (A) H$_2$O$_2$ significantly reduced QD uptake by CD11b+ cells isolated from the brains of APPswe/PS1dE9 mice that received rosiglitazone by oral administration (*p<0.05; Two-way ANOVA; n=5). (B and C) There was no effect of H$_2$O$_2$ on the number of CD11b+IE+$^+$ or CD11b+CD80+ cells in the brains of wild-type or APPswe/PS1dE9 mice. Data are expressed as means ± SEM and are given as arbitrary values.
Chapter 6 APPswe/PS1dE9 study

A

Control-treated

H2O2-treated

CD11b+QD+ cells

300

150

0

Wild-type

APPswe/PS1dE9

Rosiglitazone

B

CD11b+IA/IE+ cells

500

250

0

Wild-type

APPswe/PS1dE9

Rosiglitazone

C

CD11b+CD80+ cells

600

300

0

Wild-type

APPswe/PS1dE9

Rosiglitazone
Figure 6.24: Aβ plaques are visible in the dentate gyrus and hippocampus of control- and rosiglitazone-treated APPswe/PS1dE9 mice.

Cryostat sections were prepared from the brains of control- and rosiglitazone-treated wild-type and APPswe/PS1dE9 mice and were stained with an anti-human pan Aβ_{15-30} primary antibody and an Alexa 488nm secondary antibody to identify Aβ plaques (green). Sections were stained with an antibody against CD11b (red) and with DAPI to identify the nuclei of nearby cells (blue). Aβ plaques were clearly visible in the hippocampus and dentate gyrus of the APPswe/PS1dE9 control-treated and the APPswe/PS1dE9 rosiglitazone-treated mice. Representative images acquired at 10 x, 20 x and 40 x magnification from each treatment group are shown. Scale bars are 100 μm.
Cryostat sections were prepared from the brains of control- and rosiglitazone-treated wild-type and APPswe/PS1dE9 mice and were stained with an anti-human pan Aβ_{15-30} primary antibody and an Alexa 488nm secondary antibody to identify Aβ plaques (green). Sections were stained with an antibody against CD11b (red) and with DAPI to identify the nuclei of nearby cells (blue). Aβ plaques were clearly visible in the cortex of the APPSwe/PS1dE9 control-treated and the APPswe/PS1dE9 rosiglitazone-treated mice. Representative images acquired at 20 x and 40 x magnification from each treatment group are shown above. Scale bars are 100 μm.
Chapter 6 APPswe/PS1dE9 study

Magnification:

Wild-type
Control

Wild-type
Rosiglitazone

APPswe/PS1dE9
Control

APPswe/PS1dE9
Rosiglitazone
Figure 6.26: Aβ plaques in the cortex and hippocampus of APPswe/PS1dE9 mice.

Cryostat sections prepared from the brains of APPswe/PS1dE9 mice were stained with an anti-human pan Aβ_{15-30} antibody (green), an antibody against CD11b (red) and with DAPI to identify the nuclei of nearby cells (blue). Representative images of Aβ plaques in the (A) hippocampus and (B) cortex acquired at 60 x magnification are shown above. Scale bars are 100 μm.
6.3 Discussion

The overall objectives of this study were to investigate plaque deposition and microglial activation in the APPswe/PS1dE9 mouse model of AD and to assess their effects on learning and memory in the Morris water maze. In addition, the ability of rosiglitazone to attenuate the changes observed in the brains of APPswe/PS1dE9 mice was assessed. The significant findings of this study were that APPswe/PS1dE9 mice showed plaque deposition in the hippocampus and cortex at 7 months of age, that was accompanied by increased microglial activation and impaired learning in the reversal phase of the Morris water maze. Furthermore, rosiglitazone attenuated the genotype-related impairment in learning as well as the increased level of Aβ1-42 observed in the brains of APPswe/PS1dE9 mice.

During the acquisition phase of the Morris water maze, there was no effect of genotype on learning and both wild-type and APPswe/PS1dE9 mice showed reduced pathlength and time taken to find the platform over five days of training. In contrast, during the reversal phase of the Morris water maze, there was a difference in learning between the wild-type and APPswe/PS1dE9 mice. This difference was observed in both pathlength and time taken to find the platform and is consistent with previous data which showed that 8 month old APPswe/PS1dE9 mice exhibited impaired memory in the Morris water maze (Cao et al., 2007; Jankowsky et al., 2005). In addition, Savonenko and colleagues (2005) showed that in APPswe/PS1dE9 mice, memory declined as a function of age. They reported that APPswe/PS1dE9 mice were indistinguishable from non-transgenic animals in the Morris water maze and radial arm water maze at 6 months of age but showed impairments at 18 months of age. The impairments
observed in the reversal phase of the Morris water maze by the 7-8 month-old APPswe/PS1dE9 mice used in this study indicated that changes occurred in learning at an earlier time than previously reported and, significantly, that these were accompanied by microglial activation and Aβ accumulation.

Oral administration of rosiglitazone reduced pathlength and time taken to find the platform by APPswe/PS1dE9 mice during the reversal phase of the Morris water maze. This is consistent previous findings of Pederson and colleagues (2006) who found that rosiglitazone attenuated learning and memory deficits in Tg2576 mice in the 8-arm radial maze system. Pederson and colleagues (2006) proposed that the effects of rosiglitazone were due to it’s glucocorticoid-lowering actions since glucocorticoid receptors are highly expressed in the hippocampus whereas, in the current study it is proposed that the effects of rosiglitazone are mediated by it’s anti-inflammatory properties (Jiang et al, 1998; Ricote et al, 1998; Loane et al, 2009) which may impact on Aβ clearance from the brain.

