



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.

A study of neuroinflammation in a mouse model of Alzheimer's disease.



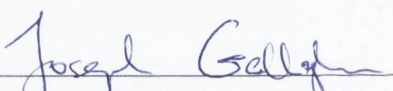
**Thesis submitted for the degree of Doctor of
Philosophy at University of Dublin, Trinity College**

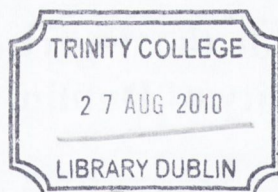
2009

Joseph J. Gallagher

I Declaration of Authorship

I declare that this thesis is entirely my own work with the following exceptions: certain results were kindly donated to me by Dr. Joanna Collingwood and Belinda Grehan. This work has not been previously submitted for a degree to this or any other university. I give my permission to the library to lend or copy this thesis.


Joseph J. Gallagher



THESIS
8928.

II Acknowledgements

I would like to thank my parents for the constant encouragement in everything I've ever done in my life and especially during this PhD, when it was needed more than ever. I'd like to thank my brothers, Mike and Andrew, and my sister, Nuala for all the support you've given me during this PhD and beforehand. I've been especially lucky to have you guys on the end of the phone whenever needed.

I'd like to thank my supervisor, Prof. Marina Lynch, for all her help and encouragement in this PhD. I probably would have walked away from Trinity without a PhD if it wasn't for her involvement and it is greatly appreciated.

I would also like to thank my collaborators Dr. Joanna Collingwood and Prof. Jon Dobson for all their help with the XRF aspect of this work. I would like to especially thank Joanna for all her help and assistance when needed, which made sure the collaboration went ahead. Much thanks to Dr. Martin Lees at Keele University who was incredibly helpful in all SQUID measurements taken. I would also like to thank Dr. Fred. Mosselmans and Dr. Tina. Geraki at the Diamond Light Source for letting me have a go on the synchrotron.

I'd like to say thanks to all the HRB people I started with... Kev, Kim, JR, Joanna, Dana and Stephen. I hadn't a clue what I was doing when I started and ye guys probably helped me out with something almost every single day. Kim thanks for all the cards, of all the things I'll take with me from TCIN, I know they'll take pride of place wherever it is I end up. A massive thanks to Mike, Jennie and Chris whose company I couldn't have done without, the lengthy cups of tea were usually the most enjoyable part of the week. Major shout outs to Briano, Keitho, RJK, Lorna, Anto, Eric, dRock, Thelma, Noreen and the pirates who will no doubt reform to put Merrion in their box.

Thanks to Cian, Phil and Deccy who head's I probably wrecked talking about neuroscience when I lived with them. Cheers to the Thursday night football crowd and thanks to Tommy, Fergie, Trev, Erica, Mary and Siobhan for all the pints... I swear I'm good for them. Finally, a major thanks to Neal who I owe a lot more than pints to. Apologies for the house.

The only reason an engineer could actually do anything in a neuroscience institute is because lots of generous and smart people were kind enough to give up their time to help me out. I can only hope to have such good fortune in the future. I'd also like to thank everyone involved in the HRB programme. I hope that I've convinced you that neuroscience can do with more engineers.

Finally, I'd like to thank Anne-Marie for always being there, all the encouragement, cups of tea and for being my girlfriend and a light that never goes out.

III Abstract

Microglia are the principal immune-competent cell of the central nervous system (CNS) and there is evidence to suggest microglia are the co-ordinating cells of the CNS inflammatory response. The blood brain barrier (BBB) isolates the CNS from the rest of the body and limits the delivery of therapeutic agents to the CNS.

Nanotechnology-based CNS drug delivery techniques, designed to bypass the restrictive nature of the BBB, often involve several constituents to mediate passage across the BBB. Little is known about the microglial response to such materials. A polyelectrolyte coated magnetic fluorescent nanostructure was evaluated in this study. The nanostructure exhibited no cytotoxic effects, internalization of the nanostructure did not induce a robust microglial activation and no meaningful increase in cytokine production was observed, although phagocytosis did occur. These results suggest the nanostructure could be used to deliver therapeutic agents to CNS cells while minimising inflammation due to microglial internalization. The potential of the nanostructure to be used as a bimodal contrast agent, generating T2-weighted MRI contrast *in vivo* and confocal microscopy contrast in brain sections, was assessed. *In vivo* experiments indicate that the nanostructure is ideally suited for use in cell tracking experiments that involve the administration of pre-labelled cells. The nanostructure generates T2-weighted MRI contrast due to the iron oxide core. Similarly, human amyloid-beta ($A\beta$) plaques are known to contain iron and it has been suggested that $A\beta$ plaque attenuation of T2 relaxation time may used a biomarker for Alzheimer's disease (AD). Copper and zinc are also known to be present in $A\beta$ plaques but little is known about the metal composition of $A\beta$ plaques in transgenic mouse models of AD and how they compare with human $A\beta$ plaques. X-ray fluorescence (XRF) analysis, adapted for use with biological tissue, was performed on $A\beta$ plaques in brain tissue prepared from 8 – 9 month old APP/PS1 transgenic mice. Iron was increased in plaques from transgenic mice but no increase in copper or zinc was observed. The increase in iron was approximately 5 times lower when compared with reported levels in human $A\beta$ plaques. This discrepancy, in addition to the absence of copper and zinc, suggests that metal colocalization to $A\beta$ plaques may occur *in vivo* as plaques mature.

Activated microglia are known to surround A β plaques and neuroinflammation, with an emphasis on microglial activation, was assessed in the APP/PS1 mouse model of AD at 8 – 9 months of age. Quantification of amyloid deposition indicated that plaque number and insoluble A β_{1-42} was greater in the brains of female APP/PS1 transgenic, compared with male APP/PS1 transgenic mice. Microglial activation characterized by increases in MHC II, CD11b and CD68 were measured in the transgenic mice and female transgenic mice exhibited increased expression of markers of microglial activation compared with male transgenic mice. Increases in the concentration of TNF- α and the expression of IL-1 β mRNA were observed in female transgenic mice only. This gender bias extended to performance in a spatial memory task where performance of APP/PS1 transgenic mice was impaired compared with the performance of wildtype mice, but female transgenic mice performance was markedly worse than that of male transgenic mice. Overall the data provide support for the proposal that activation of microglia, together with the upregulation of proinflammatory cytokines, mediate the impairment in hippocampal function in APP/PS1 transgenic mice.

IV Contents Page

I Declaration of Authorship	i
II Acknowledgements	ii
III Abstract	iv
IV Contents Page	vi
V List of Figures	xi
VI List of Tables	xiv
VII List of Abbreviations	xv

Chapter 1 Introduction

1.1 The central nervous system	2
1.1.1 Astrocytes and oligodendrocytes	2
1.1.2 Microglia	2
1.1.3 Blood brain barrier	4
1.2 Alzheimer's disease	4
1.2.1 Neurofibrillary tangles	5
1.2.2 Senile Plaques	6
1.2.3 Amyloid precursor protein	7
1.2.4 APP processing	7
1.2.5 Amyloid-beta	8
1.2.6 The amyloid cascade hypothesis	9
1.3 Alzheimer's disease mouse models	10
1.3.1 APP-based transgenic mouse models	10
1.3.2 APP/PS1 transgenic mouse models of AD	11
1.3.3 Memory deficits in APP/PS1 transgenic mouse models of AD	12

1.4 Neuroinflammation in AD	12
1.5 Vascular dysfunction in AD	14
1.6 Magnetic resonance imaging	16
1.6.1 MRI relaxometry in APP/PS1 transgenic mouse models of AD	17
1.6.2 ASL in APP/PS1 transgenic mouse models of AD	18
1.6.3 MRI contrast agents	20
1.7 Physiological role of iron	22
1.6.1 Iron in AD	22
1.6.2 Zinc and copper – physiological role and association with AD	23
1.6.3 A β interactions with metals	24
1.6.4 SQUID magnetometry	26
1.6.5 X-ray fluorescence	26
1.6.6 X-ray absorption near edge spectroscopy	27
1.6.7 XRF & XANES biological applications	28
1.8 Aims of study	29

Chapter 2 Methods

2.1 Materials	37
2.1.1 Synthesis and advanced characterization of nanostructures	37
2.1.2 Photoluminescence spectroscopy	37
2.1.3 Nanostructure behaviour in a magnetic field	37
2.2 Animals	38
2.2.1 APP ^{swe} /PS1 ^{de9} Genotyping	39
2.2.1.1 Genomic DNA isolation	39
2.3.1.2 Polymerase chain reaction for APP ^{swe} and PS1 ^{de9} genes	39

2.2.2 Animal Treatments	41
2.2.1.1 Intravenous injection	41
2.3.1.2 Induction of EAE in C57DM/6 mice	41
2.3 Behavioural	41
2.3.1 Hang-wire	41
2.3.2 Inverted screen	42
2.3.3 Morris water maze	42
2.3.3.1 Morris water maze apparatus	42
2.3.3.2 Habituation	42
2.3.3.3 Training & reversal assessment	43
2.4 MRI scanner	44
2.4.1 MRI Animal setup and maintenance	44
2.4.2 MRI protocols	44
2.4.2.1 Relaxometry scans	44
2.4.2.2 High-Resolution T2-weighted scan	45
2.4.2.3 Arterial Spin Labelling (ASL)	45
2.4.3 MRI analysis	47
2.4.2.1 Relaxometry	47
2.4.2.2 Volumetric Based Morphometry	47
2.4.2.3 ASL	48
2.5 Tissue preperation	49
2.5.1 <i>In-vivo</i>	49
2.5.1.1 Cervical dislocation and brain dissection	49
2.5.1.2 Surgical equipment and surface trace-metal decontamination	49
2.5.1.3 Intracardial perfusion	50
2.5.1.4 Cryosectioning	50
2.5.1.5 Protein quantification	51
2.5.2 <i>In-vitro</i>	51
2.5.2.1 Primary mouse mouse mixed glia preperation	51

2.5.2.2 Blood brain barrier cell model	52
2.6 Nanostructure cellular interaction methods	52
2.6.1 Confocal microscopy	52
2.6.2 Light microscopy	52
2.6.3 Live cell imaging	53
2.6.4 Flow Cytometry Analysis	53
2.6.5 Cytotoxicity	53
2.6.6 Analysis of cytokine expression	54
2.6.7 Live cell imaging	54
2.6.8 MRI phantoms and measurements	55
2.6.9 <i>In vitro</i> BBB model nanostructure interaction	55
2.6.10 DAB-enhanced Prussian blue staining of brain sections	56
2.6.11 Rhodamine detachment experiments	56
2.7 Metal and Iron analysis techniques	57
2.7.1 SQUID magnetometry	57
2.7.1.1 Tissue preparation	58
2.7.1.2 SQUID magnetometry experimental procedure	57
2.7.2 ICP-MS	58
2.7.2.1 Tissue preparation	58
2.7.2.2 ICP-MS experimental procedure	58
2.7.3 X-Ray analysis	59
2.7.3.1 Tissue preparation	59
2.7.3.2 Synchrotron hutch setup	59
2.7.3.3 XRF acquisition and analysis	60
2.7.3.4 XANES acquisition and analysis	61
2.8 Molecular analysis techniques	61
2.8.1 Analysis of soluble and insoluble A β concentrations	61