Aβ production and accumulation is considered central to the pathogenesis of AD and studies using transgenic mouse models of AD provide the opportunity to link evidence of these changes with evidence of cognitive impairment. In this study, there was no difference in the levels of soluble Aβ observed in the brains of wild-type and APPswe/PS1dE9 mice which is broadly consistent with previous data from Westerman and colleagues (2002); this group reported that the presence of soluble Aβ in the brains of Tg2576 mice under the age of 6 months had no impact on learning and memory in the Morris water maze. However, in this study increased insoluble Aβ1-42 levels were observed in cerebellar tissue isolated from the brains of APPswe/PS1dE9 mice and increased Congo red-positive Aβ plaque
burden was also observed in the hippocampus and cortex of APPswe/PS1dE9 mice. These changes parallel the behavioural deficit in the Morris water maze. Consistent with this, Westerman and colleagues (2002) reported that after 6 months of age, increased levels of insoluble Aβ were observed alongside impairments in the Morris water maze. Furthermore, Liu and colleagues (2003) also correlated the level of hippocampal insoluble Aβ_{1-42} with impairments in spatial learning and memory in APP/PS1 mice. In support of the hypothesis that insoluble Aβ_{1-42} levels correlate with behavioural deficits, the present findings indicate that rosiglitazone attenuated the increase in insoluble Aβ_{1-42} levels observed in cerebellar tissue isolated from APPswe/PS1dE9 mice and reduced the Aβ plaque burden in the cortex in parallel with attenuating the behavioural deficit in the Morris water maze.

The amyloid cascade hypothesis postulates that the accumulation of Aβ in the brains of AD patients leads to microglial activation and subsequent neuronal death (Hardy and Higgins, 1992). In this study, microglial activation was assessed by analysis of mRNA expression of cell surface activation markers and cytokines in hippocampal tissue from wild-type and APPswe/PS1dE9 mice. CD11b mRNA expression was unchanged in APPswe/PS1dE9 mice which may be because the mice in this study were relatively young. A similar finding was reported by Jimenez and colleagues (2008); in this case, CD11b was also not up-regulated in the hippocampus of APP/PS1 mice at 6 months of age but it was increased at 18 month of age. However, Manczak and colleagues (2009) reported an increase in CD11b mRNA in the cortex of 10 month-old Tg2576 mice. This discrepancy in expression levels of CD11b mRNA could be the result of regional
variations in CD11b expression or small differences in the ages of the animals but particularly, the type of transgenic mouse being examined.

In contrast to the lack of change observed here in CD11b, mRNA expression of the proinflammatory cytokine IL-1β was increased in hippocampal tissue isolated from the brains of APPswe/PS1dE9 mice and this is in agreement with a previous report by Ruan and colleagues (2009). IA/IE which is an epitope of MHC II was also assessed in tissue prepared from these animals and the data showed an increase in CD11b⁺/IA/IE⁺ cells in tissue prepared from APPswe/PS1dE9 mice. To the author’s knowledge, no previous report detailing expression of MHC II in the brains of APPswe/PS1dE9 mice exists. However, MHC II expression by microglia has been reported to be increased in the brain with ageing (O’Reilly et al., 2009; Henry et al., 2009) and therefore would be anticipated to be increased in the brains of AD patients or mouse models of AD. In addition to the change in IA/IE expression, CD68 mRNA expression was increased in hippocampal tissue prepared from APPswe/PS1dE9, compared with wildtype, mice. This is a marker of microglia and considered to be useful in identifying phagocytic microglia (Wong et al., 2005).

In addition, TLR4 and TLR2 mRNA expression was increased. TLR4-dependent upregulation of proinflammatory cytokines in the brain of a transgenic mouse model of AD has previously been reported (Jin et al., 2008) and TLR2 has been reported to mediate the effect of fibrillar Aβ on microglia activation; in the latter case, the Aβ-induced expression of proinflammatory molecules was inhibited by TLR2 antibodies (Jana et al., 1998). These data indicate that TLR4 and TLR2 could mediate the proinflammatory environment observed in the brains of the APPswe/PS1dE9 mice. GFAP mRNA expression was also increased in
hippocampal tissue from the brains of APPswe/PS1dE9 mice which indicated that astrocytes were activated alongside microglia.

However, flow cytometric analysis revealed that there was no difference in phagocytic activity of CD11b^+ cells isolated from the brains of APPswe/PS1dE9 mice compared with wild-type mice although there was an increase in the number of CD11b^+/IA/IE^+ cells. This was somewhat surprising since a positive correlation between MHC II mRNA expression by microglia and QD uptake was observed (See Chapter 5). CD68, which is generally considered to be indicative of phagocytic activity (Wong et al, 2005) was increased in transgenic mice albeit at the mRNA level. In addition, MHC II mRNA has been reported to be increased in the brain with ageing (O’Reilly et al, 2009; Henry et al, 2009) and therefore, it was predicted that it may also increase in the brains of these transgenic animals. It is possible that this finding uncouples 2 identifiers of microglial activation, cell surface expression of antigens and phagocytic activity. However, it is also possible that a positive correlation between mRNA expression of cell surface markers of microglial activation and phagocytosis does not translate to a correlation between protein expression and phagocytosis. However, another possible explanation for the apparent discrepancy is that the CD11b^+ cells isolated from the brains of APPswe/PS1dE9 mice were undergoing phenotypic changes to develop into phagocytic cells and that at an older age, phagocytic activity of the CD11b^+ cells may have been increased.

Contrary to expectations, increased expression of IA/IE was observed on cells isolated from the brains of wild-type mice that had received oral administration of rosiglitazone. The meaning of this finding is unclear as previous reports detail only the anti-inflammatory effects of PPARγ agonists

234
Chapter 6 APPswe/PS1dE9 study

(Bernardo et al., 2000; Jiang et al., 1998; Ricote et al., 1998). Therefore, it is evident that further studies are required to determine the mechanisms by which PPARγ agonists modulate inflammatory responses in the CNS.

To assess the impact of an additional stressor on microglial activation of cells isolated from the brains of APPswe/PS1dE9 mice, cells were stimulated acutely ex vivo with Aβ1-42. Treatment with Aβ1-42 had no effect on phagocytic activity. However, expression of IA/IE, by CD11b+ cells isolated from the brains wild-type and APPswe/PS1dE9 mice, was increased following treatment with Aβ1-42. These data further emphasise that phagocytic cells do not necessarily express cell surface markers of activation. It is interesting that CD11b+ cells isolated from the brains of wild-type and APPswe/PS1dE9 mice that had received oral administration of rosiglitazone for 4 weeks were unable to respond to Aβ1-42 and showed no change in expression of IA/IE. These data indicate that the protective anti-inflammatory effect of rosiglitazone acts to limit inflammation in the brains of the APPswe/PS1dE9 mice and to prevent external stimuli from exacerbating the inflammatory response. Treatment with Aβ1-42 also increased expression of CD80 by CD11b+ cells isolated from the brains of wildtype control, but not rosiglitazone-treated, mice. These data suggest that rosiglitazone exerted a protective affect against additional stimulation of CD11b+ cells by Aβ1-42.

The data presented in this study indicate that parallels exist between Aβ accumulation in the brain of APPswe/PS1dE9 mice, and the impairments observed in spatial learning, and that this may be influenced by the inflammatory environment in the brain. An attenuation of the Aβ accumulation by rosiglitazone was coupled with a beneficial affect on cognition. Rosiglitazone also exerted a protective effect within the brains of wildtype and APPswe/PS1dE9 mice against
increased microglial activation by an additional stimulus of exogenous Aβ_{1-42}. These data suggest that rosiglitazone may represent a useful treatment to prevent self-perpetuating inflammation in AD.
Chapter 7

General Discussion
7.1 General Discussion

AD is the most common neurodegenerative disease and its incidence rises exponentially with age (Benveniste et al, 2001; Lacor, 2007; Hasegawa, 1998). There is no known cure for AD and with the increase in the ageing population the financial and societal burden of caring for AD patients is enormous and increasing (Husain et al, 2008). Therefore, it is crucial to find therapies that will halt disease progression and cognitive decline. Current treatments, at best, marginally decrease the progression of cognitive deficits but are unable to prevent or reverse the underlying pathophysiology of AD (Rafii and Aisen, 2009; Hussain et al, 2008). These treatments include acetylcholinesterase inhibitors such as rivastigmine and galanthamine, which prolong the action of the neurotransmitter acetylcholine by reducing it’s metabolism in the synaptic cleft, and N-methyl-D-aspartate receptor antagonists such as memantine or AP7, which interfere with the action of glutamate, thereby preventing excitotoxic cell death (Hussain et al, 2008).

Given the acknowledged importance of inflammation in the pathogenesis of AD, the overall objective of this study was to examine the role played by microglia under different experimental circumstances which may be relevant in increasing an understanding of their role in AD. Particular emphasis was placed on assessing factors which modify phagocytosis. It was argued that, since age is the greatest risk factor in developing AD, it was important to compare phagocytic activity of microglia prepared from aged and young rats. Similarly, since oxidative changes are known to occur with age, it was considered that this might be a critical factor in modulating phagocytic activity; therefore, the modulatory effect of H$_2$O$_2$ on phagocytic activity was also investigated. Finally, because of the pivotal role played by Aβ in microglial function, as well as neuronal function, the effect of acute Aβ exposure on phagocytic
activity of cultured glia and glia prepared from young and aged rats was investigated; the effect of chronic exposure was investigated by comparing phagocytic activity in glia prepared from wildtype and APPswe/PS1dE9 mice. Furthermore, using the APPswe/PS1dE9 mouse model of AD, it was possible to investigate the link between microglial activation, Aβ accumulation and cognition, and to assess the ability of rosiglitazone, which possesses anti-inflammatory properties, to modulate these factors.

The development of the assay to investigate phagocytosis was the cornerstone of these studies. Although a variety of other options exist for examining phagocytosis, the QD-based phagocytic assay was developed as QD possess a number of properties that make them superior to conventional methods. For example, QD are superior to conventional organic due to their high degree of photostability which results in a long-lived signal. They also possess tuneable emission spectra and broadband excitation over a wide range of wavelengths. Due to their size, QD are also suitable for tagging to a range of biological targets that are of interest to researchers. Although QD are extremely useful, novel and exciting, it is important to recognise the limitations of the phagocytic assay. For example, in the current situation, the size of the QD leads to some debate regarding the mechanism utilised by the cells to ingest them. Although two known inhibitors of phagocytosis, NaF and H2O2 inhibited QD uptake, cytochalasin B (an inhibitor known to paralyse reorganisation of the actin cytoskeleton) failed to exert a clear effect on QD uptake by the CD11b+ cells in a mixed glial culture. However, it is important to note that a comparison of QD uptake to the uptake of commercially-available fluorescent latex particles that are routinely used to assess phagocytosis revealed that cytochalasin B also failed to prevent uptake of the fluorescent latex particles. In the current situation, considering that the other
inhibitors of phagocytosis that were used exerted identical effects on the uptake of both the QD and the fluorescent latex particles, it is reasonable to conclude that QD are undergoing internalisation via a phagocytic pathway. In the future, it could be useful to compare QD uptake to the classical zymosan-test and to determine if both assays respond to the modulators in the same manner. This would further confirm QD uptake as a useful phagocytic assay.

Since microglia are believed to play an important role in the pathogenesis of AD, it is important to understand the effect of Aβ on microglial activation (Nakamura, 2002). Aβ peptides have been shown to be potent activators of microglia (Szczepanik et al, 2001) and in vitro stimulate production of proinflammatory mediators (Ransohoff and Perry, 2009) including neurotoxic substances such as NO and ROS (Weldon et al, 1998). Fibrillar Aβ can induce phagocytosis by microglia both in vitro and in vivo (Szczepanik et al, 2001; Weldon et al, 1998; Casal et al, 2002).