2.8.1.1	Extraction of insoluble and soluble A β	61
2.8.1.2	Detection of SDS-soluble and insoluble A β by multi-spot ELISA	62
2.8.2	ELISA	62
2.8.2.1	Determination of cytokine concentrations by ELISA	62
2.8.3	Immunofluorescence staining for A β and MHC II	63
2.8.4	Immunohistochemistry	64
2.8.4.1	CD68 expression quantification	65
2.8.5	PCR	65
2.8.5.1	Isolation of RNA from brain tissue	65
2.8.5.2	Reverse transcription of cDNA synthesis	66
2.8.5.3	Real time PCR	67
2.8.5.4	PCR Quantification	67

Chapter 3 Results

3.1	Introduction	75
3.2	Results	76
3.3	Discussion	81

Chapter 4 Results

4.1	Introduction	110
4.3	Results	111
4.4	Discussion	120

Chapter 5 Results

5.1 Introduction

150

5.3 Results

151

5.4 Discussion

158

Chapter 6 Discussion

187

Chapter 7 References

195

VIII Appendix I: Methods

xix

IX Appendix II: XANES spectra

xxiv

X Appendix III: APP/PS1 characterization data

xix

XII Appendix IV: Companies

xxiv

XIII Appendix V: Solutions

xxiv

List of figures

Figure 1.1. Schematic of the APP processing pathway.

Figure 1.2. APP points of mutation.

Figure 1.3. Schematic representation of MRI signal generation.

Figure 1.4. Schematic illustration of ASL.

Figure 1.5. Schematic illustration of X-ray fluorescence.

Figure 1.6. XRF map creation.

Figure 2.1. Schematic illustration of Morris water maze apparatus.

Figure 2.2. ASL pulse sequence schematic and representative fit of the non-compartmental model of cerebral perfusion.

Figure 2.3. Representative ROIs drawn on a T2-weighted image.

Figure 2.4. Brain extraction and gray matter segmentation.

Figure 2.5. Schematic illustration and photograph of SQUID magnetometry sample setup.

Figure 2.6. Schematic of I18 beamline and sample placement.

Figure 3.1. Schematic illustration of fabrication and characterization of fluorescent magnetic nanostructures.

Figure 3.2. Effect of magnetic field on the orientation of nanostructures.

Figure 3.3. Effect of magnetic field on the orientation of nanostructures.

Figure 3.4. TEM images confirm the tendency of nanostructures to align with the direction of an applied magnetic field.

Figure 3.5. Sub-cellular distribution of nanostructures in mixed glial cells.

Figure 3.6. Nanostructure cellular uptake in mouse mixed glial culture is concentration dependant.

Figure 3.7. Light microscopy confirms cellular internalization of iron oxide core of nanostructure.

Figure 3.8. Nanostructure cellular uptake in mouse mixed glial culture is concentration dependant.

Figure 3.9. Z-stacking identifies the cell nucleus.

Figure 3.10. Individual confocal microscopy z-sections enables investigation of delineation of nuclei through absence of nanostructure fluorescence.

Figure 3.11. Live cell imaging of mouse mixed glial cells incubated with nanostructures.

Figure 3.12. Flow cytometric analysis of non-treated and nanostructure-treated mouse mixed glial cells.

Figure 3.13. Cell viability was not affected by nanostructures.

Figure 3.14. Effect of nanostructures on cytokine release.

Figure 3.15. MR contrast (ΔB) in agarose phantoms increases with increasing numbers of labelled cells.

Figure 3.16. Application of a magnetic field allowed nanostructures to cross an endothelial cell layer.

Figure 3.17. Rhodamine fluorescence is detected in the hippocampus and cortex of mice with EAE.

Figure 3.18. T2-weighted images are similar in control mice and mice with EAE following intravenous injection of nanostructures 24 hours previously.

Figure 3.19. DAB-enhanced prussian blue staining identified small deposits of iron in brain sections of mice with EAE intravenously administered nanostructures.

Figure 3.20. Time-related detachment of rhodamine from nanostructure.

Figure 3.21. Control mice and mice with EAE display T2-signal voids 24 hours after intravenous administration of non-fluorescent nanostructures.

Figure 3.22. Iron is present in brain sections prepared from control mice and mice with EAE.

Figure 4.1. Metal content was similar in brain tissue prepared from APP/PS1 transgenic and wildtype mice.

Figure 4.2. Isothermal remanent magnetization, measured at 5 K, did not saturate following exposure to a 10,000 Oe field.

Figure 4.3. IRM was not altered in APP/PS1 transgenic mice.

Figure 4.4. XRF map normalization.

Figure 4.5. Calcium XRF maps enabled identification of creased brain sections.

Figure 4.6. Identification of exogenous non-biological contaminant material.

Figure 4.7. Amyloid deposition was identified in an XRF-assessed brain section prepared from a female APP/PS1 transgenic mouse.

Figure 4.8. XRF-assessed amyloid plaques exhibited decreased congo red intensity.

Figure 4.9. Increased iron deposition in brain sections obtained from APP/PS1 transgenic mice does not colocalize with nickel deposition.

Figure 4.10. Comparison of metal XRF contrast with MRI contrast in a brain section prepared from a female APP/PS1 transgenic mouse.

Figure 4.11. Comparison of metal XRF contrast with MRI contrast in a brain section prepared from a wildtype mouse.

Figure 4.12. Analysis of amyloid deposition in the auditory cortex of a female APP/PS1 transgenic mouse.

Figure 4.13. Analysis of amyloid deposition in the CA2 field of a female APP/PS1 transgenic mouse.

Figure 4.14. Analysis of amyloid deposition in the CA1 field of a female APP/PS1 transgenic mice.

Figure 4.15. Microfocus mapping locations in an XRF-assessed brain section prepared from a female APP/PS1 transgenic mouse.

Figure 4.16. Microfocus XRF analysis in the dorsal retrosplenial cortex of a female APP/PS1 transgenic mouse.

Figure 4.17. XANES spectra and linear-combination fits in Area A.

Figure 4.18. Microfocus XRF analysis in the CA2 field of the hippocampus of a female APP/PS1 transgenic mouse.

Figure 4.19. XANES spectra and linear-combination fits in Area B.

Figure 5.1. Genotype analysis of APP^{swe} and PS1^{dE9} presence.

Figure 5.2. Animal ages and weights at experiment timepoints.

Figure 5.3. Muscle strength and coordination was similar in APP/PS1 transgenic and wildtype mice.

Figure 5.4. APP/PS1 transgenic and wildtype mice learn the Morris water maze.

Figure 5.5. Female APP/PS1 transgenic mice display an impaired performance in the reversal phase of the Morris water maze task.

Figure 5.6. Swim speed of female wildtype mice was greater than that of female APP/PS1 transgenic mice in the training, but not the reversal phase, of the MWM task.

Figure 5.7. Female APP/PS1 transgenic mice exhibit higher levels of plaque deposition than male APP/PS1 transgenic mice.

Figure 5.8. Brain sections prepared from female APP/PS1 transgenic mice display amyloid immunofluorescence in the cortex and hippocampus.

Figure 5.9. DAB-enhanced prussian blue staining confirms the presence of iron in congo red-positive plaques.

Figure 5.10. Diffuse and compact plaques display distinct morphology.

Figure 5.11. Modest vascular amyloid deposition was observed in brain sections prepared from 8-9 month old APP/PS1 transgenic mice

Figure 5.12. Soluble A β peptides were increased in brain tissue prepared from APP/PS1 transgenic mice compared with wildtype mice.

Figure 5.13. Insoluble A β peptides were increased in brain tissue prepared from APP/PS1 transgenic mice compared with wildtype mice.

Figure 5.14. Cells proximal to amyloid plaques display MHCII immunofluorescence.

Figure 5.15. CD68 expression was increased in brain sections prepared from a female APP/PS1 mouse compared with brain sections prepared from a wildtype mouse.

Figure 5.16. Expression of markers of microglial and astrocytic activation are upregulated in APP/PS1 transgenic mice.

Figure 5.17. Upregulation of proinflammatory cytokines in brain tissue prepared from female APP/PS1 transgenic mice.

Figure 5.18. No age-related decline in gray matter volume was observed.

Figure 5.19. Male APP/PS1 mice displayed an increased cortical and hippocampal T1 relaxation time that declined with age.

Figure 5.20. Female APP/PS1 and wildtype mice did not exhibit an age-related decline in T2 relaxation time in the hippocampus or cortex.

Figure 5.21. No age-related changes in rCBVlw were observed.

Figure 5.22. The rCBF in the hippocampus of female APP/PS1 transgenic mice decreased with age.

Appendix II Figure 1. XANES spectra of standards.

Appendix II Figure 2. Linear-combination fits components of Area A1.

Appendix II Figure 3. Linear-combination fits components of Area A2.

Appendix II Figure 4. Linear-combination fits components of Area B1.

Appendix II Figure 5. Linear-combination fits components of Area B2.

List of tables

Table 2.1 Primers used for DNA amplification

Table 2.2 Variations of D1, D2 and τ for 1.5 s, 2.0 s and 3.0 s bolus

Table 2.3 Gene expression assay numbers of PCR primers

Appendix III Table 1 MTT and CTT data

List of abbreviations

AD	Alzheimer's disease
APP	Amyloid precursor protein
ASL	Arterial spin labelling
A β	Amyloid-beta
BBB	Blood brain barrier
CAA	Cerebral amyloid angiopathy
CBF	Cerebral blood flow
CBV	Cerebral blood volume
CBV _{lv}	Cerebral blood volume (with extravascular component)
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTT	Capillary transit time
DS	Down's syndrome
EAE	Experimental autoimmune encephalitis
FAD	Familial alzheimer's disease
GFAP	Glial fibrillary acidic protein
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
LTP	Long-term potentiation
MRI	Magnetic resonance imaging
MTT	Mean transit time
MWM	Morris water maze
NFTs	Neurofibrillary tangles
PCR	Polymerase chain reaction
PD	Parkinson's disease
PrP	Prion promotor
PS1	Presenilin 1
PS2	Presenilin 2
ROS	Reactive oxygen species

SPF	Specific pathogen free
SQUID	Superconducting quantum interference device
TE	Echo time
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor alpha
TR	Repitition time
VBM	Volume based morphometry
XANES	X-ray near edge spectroscopy
XAS	X-ray absorption spectroscopy
XRF	X-ray fluorescence

Chapter 1

Introduction

The central aims of this thesis can be broadly classified as separate investigations into,

- Assessment of the neuroinflammation associated with a mouse model of Alzheimer's disease (AD) and longitudinal assessment of magnetic resonance imaging (MRI) relaxometry and cerebral blood perfusion changes in such a model.
- The development and application of a technique to map iron, copper and zinc in absolute quantities.
- The use of a magnetic and fluorescent contrast agent to track cell migration.

In order to familiarise the reader with the topics under investigation this introduction will introduce the central nervous system and its cellular constituents. Alzheimer's disease will be described and the mouse models developed to investigate this disease will be outlined; specific attention will be paid to the mouse model used in this thesis and the role of neuroinflammation in AD. The physical basis of MRI will be briefly considered, while MRI use in AD research and MRI-derived measurements of cerebral perfusion will be outlined.

In the subsequent sections the relationship between AD and metals, iron in particular, will be described. An introduction into several novel methods of assessing metal, focusing on iron's magnetic properties, will be discussed. A final section will outline the use of MRI contrast agents and recent advances in contrast agents of a bi-modal nature, specifically magnetic and fluorescent agents.

A more detailed outline of the specific aims of this thesis will close the chapter.

1.1 The central nervous system

The brain is isolated from the rest of the body by the blood brain barrier (BBB), which selectively restricts passage to the central nervous system (CNS). Two main cell types comprise brain tissue. Neurons, considered the computationally-competent cell type, and glial cells. Neurons communicate with each other at synapses and each neuron can make up to 7,000 synaptic connections with others neurons. In this manner, complex circuitry between neurons are established. The role of these circuits in processing information is poorly understood. It is estimated that neurons represent only 10% of the cellular population in brain tissue, with glia outnumbering them. Glial cells do not communicate in the manner that neurons do, but are key to aiding neuronal function and maintaining homeostasis. Glial cells can be divided into 3 main cell types: oligodendrocytes, astrocytes and microglia. In this thesis, emphasis will be placed on microglia

1.1.1 Astrocytes and oligodendrocytes

Oligodendrocytes are analogous in function to Schwann cells, resident in the peripheral nervous system. The primary function is the myelination of axons in the CNS. However, in contrast to Schwann cell myelination of a single axon only, oligodendrocytes can myelinate up to 50 axons (Fiacco et al., 2009).

Astrocytes are characterized by a distinct star-shaped morphology and are identified by staining positive for an intermediate filament protein named glial fibrillary acidic protein (GFAP). Although initially considered to be involved only in structural and metabolic support to neurons, astrocytes are now known to be involved in numerous processes in the CNS (Volterra and Meldolesi, 2005). They modulate neurotransmitter concentrations and can both release and take up neurotransmitters. They provide nutritional support to neurons and can also participate in the release of neurotrophins which promote neuronal function (Hughes et al., 2009). They have a role to play in the immune response of the CNS, becoming activated in response to neuroinflammation (Rossi and Volterra, 2009) and astrogliosis is characterized by an

increase in GFAP expression due to glial cell process, and cell number, increases. Astrocytes are an anatomical component of the BBB. Astrocytic endfeet process onto endothelial cells and are fundamentally involved in signalling across the BBB.

1.1.2 Microglia

Microglia are the principal immune competent cell of the CNS, displaying the ability to migrate to areas of inflammation, phagocytose cellular debris and pathogens and modulate inflammation via cytokine release. Microglia have a common lineage with macrophages and express cell surface markers more commonly associated with macrophages, including CD11b, and CD45 (Garden and Moller, 2006). Microglia comprise approximately 10% of the adult CNS cell population and occur throughout brain tissue maintaining a ramified morphology under normal conditions which allows the cells to assess brain tissue status. Microglia in this so called quiescent state display low levels of MHC class I and II and are not phagocytic (Pivneva, 2008). The ramified morphology displayed by microglia is constantly being reorganised while the cells probe their microenvironment for signs of injury or insult. Upon stimulation, in response to identification of infection or injury to the CNS, microglia retract their processes, become motile and can become phagocytic (Kim and Joh, 2006). Microglia respond to proinflammatory cytokines, cell necrosis factors, changes in extracellular potassium concentrations (Abraham et al., 2001), viral infections (Gonzalez-Scarano and Martin-Garcia, 2005) and a variety of proteins associated with neural pathologies (Garcao et al., 2006, Perry et al., 2002). Activated microglia can present phagocytosed and subsequently processed antigens on their cell surface and act as antigen-presenting cells (Aloisi, 2001), they express cell surface molecules and they can release proinflammatory cytokines. However, the stimulatory environment, within which microglia are resident, influences the extent of these changes and their phagocytic capability. Microglia, initially considered either resting or active, are now thought to be capable of occupying and progressing through many states of activation, possibly displaying distinct behaviour at each state, although it is now believed that these states may exhibit overlapping phenotypes.

Microglia, when activated through Toll-like Receptors (TLR), produce interleukin-1beta (IL-1 β), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF- α) cytokines. These are proinflammatory cytokines and have been implicated in the development of CNS inflammation (Landreth and Reed-Geaghan, 2009). Direct injection of these cytokines into the CNS exacerbates neurodegeneration in a mouse model of Parkinson's disease and sustained expression of some or all these cytokines is a known feature of many neurodegenerative pathologies (Godoy et al., 2008). Activated microglia, at the site of an injury, can produce chemokines leading to monocytic chemoattraction, due to the common lineage. The attenuation of the synthesis of chemokines by activated microglia results in lower levels of microglial recruitment to a site of injury (Kielian, 2004).

1.1.3 Blood brain barrier

The BBB is formed by tight junctions of transmembrane proteins between adjacent endothelial cells and associated astrocytic end feet and restricts passage of substances from the bloodstream much more than endothelial cells in capillaries elsewhere in the body. Proteins involved include the occludins, claudins and junctional adhesion molecule (Abbott et al., 2006). Endothelial cells restrict the diffusion of microscopic molecules (like bacteria) and large or hydrophilic molecules, while allowing the diffusion of small hydrophobic molecules. Endothelial cells of the barrier actively transport metabolic products such as glucose across the barrier. It is important to emphasise that the delivery of therapeutic agents to the CNS is severely limited by the BBB and methods ensuring adequate delivery of agents to the CNS is a object of active research (Edwards, 2001).

1.2 Alzheimer's disease

Alois Alzheimer described the first case of Alzheimer's Disease (AD) in 1906 (Derouesne, 2008). It is now the most common neurodegenerative disease, accounting for almost two thirds of all cases with increasing prevalence with age. It is

anatomically characterized by a slow but irreversible loss of neurons, particularly in the cortex and hippocampus. The disease is characterized functionally by initial deficits in episodic memory, which accumulate with time leading to marked dementia, comprising of impaired judgement and memory, altered behaviour, and a general decline in cognitive function (Keller, 2006). Clinical diagnosis of AD rests largely on excluding other dementias (Lopez et al., 2000), with a definitive diagnosis only possible from post-mortem analysis of brain tissue. Neuronal loss, senile plaques comprised of an amyloid-beta ($A\beta$) core and neurofibrillary tangles (NFTs) are recognised as pathological hallmarks of AD (Mott and Hulette, 2005). The pathogenesis of AD is poorly understood with recent research indicating that senile plaques and NFTs may be involved. However, there are contradictory reports relating to correlations between disease status and the prevalence of hallmark diagnostic features, further confusing the role of senile plaques and NFTs in AD pathogenesis. In addition to their presence, numerous other structural and functional alterations occur, including evidence of neuroinflammatory and oxidative changes (Lopez and DeKosky, 2003). The combined consequences of the presence of all these factors, disregarding any putative meditative effects one factor may have on another, is severe neuronal and synaptic dysfunction and loss.

Approximately 1% of AD cases are familial AD (FAD) and display an autosomal-dominant pattern of inheritance and can be attributed to mutations in one of three genes: amyloid precursor protein (APP) and presenilins 1 and 2 (Sorbi et al., 2001). Most mutations in the APP and presenilin genes increase the production of a protein called $A\beta_{1-42}$, which is the main component of senile plaques (Shepherd et al., 2009). In contrast, AD cases not associated with autosomal-dominant inheritance (i.e. sporadic AD) do not display alterations in the genes previously mentioned (Van Broeckhoven, 1998). Risk factors for sporadic AD include obesity, diabetes, atherosclerosis, smoking, gender, and coronary heart disease (Stozicka et al., 2007). Although numerous clinical trials have been conducted and are ongoing, no effective treatment has been found.

1.2.1 Neurofibrillary tangles

NFTs consist of aggregated straight or paired helical filaments, twisted ribbons or other conformations of aberrantly phosphorylated forms of the microtubule-associated protein, tau. The primary function of tau, which is particularly abundant in the axons of neurons, is to stabilize microtubules. Increases in tau phosphorylation are associated with depolymerization of microtubules mediating an increase in cytosolic tau concentration and increasing the opportunity for aggregation (Duff, 2006). As microtubules enable signalling molecules, trophic factors, vesicles, and other essential cellular constituents, to engage in axonal transport, alterations in tau influence greatly the status, performance and viability of neurons (Ballatore et al., 2007, Arriagada et al., 1992). Several studies have suggested that cerebro spinal fluid- (CSF) concentrations of tau may be indicative of AD disease severity (Andreasen et al., 2001, Arriagada et al., 1992). Transgenic mice overexpressing P301S mutant human tau developed filamentous tau lesions at 6 months of age, which increased in number with age. Microglial activation and impaired synaptic function in the hippocampus were observed prior to tangle formation at 3 months of age in these mice (Yoshiyama et al., 2007) suggesting that inflammation, ongoing from microglial activation, may be a precipitating factor in tangle formation.

1.2.2 Senile plaques

Senile plaques are lesions comprised of a core of aggregated A β surrounded by reactive astrocytes, dystrophic neurites and activated microglia and in the case of dense plaques, are approximately 50 μ m in size (Ishihara et al., 1991). A β peptide ranges in length from 39 to 43 amino acids. In dense senile plaques, A β_{1-42} is the major constituent, whereas under normal conditions, A β_{1-40} is prevalent (Yoshikawa, 1993). Histochemical staining, in addition to more quantitative techniques, has demonstrated the presence of iron, copper and zinc in senile plaques from end-stage AD brain tissue (Lovell et al., 1998, Miller et al., 2006). Plaque quantity increases with age but plaque size remains restricted to approximately 50 μ m (Kato et al., 1998). Diffuse plaques characterized by reduced A β concentrations in the plaque core have also been found in brains of AD patients but, it is unclear whether these plaques mature to the dense plaque phenotype (Armstrong et al., 1998). Cerebral amyloid

angiopathy (CAA), where A β deposition occurs in the walls of blood vessels in the CNS, also occurs in AD (Weller et al., 2009). There is no distinct correlation between senile plaque prevalence and disease severity but several reports suggest a general correlation between total levels of A β and cognitive decline (Naslund et al., 2000).