ROS and Aβ are upregulated in the brains of AD patients (Radak et al, 2004) and for this reason, the modulatory effects of Aβ1-42 and H2O2, a producer of ROS, on microglia which were identified as being CD11b+, were investigated in mixed glial cultures. Aβ1-42 had no effect on phagocytic activity of cells but increased secretion of IL-1β and expression of OX-6 and CD68. H2O2 however, inhibited phagocytic activity, increased expression of IL-1β and decreased expression of OX-6 and CD68. These results were also replicated by NaF, another inhibitor of phagocytosis, suggesting that if microglial cells were actively producing the proinflammatory cytokine IL-1β, their phagocytic activity was inhibited. It also appeared that if the phagocytic activity of microglial cells was inhibited, expression of OX-6 which is involved in antigen presentation, and CD68 which is implicated in phagocytosis, was decreased. These findings suggest that microglia can undergo different modes of
activation and that, in a proinflammatory environment, when proinflammatory cytokines are being produced, microglia are not capable of phagocytosis. However, it is possible for microglia to express cell surface activation markers alongside secretion of proinflammatory cytokines. This is interesting to consider in the context of the chronic inflammation that is often observed in neurodegenerative diseases; in this proinflammatory environment cell death can occur releasing neurotoxic molecules which stimulate microglial activation and the further production of proinflammatory mediators. It is possible that the inhibitory effect of this proinflammatory environment on phagocytic activity results in cells becoming trapped in a vicious cycle of inflammation.

The principal risk factor for AD is age and therefore it was considered essential to assess age-related changes in microglial activation. Phagocytosis and expression of OX-6 were increased in parallel in cells isolated from the brains of aged rats while expression of IL-1β mRNA was decreased in the cortex and hippocampus of aged rats. These data further support the theory that, phagocytic activity and expression of cell surface activation markers are directly related while an inverse relationship exists between phagocytic activity and synthesis of proinflammatory cytokines. These data provide a potential explanation for the inefficiency of microglial cells in removal of Aβ from the brains of AD patients by phagocytosis – it is possible that the inflammatory environment in the brains of AD patients prevents phagocytosis by microglia.

Since ROS and Aβ are both upregulated in the brains of AD patients (Radak et al, 2004), and Aβ1-42 and H2O2 had been shown to exert such definitive effects on microglial activation in vitro, the effects of these modulators were assessed on cells isolated from the brains of young and aged rats. Contrary to the data obtained in
vitro, both Aβ₁₄₂ and H₂O₂ caused a significant increase in phagocytic activity in the cells isolated from the brains of young rats. This effect of Aβ is not surprising considering it is produced constitutively during normal cell metabolism and is present in small quantities in normal healthy individuals (Weldon et al, 1998). It is likely that in healthy, young adults, Aβ is routinely phagocytosed, metabolised by the cells and cleared from the brain. H₂O₂ is a producer of ROS, which can cause tissue damage and cell death (Shen et al, 2008), and dying cells may produce oxidative signals that stimulate phagocytosis.

The most interesting results of this study were the effects of Aβ₁₄₂ and H₂O₂ on cells isolated from the brains of aged rats. Aβ₁₄₂ had no effect on phagocytic activity of cells isolated from the brains of aged rats. This reflects the in vitro situation and when considered alongside the data from the young rats, may represent a realistic model of what happens in vivo. The cells isolated from the brains of the aged rats displayed increased phagocytic activity and it is possible that in vivo this elevated phagocytosis facilitates clearance of cellular debris and maintenance of CNS tissue homeostasis. However, the data indicate that if the cells are stimulated by an additional stressor such as Aβ₁₄₂ that they are unable to respond to this insult. This could be described as a ceiling effect on the phagocytic activity of these cells rather than a defect and it could result in serious deleterious effects in the brains of aged individuals.

H₂O₂ also exhibited differential age-related effects on the phagocytic activity of microglia. H₂O₂ inhibited the phagocytic activity of cells isolated from the brains of aged rats and this reflects the results that were obtained in the in vitro study. There are conflicting data in the literature regarding the effect of H₂O₂ on phagocytosis. One group reported that H₂O₂ decreased phagocytosis by rat alveolar macrophages in
vitro (Oostings et al, 1990) while another reported that it increased phagocytosis by human neutrophils in vitro (Bejarano et al, 2006). These differences in the effects of H₂O₂ may be species-dependent. One mechanism by which H₂O₂ may inhibit phagocytosis is by decreasing cellular ATP (Oostings et al, 1990) but it also induces the NFκB signalling pathway resulting in production of TNF-α (Dimayuga et al, 2001) which promotes a proinflammatory environment. That ROS production is upregulated in the brains of aged rats is well established (Murray and Lynch, 1998) and it has been reported that the oxidative state of the cell regulates microglial activation (Shih et al, 2006). It is possible that the addition of H₂O₂ to the cells isolated from the brains of aged animals induced an excessive oxidative stress thereby essentially paralysing phagocytic function (Shih et al, 2006). Overall, it is possible that cells prepared from aged rats are more vulnerable to changes in the microenvironment.

The amyloid cascade hypothesis was proposed in the 1990's by Hardy and Higgins as the key mechanistic pathway in the development of AD (Hardy and Higgins, 1992). It proposes that accumulation of Aβ is the fundamental, initiating event in AD, leading to NFT formation, microglial activation and neuronal loss, resulting in cognitive decline (Lacor, 2007; Hardy and Selkoe, 2002). The focus of AD research on the amyloid cascade hypothesis is supported by strong genetic evidence implicating the primacy of the amyloid pathway (Rafii and Aisen, 2009). There are currently 4 genes (APP, PS1, PS2 and ApoE4) known to be involved in the development of AD and all of these genes are involved in Aβ production or deposition (Hardy and Selkoe, 2002). Aβ deposition has been shown to precede the appearance of NFT in human brains (Yamaguchi et al, 2001) and more recently, Oddo and colleagues (2006) showed that Aβ immunotherapy reduced plaque load
resulting in reduced tau pathology in their triple transgenic animal model of AD. This suggests that tau pathology is a direct consequence of Aβ accumulation and amyloid plaque formation (Oddo et al, 2006).

In this study, increased levels of insoluble Aβ1-42 were observed in tissue isolated from the brains of the APPswe/PS1dE9 mice and this was accompanied by significant plaque deposition in the cortex and hippocampus. This increased level of Aβ in the brain was accompanied by increased expression of the cell surface marker of activation MHC II and the proinflammatory cytokine IL-1β indicative of a proinflammatory microenvironment in the brains of the APPswe/PS1dE9 mouse model of AD. Importantly, a blunted phagocytic response was observed suggesting that the increased expression of proinflammatory mediators negatively impacted on phagocytic activity which, in turn, resulted in increased Aβ. These changes in the APPswe/PS1dE9 mice were accompanied by an impairment in the reversal phase of the Morris water maze. The changes seen both in the in vitro and ex vivo experiments following treatment of cells with Aβ1-42 indicate that accumulation of Aβ leads to altered microglial activation, resulting in upregulation of mediators of inflammation, impaired microglial phagocytic function and further upregulation of Aβ. It might therefore be suggested that, in neurodegenerative diseases, microglial cells can become trapped in a self-perpetuating cycle of damage where the initial immune response further enhances the damage.

The finding that there was no difference in the phagocytic activity of CD11b+ cells isolated from the brains of APPswe/PS1dE9 mice compared with wild-type mice, despite an increased number of plaques, is very important to consider in light of the knowledge that microglia are very efficient phagocytes and have been shown to phagocytose amyloid aggregates and senile plaques in vitro (Bard et al, 2000; DeWitt
et al, 1998). It must be contemplated whether these microglia possess an intrinsic deficit in their phagocytic activity or whether there is an alternative explanation for their lack of activity. Previous finding suggest the later (Koenigsknecht and Landreth, 2004). For example, studies utilising vaccination approaches to develop a therapy for AD revealed that the introduction of anti-Aβ antibodies to the brain resulted in rapid removal of deposited Aβ from the brain following a robust phagocytic response of plaque-associated microglia (Schenk et al, 1999; Bard et al, 2000). These findings suggest that plaque-associated microglia are capable of phagocytosis and Aβ clearance but that their activity is paralysed or unelicited. It is possible that microglia do not recognise amyloid plaques as substances that require removal. Schenk and colleagues (1999) argued that the FcR on the surface of the microglial cells recognised the Aβ-antibody complex.

It seems reasonable to suggest that the most logical way to attempt to end the self-perpetuating cycle of inflammation and damage would be to prevent or reduce the inflammation, possibly allowing stimulation of phagocytic activity of the microglia leading to Aβ clearance and improved functioning. Rosiglitazone, a PPARγ agonist that has reported anti-inflammatory effects (Jiang et al, 1998; Ricote et al, 1998), was used to test this theory. Rosiglitazone successfully reversed the impairment in spatial learning in the Morris water maze, reduced the levels of insoluble Aβ_{1-42} and reduced the plaque load in the brains of APPswe/PS1dE9 mice. Rosiglitazone increased expression of IA/IE by microglia isolated from wild-type mice and therefore, the mechanism of action of rosiglitazone may involve stimulation of expression of IA/IE and phagocytic activity of microglia which is consistent with the earlier results that indicated a direct relationship between expression of cell surface activation markers and phagocytosis. Regardless of the pathway involved in mediating the effects of
rosiglitazone, the reduction in Aβ was accompanied by an improvement in learning, establishing a strong link between Aβ and cognition.

In this study, the data showed that Aβ_{1-42} treatment of cells isolated from the brains of wildtype and APPswe/PS1dE9 mice increased the expression of IA/IE but failed to increase phagocytosis. Although the effect was observed in cells isolated from both wildtype and APPswe/PS1dE9 mice, the response was depressed in the cells isolated from the APPswe/PS1dE9 mice. Aβ_{1-42} also increased expression of CD80 by cells isolated from wild-type mice but failed to elicit the same response from cells isolated from the brains of APPswe/PS1dE9 mice. This indicates that the cells from the APPswe/PS1dE9 mice were unable to respond to an additional external stimulus and these findings pose an interesting parallel with cells prepared from aged rats. An interesting result from this study was that rosiglitazone exerted a protective effect against addition of exogenous Aβ_{1-42}. Cells isolated from wild-type and APPswe/PS1dE9 mice that had received oral administration of rosiglitazone did not respond to exogenous Aβ_{1-42}. This suggests that rosiglitazone exerted an anti-inflammatory effect on the cells and succeeded in maintaining the cells in this anti-inflammatory state following exposure to an additional insult. The mechanism by which rosiglitazone exerts its effect is not fully understood but it appears to exert a beneficial effect in the APPswe/PS1dE9 mouse model of AD.

The data acquired in this study support the amyloid cascade hypothesis of AD and suggest that the proinflammatory environment which exists in the brains of AD patients is likely to exert an inhibitory effect on the phagocytic activity of microglia, resulting in accumulation of Aβ. New therapies for AD are currently being developed aimed at preventing Aβ formation, blocking its aggregation into plaques, lowering its soluble levels in the brain, and disassembling existing amyloid plaques (Rafii and
Aisen, 2009; Husain et al, 2008). The data acquired in this study suggest that a potential therapeutic strategy may involve modulating the proinflammatory environment of the brains of AD patients, facilitating the activation of phagocytic microglia and thereby increasing Aβ clearance.