1.2.3 Amyloid precursor protein

A β is derived from the proteolytic cleavage of the amyloid precursor protein (APP), a type I 751 – 770 residue glycosylated transmembrane protein with a large hydrophilic extracellular domain, a hydrophobic 23 residue transmembrane domain and a small cytoplasmic domain (Walsh and Selkoe, 2007); the 695 isoform is expressed mainly in neurons (Haass et al., 1991). Some confusion still exists over the normal physiological role of APP but, several functions have been proposed; they include a role in cell adhesion, copper homeostasis, growth promotion, wound healing and coagulation, and neuronal migration during development (Heber et al., 2000, Herms et al., 2004, Muller et al., 1994, Young-Pearse et al., 2007). Studies using APP knock-out transgenic mice identified the importance of APP by showing that knockouts exhibited impairments in spatial learning (Dawson et al., 1999) and impaired long-term potentiation (LTP) (Seabrook et al., 1999).

1.2.4 APP processing

There are 2 proteolytic processing pathways of APP: the non-amyloidogenic pathway, which cleaves APP to form soluble A β , and the amyloidogenic pathway which produces A β peptides, where APP is cleaved initially by α -secretase and β -secretase, respectively. Both pathways are then acted upon by an aspartyl protease referred to as the γ -secretase and are represented schematically in Figure 1.1. The α -secretase pathway generates a soluble APP α peptide and a 10 kDa C-terminal APP fragment that can be further processed by γ -secretase to generate the P3 peptides. A β is derived from the APP by the sequential action of β - and γ -secretases (Walsh and Selkoe, 2007). APP cleavage by β -secretase results in shedding of its large ectodomain to be shed into the luminal and extracellular fluid, leaving a membrane

bound C-terminal fragment. This is 99 amino acids in length and is subsequently cleaved by γ -secretase, causing A β to be released. Depending on the point of cleavage by γ -secretase, three principal forms of A β , comprising of 38, 40 or 42 amino acid residues, are produced. γ -secretase has been identified as a complex of enzymes composed of presenilin 1 or 2, (PS1 and PS2), nicastrin, anterior pharynx defective and presenilin enhancer 2 (Levitan et al., 2001, Steiner et al., 2002, Wolfe et al., 1999). In individuals where an increased proportion of A β_{1-42} is produced, early onset AD occurs (Kumar-Singh et al., 2006, Rovelet-Lecrux et al., 2006), suggesting this alone is sufficient to cause the disease.

1.2.5 Amyloid-beta

A β is produced in healthy CNS tissue and does not appear to be detrimental until it aggregates. Senile plaques have been determined to be insoluble fibrillar aggregates of A β , specifically A β_{1-42} . A β monomers can rapidly aggregate in normal circumstances to form oligomers and subsequently insoluble dense fibrillar aggregates, presumably forming the base of senile plaques; A β_{1-42} exhibits the greatest tendency to aggregate. The kinetics of senile plaque formation *in vivo* are surprisingly fast, with plaque formations occurring in 24 hours (Meyer-Luehmann et al., 2008). Aggregated A β -induced neurotoxicity is well established *in vitro* with A β_{1-42} exhibiting greater potency than other peptides (Chiang et al., 2008). This has been replicated *in vivo* in experiments involving the cerebral infusion of A β (Nakamura et al., 2001). The detrimental effects of oligomeric forms of A β have been investigated and soluble pre-fibrillar oligomeric forms of A β have been shown to mediate synaptic dysfunction *in vitro* and *in vivo* (Walsh et al., 2002). It has also been reported that soluble A β concentrations match AD severity much more closely than senile plaque numbers (Lue et al., 1999). It has also been demonstrated that the presence of oligomeric A β_{1-42} intracellularly results in inhibition of synaptic transmission (Pigino et al., 2009). Although A β aggregation can occur under normal circumstances, it has been shown to be accelerated *in vitro* in the presence of iron, copper and zinc (Garzon-Rodriguez et al., 1999, Khan et al., 2006) and perhaps predictably studies have confirmed the presence of low and high affinity binding sites for copper, zinc

and iron (Atwood et al., 2002, Atwood et al., 2000). When A β binds copper and iron, redox reactions take place (Barnham et al., 2004, Opazo et al., 2002) and isolated senile plaques generate reactive oxygen species (ROS) in a manner dependent upon copper and iron (Sayre et al., 1999).

1.2.6 The amyloid cascade hypothesis

A central hypothesis to explain AD pathogenesis was proposed which states that the process of AD pathology is the result of an imbalance between the production and clearance of A β , leading to an overproduction of A β and subsequent formation of soluble oligomers and insoluble fibrils (Hardy and Allsop, 1991). Although the initial focus on the role of the insoluble fibrils in AD pathogenesis proved compelling, recent work implicating soluble forms of amyloid broadens the discussion of the underlying mechanism suggested by the cascade hypothesis, but does not negate the involvement of A β .

The APP gene is localized to chromosome 21, trisomy of which occurs in Down's syndrome (DS), and interestingly AD-like neuropathology has been described in persons with DS, further supporting A β as the primary causative agent in AD (Lemere et al., 1996). Mutations in the APP gene also leads to AD-like pathology, suggesting alterations in A β processing, in addition to overall A β concentration levels, can lead to AD pathology. Inherited mutations with PS1 and PS2 increase A β_{1-42} /A β_{1-40} ratio leading to early onset AD (Bentahir et al., 2006). The demonstrated neurotoxic effects of different species of synthetic A β , both *in vitro* and *in vivo*, in addition to the ability of A β to activate microglia resulting in the production of proinflammatory cytokines and oxidative stress associated with AD pathology, suggest A β is capable of inducing widespread effects similar to those observed in AD brains.

Although the mechanism by which A β mediates its neurotoxic potency is unknown, it has been demonstrated that NFT-induced neuronal death is potentiated by A β (Lewis et al., 2001) and NFT were significantly increased in the amygdala of the P301L tau transgenic mouse cerebrally administered A β_{1-42} fibrils (Gotz et al.,

2001). However, increasing tau expression levels and hyperphosphorylation in a transgenic mouse model of AD had no effect on AD pathology onset or progression (Oddo et al., 2007). These data provide clear rationale for focusing on A β as the primary causative agent in AD, as tau abnormalities although responsible for some level of neuronal death in AD, appear subsequent to the presence of A β and appearance of A β -mediated detrimental effects.

1.3 Alzheimer's disease mouse models

Animals models of AD have attempted to model the increase in A β concentrations by intracerebral or intracerebroventricular infusions of synthetic A β peptides (Snow et al., 1994). Data from models such as these provided evidence of the neurotoxic effects of A β ₁₋₄₂ and impairments in memory (Frautschy et al., 1992, Games et al., 1992). However, the difficulty in replicating the aggregation state and dose of A β infused, and the confounding effects of variability in surgical techniques, delivery of A β and the variability in animal strains prompted the investigation of alternative animal models of AD. The identification of genes associated with AD led to the development of numerous genetically-modified mouse models of AD. These models replicate different aspects of AD pathology such as A β plaque formation, microglia and astrocyte activation and cognitive deficits. Due to the complex nature of AD pathology, no single mouse model can be considered to accurately model all the pathological and behavioural symptoms associated with AD.

1.3.1 APP-based transgenic mouse models

The first reported transgenic mouse model to successfully develop senile plaques and display AD-like neuropathology, such as dystrophic neurites and neuroinflammation, was the PDAPP transgenic mouse (Games et al., 1995), which overexpresses the mutant form of human APP favouring the production of A β ₁₋₄₂ under the control of human platelet derived growth factor b (PDGF-b) chain as gene promoter. However, no evidence of neuronal loss in areas displaying senile plaque deposition have been

observed in these animals (Irizarry et al., 1997). Spatial memory deficits observed in these animals correlate with plaque deposition (Chen et al., 2000).

More recently transgenic animals have been developed which overexpress mutant forms of APP identified from human studies, Figure 1.2 schematically illustrates the point of mutation associated with the most common APP transgenic mice. The Tg2576 transgenic mouse model of AD overexpresses the Swedish double mutation of APP under the control of the prion protein (PrP) promoter (Hsiao et al., 1996). These mice display senile plaque deposition, beginning at 9 – 12 months of age, neuroinflammation, oxidative stress and cognitive impairments in spatial and working memory associated with A β deposition (Zhuo et al., 2008, Apelt and Schliebs, 2001, Pratico et al., 2001). However, these mice display no neuronal loss (Eriksen and Janus, 2007). Numerous other transgenic mouse models of AD have been studied, however the model used in the work presented here is a double transgenic mouse model, where the Swedish mutant APP is overexpressed in tandem with increased PS1 expression.

1.3.2 APP/PS1 transgenic mouse models of AD

Overexpression of mutant, but not wildtype, PS1 was found to increase the amount of A β ₁₋₄₂ in the brain of mice, although senile plaques did not develop (Duff, 2006). Crossing this PS1 transgenic mouse with the Tg2576 transgenic mouse generated viable progeny with 3 – 5 fold increase in concentrations of A β ₁₋₄₀ and A β ₁₋₄₂, compared with single-transgenic littermates, and senile plaques deposition, accompanied by neuroinflammation, by 6 – 8 months of age (Holcomb et al., 1998).

More recently several investigators have undertaken research using an APP^{swe}/PS1^{dE9} transgenic mouse model of AD. This mouse was developed by co-injection of the APP695 isoform containing the Swedish mutation and the exon-9-deleted PS1 into pronuclei, with each gene under the control of an independent PrP promoter (Jankowsky et al., 2001). A β plaques have been observed in these mice as early as 4 months of age, with plaque deposition observed in the hippocampus and

cortex of mice at 8-9 months of age. Concentrations of $A\beta_{1-42}$ are consistently reported as elevated above $A\beta_{1-40}$ (Gordon et al., 2002). CAA is observed in these animals from 4 months of age, and although progressing, it does so at a rate significantly slower than that observed in the Tg2576 mouse (Garcia-Alloza et al., 2006). Female transgenic mice have higher levels of plaque deposition than male transgenic mice (Wang et al., 2003).