7.2 Future Work

Future studies could include:

- Analysis of the phagocytic and cytokine profile of isolated primary microglia and astrocytes.
- Assessment of microglial phagocytosis using fluorescently-labelled Aβ.
- Analysis of the effects of purified forms of Aβ, both soluble and insoluble, on microglial activation.
- Comparison of activation states of CD11b+ cells isolated from the brains of young and aged, wild-type and APPswe/PS1dE9 mice.
Chapter 8

Bibliography
8.0 Bibliography


Lacor, P.N. 2007 Advances on the Understanding of the Origins of Synaptic Pathology in AD. *Current Genomics* 8:486-508


Lynch, A.M. and Lynch, M.A. 2001. The age-related increase in IL-1 type 1 receptor in rat hippocampus is coupled with an increase in caspase-3 activation. *Eur J Neurosci* 15:1779-1788


Appendix I

Publications

Peer-reviewed papers:


Abstracts for oral presentation:


Abstracts for Poster Presentation:

O'Reilly JA, Lynch MA. (2009) APPsw/PS1dE9 mice exhibit behavioural deficits and increased microglial activation. *Irish Journal of Medical Science*.


Appendix II

Materials

Cell culture media

Roswell Park Memorial Institute (RPMI)-1640 medium (Biosera, UK) was supplemented with 10% heat-inactivated (56 degrees Celsius (°C) for 60 minutes) foetal calf serum (FCS) (Biosera, UK) and 1% Ciprofloxacin hydrochloride (1 mg/ml; Mediatech Inc., USA) or 1% penicillin/streptomycin (100 μg/ml; Gibco, UK).

Dulbecco’s Modified Eagles Medium (DMEM) (Gibco, UK) was supplemented with 10% heat-inactivated (56°C for 60 minutes) foetal calf serum (FCS) (Biosera, UK) and 1% Ciprofloxacin hydrochloride (1 mg/ml; Mediatech Inc., USA.) or 1% penicillin/streptomycin (100 μg/ml; Gibco, UK).

Phosphate-buffered saline (PBS) 10x

80 g Sodium chloride (NaCl, 137 mM; Sigma, UK)
11.5 g di-Sodium hydrogen phosphate (Na₂HPO₄, 8.1 mM; Sigma, UK)
2 g Potassium dihydrogen phosphate (KH₂PO₄, 10 mM; Sigma, UK)
2 g Potassium chloride (KCl, 2.7 mM; Sigma, UK)
Dissolved in 1 L ddH₂O, pH 7.3.

ELISA coating buffer

0.84 g Sodium Hydrogen Carbonate (NaHCO₃)
0.36 g Sodium Carbonate (Na₂CO₃)
Dissolved in 100 ml PBS, pH to 9.5.
**ELISA Assay Diluent**

100 ml 1x PBS (Sigma, UK)

1 g Bovine Serum Albumin (Sigma, UK) for IL-1β ELISA or 10 ml of FCS for IL-6 ELISA

**ELISA Wash Buffer**

1 L 1x PBS (Biosera, UK)

500 µl Tween-20

**ELISA Substrate Solution**

5 ml colour reagent A: stabilized peroxide solution (R&D Systems, UK).

5 ml colour reagent B: stabilized chromogen solution (R&D Systems, UK).

**ELISA Stopping Solution (1 M)**

26.6 ml 18.8 M H₂SO₄

473.4 ml dH₂O

**Fluorescence Activated Cell Sorting (FACS) Buffer**

500 ml PBS

10 ml FCS

0.5 g Sodium azide (NaN₃)

**FACS Block**

50% FACS Buffer

50% FCS
**Krebs solution**

3.975 g NaCl
0.095 g KCl
0.08 g KH$_2$PO$_4$
0.135 g MgSO$_4$
0.67 g NaHCO$_3$
0.9 g Glucose

Make up to 500 ml with dH$_2$O and pH to 7.3

**Calcium chloride (stock)**

2.94 g CaCl$_2$ in 20 ml dH$_2$O

Store at 4°C

**Krebs solution containing calcium**

Add 200 µl CaCl$_2$ to 100 ml Krebs solution (1:500 dilution) just before use, for washing or short term storage. For long term storage, add 10% DMSO to Krebs/CaCl$_2$, snap freeze tissue in liquid N$_2$ and store at -80°C.

**Sodium chloride-sodium citrate (SSC) buffer 20X**

88.2 g 0.3 M Trisodium citrate
175.32 g 3 M Sodium chloride

Make up to 1 L with dH$_2$O and pH to 7.

**Guanidine buffer**

7.51 g guanidine

Dissolved in 1 L dH$_2$O, pH 8
**Digestion Buffer**

5 ml 20X SSC

200 μl 0.5 M ethylene diamine tetraacetic acid (EDTA)

2 ml 1 M Tris-HCl pH 7

92.8 ml dH₂O

**PCR Reaction Buffer (10X)**

500 mM KCl

100 mM Tris-HCl

1% Triton X-100

pH 9

**Thioflavin T stock solution (2 mM)**

500 ml dH₂O

0.319 g ThT

Filter before use.

**Glycine stock solution (100 mM)**

7.51 g Glycine

Dissolve in 1 L dH₂O, pH 8.5

**Saturated sodium chloride solution**

50 g Sodium chloride (NaCl)

500 ml 80% ethanol

Stir overnight before use.
**Congo red staining solution**

2.5 g Congo red

500 ml Saturated sodium chloride solution

Stir overnight and filter before use.

**PHEM Buffer (2X 500 ml)**

18.14 g PIPES

6.5 g HEPES

3.8 g EGTA

0.99 g MgSO₄

pH 7.0

**Homogenisation Buffer**

0.292 g sodium chloride

1 g sodium dodecyl sulphate (SDS)

Dissolve in 100 ml dH₂O, pH 10

Add: 10 µl/ml protease inhibitor cocktail

5 µl/ml phosphatase inhibitor I cocktail

5 µl/ml phosphatase inhibitor II cocktail

**Kit Buffer**

200 ml 1X Tris wash buffer (TWB)

Add: 10 µl/ml protease inhibitor cocktail

5 µl/ml phosphatase inhibitor I cocktail

5 µl/ml phosphatase inhibitor II cocktail
### Appendix III

**General Products**

<table>
<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloid β_{1-42} peptide</td>
<td>Biosource, Belgium</td>
</tr>
<tr>
<td>Base pair ladder</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Sigma, UK</td>
</tr>
<tr>
<td>CD16/CD32 FcγRIII block</td>
<td>BD Pharmingen, USA</td>
</tr>
<tr>
<td>CellTiter 96® AQueous One</td>
<td>Promega, Ireland</td>
</tr>
<tr>
<td>Solution Cell Proliferation Assay</td>
<td>Pall Corporation, UK</td>
</tr>
<tr>
<td>Cellulose acetate membrane filter</td>
<td>Macherey-Nagel, Germany</td>
</tr>
<tr>
<td>Cell lysis mastermix</td>
<td>Mediatech Inc, USA</td>
</tr>
<tr>
<td>Ciprofloxacin hydrochloride</td>
<td>Roche Applied Science, Germany</td>
</tr>
<tr>
<td>Collagenase D</td>
<td></td>
</tr>
<tr>
<td>Confocal laser microscope</td>
<td>Zeiss Ltd, UK</td>
</tr>
<tr>
<td>Congo red solution</td>
<td>Sigma, UK</td>
</tr>
<tr>
<td>Coverslips</td>
<td>Chance Propper, UK</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>Sigma, UK</td>
</tr>
<tr>
<td>Cytomation pen</td>
<td>Dako, UK</td>
</tr>
<tr>
<td>DAPI mounting medium</td>
<td>Vector, UK</td>
</tr>
<tr>
<td>Depex polystyrene</td>
<td>Electron Microscopy Sciences</td>
</tr>
<tr>
<td>DNAse I</td>
<td>Sigma, UK</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle’s Medium</td>
<td>Gibco, UK</td>
</tr>
<tr>
<td>Falcon tube</td>
<td>Sarstedt, Ireland</td>
</tr>
<tr>
<td>Fluorescent latex particles</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Foetal calf serum</td>
<td>Sigma, UK</td>
</tr>
</tbody>
</table>
Frosted microscope slides
Glass bottom dishes
Glycine
GM-CSF
Goat anti-mouse Alexa 488 nm
Goat anti-rabbit Alexa 488 nm
Goat anti-rat Alexa 546 nm
Goat anti-rat Alexa 633 nm
High-capacity cDNA archive kit
HiLyte Flour 488 labelled Aβ_{1-42}
IL-1β ELISA kit
IL-6 paired antibodies
Isopropanol
Jumpstart™ Taq DNA Polymerase
Ketamine (Velatar V)
Laminar airflow unit
Light microscope
Lipopolysaccharide
Loading buffer
Magnesium chloride
Maple syrup
Methyl green solution
Microtitre plate reader
Mouse anti-rat CD11b
Mouse anti-rat OX-6
Fischer Scientific Ltd, Ireland
MatTek Corporation, USA
Sigma, UK
R&D Systems, UK
Invitrogen
Biosciences, Ireland
Biosciences, Ireland
Biosciences, Ireland
Applied Biosystems, Germany
Anaspec Inc, USA
R&D Systems, UK
BD Biosciences, USA
Sigma, UK
Sigma, UK
Pharmacia, Germany
AGB Scientific Ltd, Ireland
Nikon, Japan
Alexis, Switzerland
Promega, USA
Promega, USA
Newforge, Canada
Sigma, UK
Labsystems, Finland
AbD Serotec, UK
AbD Serotec, UK
Mouse anti-rat CD68
MSD® 96-well multi-spot Aβ assay kit
NanoDrop Spectrophotometer
Non-toxic poster paint
Non-toxic powder paint
Normal goat serum
96-well plates
Nucleotide mix
Nylon mesh filter
OCT

Pasteur pipette
PCR reaction buffer (10X)
Percoll
Phenol:chloroform:isoamyl alcohol
Phosphatase inhibitor I cocktail
Phosphatase inhibitor II cocktail
Phosphate buffered saline
Polyethylene glycol-1,000
Protease inhibitor cocktail
Proteinase K
Protein assay kit
Quantum dots
Rabbit anti-human anti-pan β amyloid15-30
Rabbit anti-rat GFAP
Rat anti-mouse CD11b

AbD Serotec, UK
Meso Scale Discovery, USA
NanoDrop Tech. Inc, USA
Reads, Ireland
Crafty Devils, UK
Vector, UK
Nunc Immuno, Denmark
Bioline, UK
BD Biosciences, USA
VWR International Ltd, Ireland
Sarstedt, UK
Promega, USA
Sigma, UK
Fluka, Ireland
Sigma, UK
Sigma, UK
Sigma, UK
Pierce, The Netherlands
TCD, Ireland
Merck Chemicals Ltd, UK
AbD Serotec, UK
AbD Serotec, UK
Rat anti-mouse CD68  
Rat anti-mouse MHC II  
Recombinant rat IL-1β  
Red blood cell lysis buffer  
Rimadyl  
Rosiglitazone Maleate  
RPMI  
Scalpel  
Sense and antisense primers  
Sodium fluoride  
Sodium pentobarbital  
Streptomycin/penicillin  
Substrate solution  
Sucrose  
Thermocycler  
Thioflavin T  
Tetramethylbenzidine  
TNF-α paired antibodies  
Tris-HCl  
Tris wash buffer  
Trypan blue  
Trypsin-EDTA  
Ultraviolet transiluminator  
Xylazine