1.3.3 Memory deficits in APP/PS1 transgenic mouse models of AD

APP/PS1 transgenic mice showed no behavioural deficits at 3 – 4 months of age (Puolivali et al., 2002) or 6 months of age (Cao et al., 2007). Evidence of spatial memory learning impairments in APP/PS1 transgenic mice, at 8-9 months of age, has been reported (Cao et al., 2007). Hippocampal concentration of total $A\beta_{1-42}$ has been shown to correlate with performance impairment in the Morris water maze (MWM) in APP/PS1 transgenic mice at 11-12 months of age, but soluble $A\beta_{1-42}$ and total $A\beta_{1-40}$ were not correlated with the behavioural impairment (Puolivali et al., 2002). APP/PS1 mice at 15-16 months of age displayed impaired spatial learning as assessed by the MWM task. This spatial memory deficit was correlated with an increase in $A\beta$ concentration in the cortex and hippocampus (Arendash et al., 2001). More recently the visuo-spatial learning of APP/PS1 transgenic mice was assessed using the Barnes maze, 18 month old transgenic mice displayed impaired performance compared with wildtype mice; no difference was observed at 6 months of age (O'Leary and Brown, 2009). This task assesses episodic-like memory performance and previous work has indicated this form of memory may be the first to be affected in these mice, with deficits observed at 6-7 months of age (Reiserer et al., 2007). Longitudinal assessment of APP/PS1 transgenic mouse performance in a passive avoidance task and in the reversal phase of the water T-maze indicated deficits occur from 6 months of age, and that these deficits increase with age and AD-like neuropathology (Filali and Lalonde, 2009).

1.4 Neuroinflammation in AD

Initial evidence of the role of inflammation in AD arose from epidemiological studies that observed that patients treated with non-steroidal anti-inflammatory drugs exhibited decreased incidence of AD (Breitner, 1996). Evidence that microglia play a role in inflammation in the CNS of AD patients came from studies showing that IL-1 β , IL-6, and TNF- α expression were upregulated in these individuals, and they were predominantly associated with AD plaques (Griffin et al., 1989, Wood et al., 1993, Cacabelos et al., 1994). These cytokines were subsequently found to be produced predominantly by activated microglia (Lue et al., 2001, Griffin et al., 1995). Studies of brain sections prepared from post mortem tissue found microglia with marked expression of MHC class II on their surface to be associated with A β plaques (Luber-Narod and Rogers, 1988, Rogers et al., 1988, Perlmuter et al., 1992). A β plaques have been shown to recruit microglia 1 – 2 days following plaque appearance (Meyer-Luehmann et al., 2008) and these microglia are known to release a diverse range of proinflammatory molecules, such as nitric oxide, IL-1 β , and TNF- α (Floden and Combs, 2006, Li et al., 1996). It has been proposed that the activated microglia might attempt to clear amyloid deposits or possibly restrict plaque growth (Simard et al., 2006). Importantly, these changes have been observed in brain tissue prepared from APP/PS1 transgenic mouse models of AD.

A study examining the time course of plaque deposition and neuroinflammation in an APP/PS1 transgenic mouse model, observed that increases in GFAP and MHC II expression were associated with increases in plaque deposition (Gordon et al., 2002). Similarly, in an APP transgenic mouse model of AD, no CD11b-positive microglia were detected prior to plaque deposition (Dudal et al., 2004). These data suggest soluble A β may not be as effective an ‘activator’ of microglia as fibrillar A β . In this study, glial activation was not observed prior to plaque deposition. More recently, in APP/PS1 transgenic mice that displayed plaque deposition at 4 months of age, CD11b positive microglia and GFAP-positive astrocyte populations were associated with amyloid plaques and pro-inflammatory

cytokine expression, TNF- α and IL-1 β , was observed at 8 months (Zhang et al., 2009).

Microglia are capable of A β phagocytosis as demonstrated by several *in vitro* reports (Paresce et al., 1996, Ard et al., 1996, DeWitt et al., 1998). However, recent multiphoton *in vivo* data confirming the persistence of amyloid plaques following formation, indicate microglia do not clear plaques, although some level of phagocytosis may occur on the perimeter of plaques. This seemingly contradictory behaviour has led some investigators to speculate that microglial interactions with amyloid plaques *in vivo* do not effectively engage receptor systems associated with the phagocytic machinery (Bard et al., 2000). Some commentators have termed this 'frustrated phagocytosis' (Rogers et al., 2002a), while others have suggested that the microglial dysfunction is due to senescence (Streit, 2004). This may not be the case as animals genetically engineered to develop plaques early in life do exhibit plaque clearance. It is clear that persistent activation, characterized by production of proinflammatory molecules, in the absence of effective phagocytosis of A β species, is a consistent feature of microglia proximal to amyloid plaques. This has been found in all mouse models of AD where plaque deposition occurs.

Some studies have reported a beneficial role for microglia under specific circumstances. Activation of microglia induced by intracerebral injection of LPS has been shown to reduce plaque density in mouse models of AD (DiCarlo et al., 2001, Herber et al., 2007, Herber et al., 2004). While reducing microglial activation using dexamethasone attenuated LPS-induced plaque reduction. Interestingly chronic overexpression of IL-1 β in the hippocampus of APP^{swe}/PS1^{dE9} mice led to a decrease in A β plaque deposition (Shafteel et al., 2007).

The longitudinal assessment of neuroinflammation in mouse models of AD, and comparison of such findings with age-related changes in markers of neuroinflammation in wildtype mice, represents an opportunity to examine the role neuroinflammation may have to play in mediating the detrimental affects associated with AD.

1.5 Vascular dysfunction in AD

Cerebral blood flow (CBF) is integral to brain function and interruption results in brain function cessation within seconds, and irreversible damage to the cellular constituents of the brain within minutes (Hossmann, 1994). Recent work has determined that activity-induced haemodynamic responses require complex signalling mechanisms that involve not only neurons but also astrocytes and vascular cells. Imaging studies have reported reductions in CBF and glucose utilization in patients with AD, while a growing body of evidence exists indicating vascular A β depositions alter blood vessel morphology and impair function (Thomas et al., 1996, Niwa et al., 2001). Studies of cerebral blood volume (CBV) and perfusion suggest increasing prevalence of compromised brain perfusion in the temporoparietal cortex as AD progresses (Alsop et al., 2000). Transgenic mice overexpressing APP^{swe} display an alteration in the regulation of the cerebral circulation at 2–3 months of age (Niwa et al., 2002). Cerebrovascular dysregulation can be reproduced in wildtype mice by topical application of A β to the cerebral cortex, while systemic administration of A β reduces resting CBF (Niwa et al., 2000); this study found A β_{1-40} to be more potent in mediating cerebrovascular alterations. Peptide-specific immunofluorescence has revealed that A β_{1-40} is the predominant form in cerebral blood vessels, whereas A β_{1-42} is more abundant in brain parenchyma (Alonzo et al., 1998). These studies suggest cerebrovascular dysfunction is not solely due to neuropathology, but more closely linked with A β -induced effects on the cerebrovasculature.

1.6 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) has become increasingly useful in probing anatomical, biochemical and functional alterations in brain tissue due to neuropathology in a non-invasive manner and is now routinely applied in the clinic. MRI has been adopted as an investigative tool in the neurosciences, with a proliferation of techniques available; the ability to assess changes in the brain tissue of animals in longitudinal studies is especially useful in AD research.

The origin of the MR signal is the small magnetization associated with protons in the hydrogen atom. Normally the orientation of each proton's magnetization is random such that there is no net magnetization due a bulk volume of protons. However, when the protons are placed in a static magnetic field, B_0 , an excess of protons line up with the magnetic field, thus creating a small excess of magnetization associated with the protons. This excess is called the boltzmann population and it is this magnetization that forms the basis of the MR signal. This process is illustrated in Figure 1.3a where the boltzmann population is established and small net magnetization, M , is present in the same direction as the external magnetic field B_0 . To enable us to differentiate between the magnetization due to the protons and B_0 , we need to perturb the magnetization associated with the protons. Basic MRI contrast is generated by perturbing the magnetization of the boltzmann population excess, through the use of a radiofrequency (RF) pulse, and measuring the relaxation of the magnetization back to equilibrium. The application of an RF pulse to forces M , the net magnetization, to move into a different plane, this process is represented in Figure 1.3b. Upon removal of the RF pulse the perturbed magnetization, M , attempts to relax to it's equilibrium state – as defined by the Boltzmann population. This relaxation is defined by two relaxation processes, termed longitudinal relaxation time (T_1) and transverse relaxation time (T_2), see Figure. 1.3c. The regrowth of the magnetization, M_z , along the z-axis is termed T_1 relaxation. The decay of magnetization in the transverse plane, M_{xy} , is termed T_2 relaxation. Sampling the signal at different points during relaxation alters the T_1 - and T_2 -weighting of the signal thus generating contrast.

T1 and T2 relaxation times relate to different aspects of the biophysical and biochemical environment within which protons, the basis of the MRI signal, find themselves as they relax. Relaxation times can be calculated by sampling the signal at different timepoints and have been shown to be modulated in pathology; T1 hypointensities have been reported in multiple sclerosis (van Walderveen et al., 1999) and in instances of acute astrocytic activation (Sibson et al., 2008), and T2 hyperintensity has been described in ischemic tissue (Moseley et al., 1990). MRI is capable of providing high-resolution anatomical images which can be used to evaluate changes in brain structure and, using this method, brain atrophy in AD patients can be evaluated (Smith et al., 2002). T2 relaxation time is attenuated by iron, due to its paramagnetic behaviour, and this has been suggested as a possible biomarker in AD.

A study of the APP/PS1 transgenic mouse model of AD found no differences in volumes of brain structures between transgenic and wildtype mice (Lau et al., 2008). A more recent report examining the volume changes in brain structures of the APP/PS1 mouse model of AD and a wildtype cohort of mice concluded that only subtle differences were present, with none observable prior to 14 months of age (Maheswaran et al., 2009).

1.6.1 MRI relaxometry in APP/PS1 transgenic mouse models of AD

Numerous studies have confirmed that T2-weighted MRI scans can be used to detect amyloid plaques in APP/PS1 transgenic mouse models of AD, using lengthy *ex vivo* scans (Lee et al., 2004, Zhang et al., 2004). *In vivo* detection of A β plaques in APP transgenic mice using T2*-weighted MRI scans has also been reported (Jack et al., 2005, Jack et al., 2004). In one study correlation of MRI observations with a panel of different histological staining techniques indicated that only plaques that were positive for both thioflavin-S and iron were visible on the MR images (Vanhoutte et al., 2005).

Transgenic APP/PS1 mice, aged 16-23 months, displaying excessive amyloid deposition, exhibit *in vivo* reductions in T2 relaxation times measured at 7 T (Helpert et al., 2004). More recently, several groups have reported an exacerbation of the age-related decline in T2 relaxation times in areas associated with amyloid plaque deposition in APP/PS1 and Tg2576 transgenic AD mice, at 12-15 months of age (Falangola et al., 2007, El Tannir El Tayara et al., 2006, Braakman et al., 2006). However, these studies also report a reduction in T2 relaxation times in transgenic PS1 mice, in which amyloid plaques are not observed, suggesting although amyloid deposition may attenuate T2 relaxation time, it is not the only contributor to the deficit seen in these mice. There is only one report of T2 relaxation time reductions preceding plaque amyloid deposition and it was detected in the subiculum of APP/PS1 transgenic mice at 22 weeks of age measured at 7 T (El Tayara Nel et al., 2007). Also reported in this study was an age-related decline in T1 relaxation time in the parietal cortex and striatum that was independent of amyloid load and this was also reported in another study of APP/PS2 transgenic mice (Weidensteiner et al., 2009). Both increases and decreases in T2 relaxation times have been observed in AD patients (Kirsch et al., 1992, Benveniste et al., 1999). These data suggest T2 relaxation time attenuation in transgenic mouse models of AD occurs primarily due to excessive amyloid plaque deposition. However, absence of neuronal loss in AD models complicates the interpretation of the change in T2 relaxation time in human studies. An age-related decline in T2 relaxation times in the brain of wildtype mice is a consistent feature of longitudinal studies, the relevance and origin of this feature is unclear. It has been suggested that this may be due to a decrease in cell density (Weidensteiner et al, 2009). However, such a decrease would lead to an increase in extracellular space and presumably extracellular fluid, leading to an increase in T2 relaxation. This relationship may be further complicated in instances of neurodegeneration where ventricular enlargement occurs. The widely reported absence of neuronal loss in APP/PS1 transgenic mouse models of AD offers the potential to assess relaxometry changes due to ageing and amyloid deposition only. An investigation into this may be considered relevant to relaxometry changes observed in humans during early-stage AD, or the phase termed mild cognitive impairment.

1.6.2 ASL in APP/PS1 transgenic mouse models of AD

MRI can also be used to assess cerebral blood flow. Arterial spin labelling (ASL) magnetically saturates inflowing blood into the brain and uses the changes in the MRI image in this instance, compared with non-saturated blood flow images, to assess cerebral blood flow. A schematic illustration of the technique is presented in Figure. 3.4. The signal acquired from ASL experiments is proportional to cerebral blood flow. To quantify aspects of CBF the ASL signal must be used as an input to a mathematical model of blood flow. In this manner quantifiable measures of cerebral blood flow and volume can be determined. However, many researchers utilise different models leading which can lead to difficulties when comparing across studies.

This technique has shown efficiency in detecting cerebral blood flow changes with age, pathology and activation in humans (Parkes et al., 2004, Chalela et al., 2000, Gonzalez-At et al., 2000). Investigation of changes in the cerebral perfusion of AD patients has also been possible using this technique (Johnson et al., 2006) with hypoperfusion detected in the temporoparietal cortex, which is in agreement with results found using PET and SPECT imaging (Johnson et al., 2005).

Using contrast-enhanced perfusion imaging, a reduction in cortical and hippocampal cerebral blood volume (CBV) was identified in mutant APP transgenic mice at 4 months of age (Wu et al., 2004). This technique has also been used to uncover a reduced CBV in 15-month old APP/PS1 transgenic mice (Hooijmans et al., 2007). More recently, assessment of CBV in a mouse model of AD overexpressing human PS2 and a mutant human APP transgene found a decreased perfusion and CBV in the occipital cortex of transgenic mice at 10 months of age compared with wildtype mice (Weidensteiner et al., 2009). To date, studies utilising ASL to measure cerebral blood flow (CBF) are rare. However, reduced perfusion in the cortex of APP/PS1 transgenic mice at 33, 54, and 62 weeks compared with wildtype mice, as measured by ASL, has been reported (Dhenain et al., 2008).

The absence of exogenous contrast material when measuring using the ASL method makes it an attractive approach to CBF quantification and as such it represents a clinically translatable technique. Investigation of the ability of ASL models, and in this thesis the novel ASL method developed by Kelly and colleagues, 2009, to accurately track changes in CBF in a mouse model of AD is an important step in the maturation of the ASL technique.

1.6.3 MRI contrast agents

Although MRI can provide exquisite anatomical detail and can also be used to assess blood flow, it cannot, without the use of exogenously applied contrast agents, afford contrast to specific cells or on-going molecular processes. Many materials can greatly affect T1 or T2 relaxation, recent advances in the fabrication of ultra-small particles and nanotechnology approaches have the production of a range of material capable of generating MR contrast. Iron oxide-based magnetic nanoparticles have become one of the most widely used superparamagnetic contrast agents in MRI. Their effect on the relaxation time of water is measurable even at nanomolar concentrations. Iron-oxide contrast agents increase T2 relaxation time leading to hypointensity in T2-weighted images. The use of nanoparticles in animal experiments to track labeled cells using MRI has led to the visualisation of cell migration in various pathologies.

Macrophage and T-cell infiltration from the bloodstream to brain tissue, specifically the cerebellum, in animals with experimental autoimmune encephalitis (EAE), which is an animal model of multiple sclerosis (MS), has been visualised using this technique (Brochet et al., 2006). Macrophage migration to the site of a cerebral blood vessel infarct in animal stroke models has been similarly examined (Weber et al., 2005). More recently, many researchers have attempted to target more specific molecular processes through the addition of molecular process-relevant organo-compounds to existing contrast agents. Antibody-linked magnetic nanoparticles have been used to visualize the kinetics of endothelial expression of vascular cell adhesion molecule-1 in a mouse model of stroke (van Kasteren et al., 2009).

The use of bi-modal magnetic fluorescent nanostructures, enabling the capabilities of MRI and confocal microscopy to be exploited, has also been investigated and several studies assessing the application of multimodal magnetic-fluorescent nanomaterials for cellular imaging have been completed. Biocompatible iron oxide nanoparticles conjugated to a fluorescent dye and the Tat-peptide have been examined, using confocal microscopy and T2-weighted MRI, following incubation with primary human dermal fibroblast cells and Madin–Darby bovine kidney derived cells (Nitin et al., 2004). In another study, Rhodamine-labeled citric acid-capped magnetite nanostructures were utilised as fluorescent biological markers (Sahoo et al., 2005). It has also been demonstrated that several cancer cell lines can be labelled by different fluorescent superparamagnetic core-shell nanostructures containing magnetite nanoparticles and fluorescein-5-isothiocyanate (FITC) species (Becker et al., 2007, Rodriguez et al., 2006, Weissleder et al., 2005). More recently, nanoparticles coated in polyelectrolyte layers have been investigated as potentially useful in CNS drug delivery. The fabrication method affords a high level of control over the number of layers present on nanoparticles enabling degradation characteristics to be controlled (Jewell and Lynn, 2008).

The ability to characterize tracked cells by various methods of contrast simultaneously, or sequentially, offers great potential in understanding more clearly the dynamics involved in normally peripherally-resident cells infiltrating the CNS.

1.7 Physiological role of iron

Iron is essential to almost all living species. In humans its roles include oxygen transport, storage and activation, electron transport and many important metabolic processes. Iron is necessary for both neurotransmitter synthesis and myelination and, extrapyramidal regions associated with motor functions tend to have more iron than non-motor-related regions (Koeppen, 1995). As in the rest of the body, iron is stored within the ferritin protein. Ferritin is the body's primary iron storage protein, and consists of a spherical cage containing a maximum of 4,500 iron atoms as a ferrihydrite-like core up to 8nm in diameter.

Ferritin is present to the greatest degree in oligodendrocytes, although neurons, astrocytes and microglia do express ferritin (Hulet et al., 1999). Increased iron, assessed histochemically, is observed in glial cells of the cortex, cerebellum and hippocampus in older compared with younger individuals; ferritin immunoreactivity is also stronger in these cells (Connor et al., 2001). Iron can adopt either the ferric (Fe^{3+}) or the ferrous (Fe^{2+}) valence state *in vivo*. This property is utilised in uptake, transport and storage. The ferrous state of iron is highly reactive, and if free iron is not sequestered and stored as a ferric iron compound, then the iron can partake in deleterious chemical reactions that stimulate the overproduction of reactive chemical species. For instance, ferrous iron reacting with hydrogen peroxide (H_2O_2) via the Fenton reaction will produce highly reactive and potentially damaging hydroxyl radicals.

1.7.1 Iron in AD

Abnormal iron accumulation is associated with a range of neurodegenerative disorders, including AD and Parkinson's disease (PD) and accumulation of iron in the brain, particularly in cells that are associated with senile plaques, is a consistent observation in AD (Zecca et al., 2004, Connor et al., 1992). Iron has also been implicated as having a role to play in the oxidative stress seen in AD and in the brains

of AD patients, abnormal iron accumulation occurs in the absence of the normal aged-related increase in ferritin, concurrent with decreased levels of transferrin receptor expression (Thompson et al., 2001), thereby increasing the risk of oxidative stress through the loss of what is considered the primary method of iron metabolism (Castellani et al., 2007, Thompson et al., 2003). There is also evidence suggesting iron dysregulation may be a causative agent of neurodegeneration. However, it is unknown at what stage dysregulation of iron occurs in AD and it is suggested that disturbances at any of the several stages in iron metabolism including uptake and release, storage, intracellular metabolism, and regulation would prove detrimental (Ke and Ming Qian, 2003).

Though ferritin is the primary mechanism for iron storage in the brain, over the past decade experimental work has demonstrated the presence of another form of iron in human brain tissue – biogenic magnetite (Fe_3O_4). This contains alternating lattices of ferrous and ferric iron and was first found by Kirschvink and co-workers (Kirschvink et al., 1992). This finding has been replicated in several studies with efforts made to constrain any potential artifacts from the handling of post-mortem tissue (Schultheiss-Grassi and Dobson, 1999, Schultheiss-Grassi et al., 1999). The presence of magnetite is significant, as it may play a role in the progression and initiation of neurodegenerative disease through free radical production leading to tissue damage at the site of magnetite accumulation. High levels of free iron in AD-affected brain tissue have been noted as a possible cause of neuron degeneration through free radical processes via the Fenton reaction. Magnetite has been shown to have a substantial effect on free radical generation (Chignell, 1998, Scaiano, 1997).

1.7.2 Zinc and copper – Physiological role and association with AD

Zinc plays a key role in the physiology of CNS tissue, its ionic form Zn^{2+} is highly enriched at glutamatergic nerve terminals, is released upon neuronal activity and is involved in a multitude of signalling pathways through reactions with ion channels, receptors and transporters (Sensi et al., 2009). The presence of zinc in amyloid plaques and CAA has led to the suggestion that it may play a role in AD (Friedlich et

al., 2004, Miller et al., 2006). Copper, also essential to brain physiology, is similarly implicated in AD (Lovell et al., 1998).

It has been shown that zinc transporter proteins, which transport zinc ions into different intracellular compartments when intercellular zinc is elevated, are increased in AD and found in senile plaques (Lovell et al., 2006, Smith et al., 2006, Zhang et al., 2008). Ionic zinc (Zn^{2+}) is also a potent toxin and can mediate neuronal and glial death (Choi et al., 1988). Copper has also been implicated in AD with significant alterations to the concentrations of all three metals seen in brain areas associated with AD pathology (Deibel et al., 1996), with an iron and zinc increase, but a copper decrease, reported. Iron, copper and zinc have been demonstrated to accelerate A β aggregation (Garzon-Rodriguez et al., 1999) and it has also been reported that iron can alter APP processing (Rogers et al., 2002b). These findings lend support to the hypothesis that metal accumulation or dysregulation contributes to the pathogenesis of AD.