AbD Serotec, UK  
AbD Serotec, UK  
R&D Systems, UK  
Sigma, UK  
Pfizer Animal Health, USA  
Alpha Technologies, Ireland  
Biosera, UK  
Schwann-Mann, UK  
MWG Biotech, Germany  
Sigma, UK  
Merial Animal Health Ltd, UK  
Gibco, UK  
R&D systems, UK  
Sigma, UK  
Biosciences, Ireland  
Sigma, UK  
Sigma, UK  
BD Biosciences, USA  
Promega, USA  
Meso-Scale Discovery, USA  
Sigma, UK  
Sigma, UK  
Bioimaging Systems, USA  
Bayer, Germany
### Appendix IV

#### Company addresses

<table>
<thead>
<tr>
<th>Company</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexis</td>
<td>Alexis Corporation (UK) Ltd., Bingham, Nottingham NG13 8LS, UK</td>
</tr>
<tr>
<td>Amersham</td>
<td>Amersham Biosciences Ltd., P.O. Box 6757, Amersham Place, Little Chalfont, Buckinghamshire HPA 9NA, UK</td>
</tr>
<tr>
<td>Applied Biosystems</td>
<td>Frankfurter Street 129b, 64293 Darmstadt, Germany</td>
</tr>
<tr>
<td>BD Biosciences</td>
<td>Bio-Rad Laboratories Ltd., BD Biosciences, 2350 Qume Drive, Maylands Avenue, San Jose, CA, USA</td>
</tr>
<tr>
<td>Biosource</td>
<td>Biosource International, 542 Flynn Road, Camarillo, CA 93012, USA</td>
</tr>
<tr>
<td>Calbiochem</td>
<td>Calbiochem-Novabiochem Corp., 10394 Pacifica Centre Court San Diego, CA 92121, USA</td>
</tr>
</tbody>
</table>
Canopus Corporation
Grass Valley (UK) Ltd.,
Unit 1&2, The Duran Centre,
14 Arkwright Road,
Reading RG2 0LS
UK.

Carl Zeiss Ltd.,
15-20 Woodfield Road,
Welwyn Garden Centre,
Hertfordshire,
AL7 1JQ,
UK

Crafty Devils
22/23 Taw Mill Business Park,
Howard Avenue,
Barnstaple,
Devon,
EX32 8QA,
UK

Dako Diagnostics Ireland Ltd
12 Camden Row,
Dublin 8

Gibco
Gibco Ltd.
3 Fountain Drive,
Linchinnnan Drive,
Paisley PA4 9RF,
Scotland

Hycor Biomedical
Hycor,
Pentlands Science Park,
Bush Loan,
Penicuik,
Edinburgh,
EH26 0PL
UK
Invitrogen
Invitrogen Ltd.,
3 Fountain Drive,
Linchinnnan Drive,
Paisley PA4 9RF
Scotland

Labsystems
Labsystems OY,
Sorfaajankatu 15,
SF-FIN-00811,
Helsinki,
Finland

Lennox
Lennox Laboratory Supplies Ltd.,
John F. Kennedy Drive,
Naas Road,
Dublin 12,
Ireland

Macherey-Nagel
Macherey-Nagel GmbH & Co.,
KG
Postfach 10 13 52
D-52313 Duren,
Germany

Merck Chemicals Ltd.
Boulevard Industrial Park,
Padge Road,
Beeston,
Nottingham,
NG9 2JR,
UK
<table>
<thead>
<tr>
<th>Company</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merial Animal Health Ltd.</td>
<td>PO Box 327, Sandringham House, Sandringham Avenue, Harlow Business Park, Harlow, Essex CM19 5TG, UK</td>
</tr>
<tr>
<td>Meso Scale Discovery</td>
<td>9238 Gaither Road, Gaithersburg, Maryland 20877, USA</td>
</tr>
<tr>
<td>MWG Biotech</td>
<td>Anzinger Strasse 7A, Ebersberg D-85560, USA</td>
</tr>
<tr>
<td>Nanodrop Technologies Inc.</td>
<td>3411 Silverside Rd, Bancroft Bldg, Willmington, Delaware, USA</td>
</tr>
<tr>
<td>NUNC</td>
<td>Thermo Fisher Scientific, Kamstrupvej 90, Postbox 280, DK-4000, Roskilde, Denmark</td>
</tr>
<tr>
<td>Company</td>
<td>Address</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Pall Corporation</td>
<td>2200 Northern Boulevard, East Hills, NY 11548, USA</td>
</tr>
<tr>
<td>Pierce</td>
<td>Pierce Biotechnologies, 3747 N. Meridian Road, P.O. Box 117, Rockford IL 61105, USA</td>
</tr>
<tr>
<td>Promega</td>
<td>2800 Woods Hollow Road, Madison WI 53711, USA</td>
</tr>
<tr>
<td>R&amp;D Systems</td>
<td>614 McKinley Place NE, Minneapolis, MN 55413, USA</td>
</tr>
<tr>
<td>Roche</td>
<td>Roche Diagnostics Ltd., Bell lane A, Lewes, East Sussex BN7 ILG, UK</td>
</tr>
<tr>
<td>Serotec</td>
<td>22 Bankside, South Approach, Kidlington, Oxford OX5 1JE, UK</td>
</tr>
</tbody>
</table>
Sigma
Fancy Road,
Poole,
Dorset BH12 4GH,
UK

Startedt
Starstedt Ltd.,
Sinnottstown Lane,
Drinagh,
Wexford,
Ireland.

Ultra-Violet Products
Unit 1,
Trinity Hall Farm Estate,
Hullfield Road,
Cambridge CB4 1TG
UK

Vector
Vector Laboratories Inc.,
30 Ingold Road,
Burlingame,
CA 94010,
USA

Whatman
Whatman Plc
Whatman House,
St. Leonard’s Road,
Maidstone,
Kent ME16 0LS,
UK