1.7.3 A β interactions with metals

Senile plaques in AD and in transgenic mouse models of AD colocalize with iron, copper and zinc in human AD studies (Cherny et al., 1999, Lovell et al., 1998, Miller et al., 2006, Stoltenberg et al., 2005). Recent work has suggested plaques are more likely to form proximal to areas of high zinc density in the cortex but conversely plaque burden was increased in animals with a zinc-deficient diet (Stoltenberg et al., 2007). Increased iron was also found within the glial cells surrounding the plaques (Quintana et al., 2006). Excess iron is typically sequestered by the iron storage protein ferritin and it was found that expression of ferritin is associated with degeneration of microglia in AD brain (Lopes et al., 2008). Evidence of the co-localization of ferric iron and A β plaques in APP/PS1 transgenic mice has been reported (Falangola et al., 2005).

More recently work has focused on examining metal content in relation to amyloid peptide concentration and data indicate that plaques in a APP/PS1 transgenic mouse model bind less iron, copper and zinc than human AD plaques, when normalized to amyloid protein concentration in plaques assessed. This study also reported elevations in calcium concentration in transgenic mouse plaques, which is unusual as A β does not have any known binding sites for calcium (Leskovjan et al., 2009). Data from this study indicated that within plaques, zinc was elevated to the greatest level followed by calcium, iron and copper. The authors suggest the differences in plaque composition in the mouse model of AD may contribute to the absence of neuronal cell loss. It is important to note that these data were collected from tissue specimens stained with Thioflavine-S to identify amyloid plaques and no information about the redox state of the metals in plaques was collected. Plaques investigated in studies of these kind, are predominantly from human studies where AD was fully progressed or animals aged such that plaque deposition was widespread. Although iron, copper and zinc all modulate A β aggregation, it is not known if colocalization with senile plaque cores occurs upon initial formation or subsequently.

Oxidative stress has been shown to correlate with amyloid plaque deposition (Smith et al., 1998). Studies investigating the interaction of A β and metals have consistently reported the reduction of both iron ($\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$) and copper ($\text{Cu}^{2+} \rightarrow \text{Cu}^{+}$) upon A β binding, producing H_2O_2 in the presence of oxygen in vitro (Huang et al., 1999, Rotkamp et al., 2001). Alternately, A β also binds zinc, which is inert to redox chemistry. Recent studies have suggested a protective role for zinc in AD, where its binding to A β inhibits copper reduction and subsequent H_2O_2 production (Cuajungco et al., 2000). More recent work has provided support for oxidative damage in the immediate vicinity of senile plaques to be predominantly associated with A β_{1-42} -driven iron-redox cycling, which was found to be significantly attenuated in vitro in the presence of copper and/or zinc (Khan et al., 2006).

Investigations into global concentrations of iron and other metals in APP and/or PS1 transgenic mouse models of AD are rare. However, an increase in the

concentration levels of copper in APP knockout mice has been reported (White et al., 1999) and it has also been reported that overexpression of A β leads to a significant decrease in the age-dependant elevation of copper but not iron (Maynard et al., 2002).

1.7.4 SQUID Magnetometry

Although identifying iron in tissue is relatively straightforward, determining its chemical state and environment is more complex, and hampered in particular by the oxidation of iron during conventional tissue fixation and staining processes (Dobson and Grassi, 1996). Progress has been made in staining techniques and certain information can be gathered about the redox state of iron. However, these conventional approaches are limited, particularly in terms of identifying the state of precipitated iron (Smith et al., 1997).

SQUID magnetometry is the most effective and sensitive method of measuring the magnetic properties of a sample. Different iron compounds within a sample provide different responses depending on the applied magnetic field and sample temperature. Inspection of these responses allow the identification of presence of iron compounds (Dobson, 2002, Hautot et al., 2003). This approach has been applied to post mortem brain tissue obtained from persons with AD, where a higher concentration of magnetite was observed in three samples of AD tissue than in three age- and sex-matched controls (Hautot et al., 2003). These results demonstrate that magnetite is a contributing factor to the elevated levels of iron observed in AD tissue (Hautot et al., 2005, Dobson, 2001).

1.7.5 X-ray fluorescence

X-ray fluorescence (XRF) takes advantage of the intrinsic fluorescent properties of elements. The term fluorescence is applied to phenomena in which the absorption of higher-energy radiation results in the re-emission of lower-energy radiation. High energy incident X-rays (primary radiation) are used to expel inner shell electrons (such as k- or l-shell) from atoms of the material under investigation. The removal of

an electron results in the electronic structure of the atom being unstable, and electrons in higher orbitals "fall" into the lower orbital to fill the hole left behind, emitting fluorescence (secondary radiation) in the process. The energy released is equal to the energy difference of the two orbitals involved. This process is illustrated in Figure 1.5. Thus, the material emits radiation, which has energy characteristics of the atoms present; energy analysis of the secondary radiation enables identification of each characteristic radiation and the energy can be related to the amount of each element in the material.

To effectively assess what elements may be present in a sample, the energy of the primary radiation must be large enough to expel inner shell electrons from elements of interest. XRF maps can be generated by moving the sample, as opposed to the beam, and gathering data. Accurate positioning and the size of the beam determines the spatial resolution possible. This process is illustrated in Figure 1.6. Synchrotron production of X-rays and subsequent beam-focusing, through the use of specialised hardware, enables XRF mapping at sub-millimetre resolution. XRF measurements of biological samples yield quantitative information about the spatial distribution of multiple elements simultaneously with high sensitivity and low background.

1.7.6 X-ray absorption near edge spectroscopy

XRF provides information on occupied electronic bands by analysis of the re-emission of energy incident on the material under investigation. In contrast, X-ray absorption spectroscopy (XAS) evaluates unoccupied electronic states by examining the absorption of X-rays of increasing energy. X-ray absorption near edge spectroscopy (XANES) is a subset of XAS and examines the energy absorption of X-rays as they range from below the energy necessary to expel an inner core electron to beyond. The X-ray absorption profiles of elements in different oxidative states are sufficiently different to enable identification of their presence from XANES data using linear combination statistical analysis.

1.7.7 XRF & XANES biological applications

A study of brain slices from PD and age-matched controls mapped iron, copper and zinc using XRF confirmed the potential of the technique, and confirmed the histological data indicating increases in iron and zinc are associated with PD. XRF data also indicated increases in iron and zinc were mutually exclusive and did not occur in the same area; this analysis is not possible using conventional histological staining techniques (Popescu et al., 2009). Further XRF and XANES studies of PD tissue have indicated greater concentrations of iron oxides in substantia nigra compared with age-matched controls (Ide-Ektessabi and Rabionet, 2005). Imbalances of zinc, copper and iron oxides have also been determined in single neurons from PD brain tissue (Oakley et al., 2007).

The location and characterization of iron compounds in AD tissue has been investigated using XRF and XANES at sub-cellular resolution (Collingwood and Dobson, 2006, Collingwood et al., 2005). Data from these studies confirmed abnormal iron distribution and indicated the high prevalence of iron oxides in AD tissue. These techniques have also been applied to examine senile plaques and, as previously discussed, quantified the relative amount of iron, copper and zinc in plaques from human AD tissue and transgenic mouse model AD tissue. Recent advances have enabled the quantification of A β concentrations in plaque cores using synchrotron fourier transform infrared micro-spectroscopy and subsequent XRF investigation (Leskovjan et al., 2009, Miller et al., 2006).

1.8 Aims of study

The aims of these studies were to

- Evaluate the interaction of a magnetite-based polyelectrolyte-coated fluorescently tagged nanostructure with glial cells and evaluate the potential of *in vivo* bi-modal tracking of specific cell populations using this nanostructure.
- Assess the potential of XRF to perform analysis of iron, copper and zinc distribution in the brains of mice.
- Assess the colocalization of iron, copper and zinc with amyloid plaques in brain sections prepared from 8 – 9 month old APP/PS1 transgenic mice, using XRF.
- Assess the extent of amyloid deposition, neuroinflammation and the spatial memory performance of APP/PS1 transgenic mice at 8 – 9 months of age.
- Longitudinally assess MRI relaxometry and blood perfusion changes in APP/PS1 transgenic and wildtype mice, from 3 – 4 months to 8 – 9 months of age.

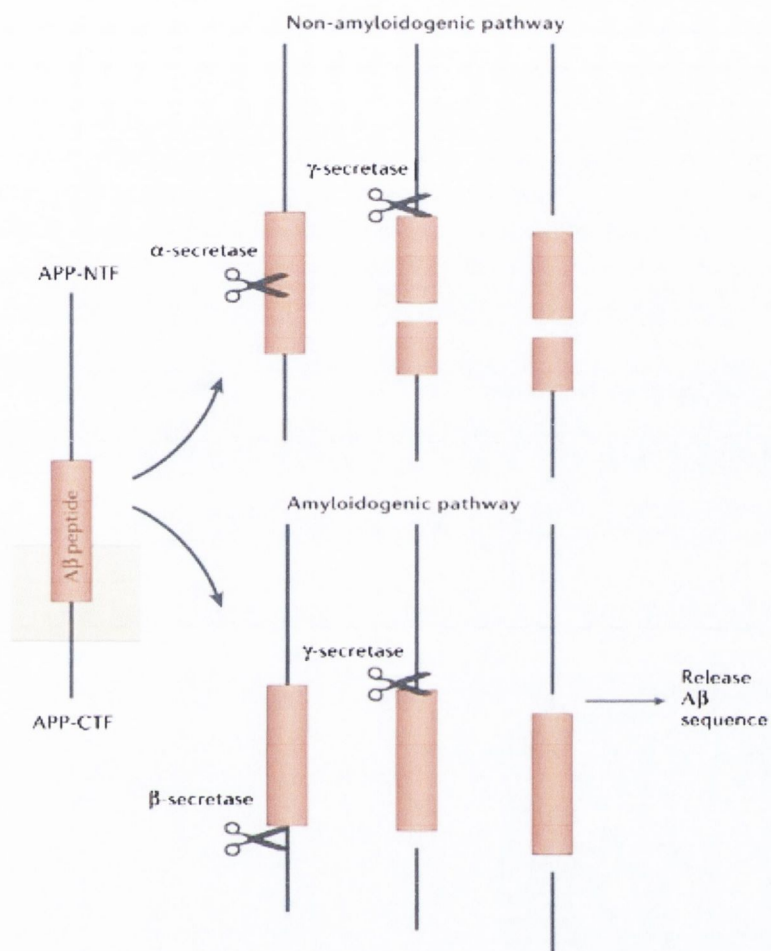


Figure 1.1. Schematic of the APP processing pathway.

A schematic representation of the APP processing pathway with secretases involved in the non-amyloidogenic pathway illustrated in the top half and the amyloidogenic pathway illustrated in the bottom half.

Adapted from Van Dam et al, 2006.

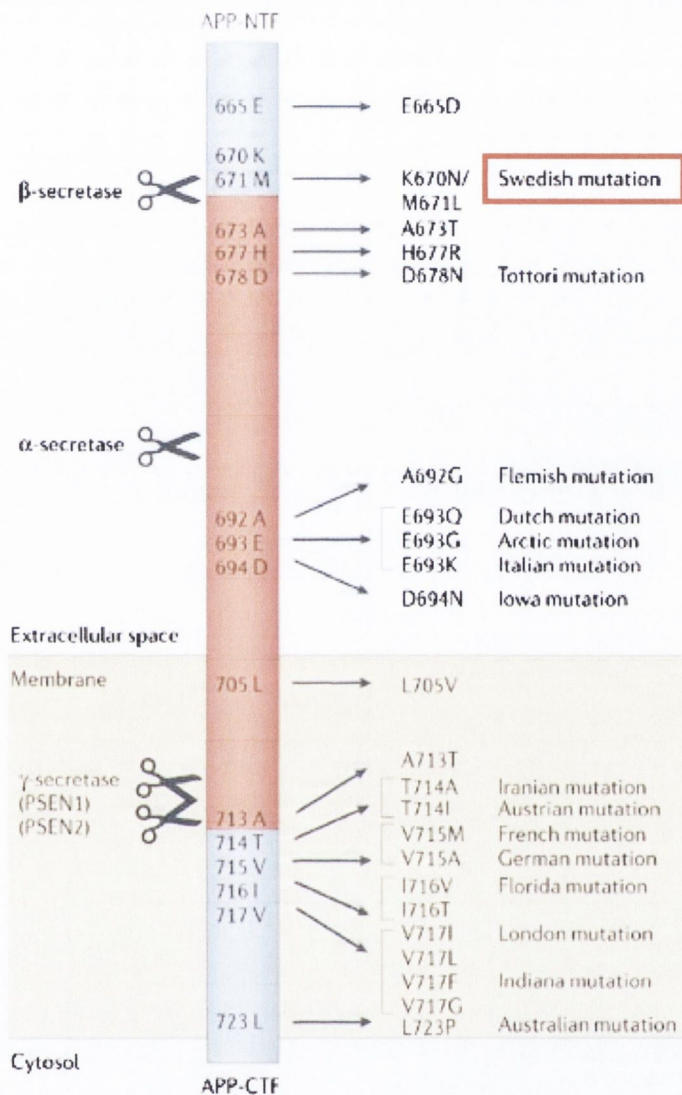


Figure 1.2. APP points of mutation.

A schematic representation of the most common points of mutation on APP. The transgenic animal utilised in this study overexpress the swedish mutation of APP (highlight with the red box).

Adapted from Van Dam et al, 2006.

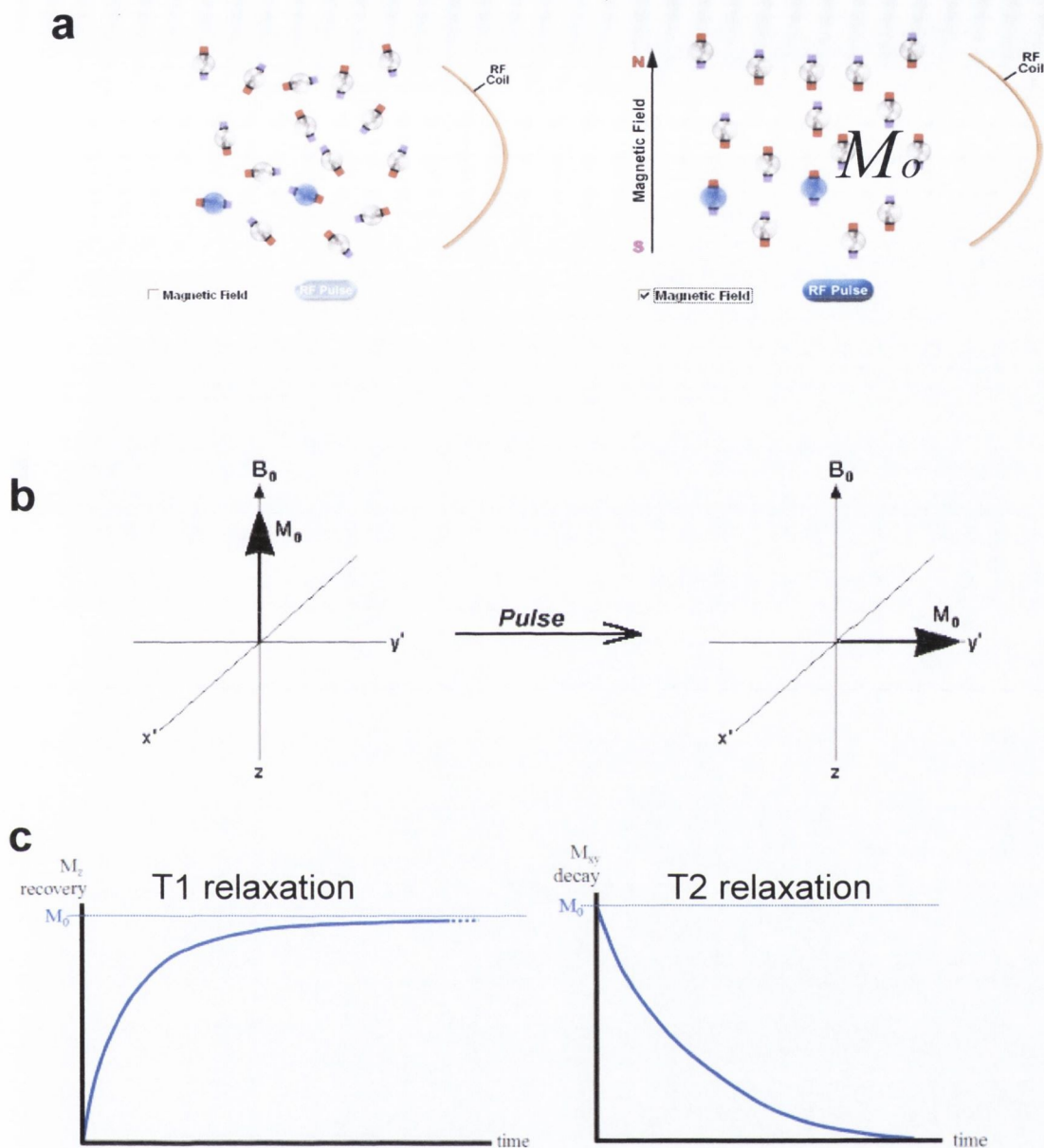
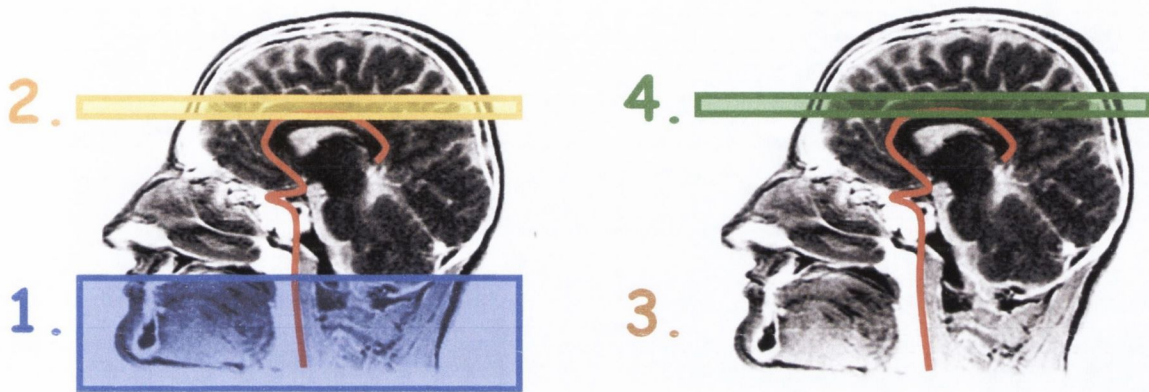


Figure 1.3. Schematic representation of MRI signal generation.

The generation of the magnetic signal, M_0 , due to the presence of a large static magnetic field, B_0 , is presented (a). The movement of M_0 , from the z-plane to the x-y plane, following the application of an RF pulse is illustrated (b). The relaxation of the perturbed magnetization to its equilibrium state is also presented (c). The increase of the magnetization along the z-axis represents T1 relaxation while the decay of magnetization along the x-y plane represents T2 relaxation.

Adapted from www.mritutor.org



1. Tag the inflowing arterial blood by magnetic inversion.
2. Acquire the tag image.
3. Repeat the experiment without tag.
4. Acquire the control image.
5. Subtract the control image from the tag image.
6. Resulting signal is proportional to cerebral blood flow.

Figure 1.4. Schematic illustration of ASL.

A schematic illustration of ASL acquisition is presented. The MR signal is first obtained with the in-flowing blood tagged (1, 2). The signal is then obtained without the in-flowing blood tagged (3, 4). The difference in these measurements is proportional to cerebral blood flow, however, a mathematical model is necessary to determine quantifiable aspects of the blood flow.

Adapted from <http://www.umich.edu/~fmri/asl.html>

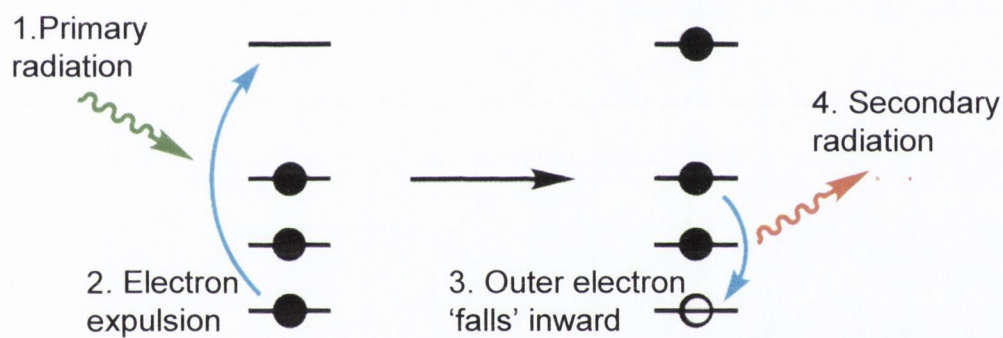


Figure 1.5. Schematic illustration of X-ray fluorescence.

A schematic representation of X-ray fluorescence is presented. Primary radiation (1) is of sufficient energy to expel an inner shell electron from its orbital (2). The electronic structure of the atom is now unstable and an electron from an outer shell has to 'fall' inward to the vacant inner shell (3). The electron expels the energy difference between the orbitals as secondary radiation (4).

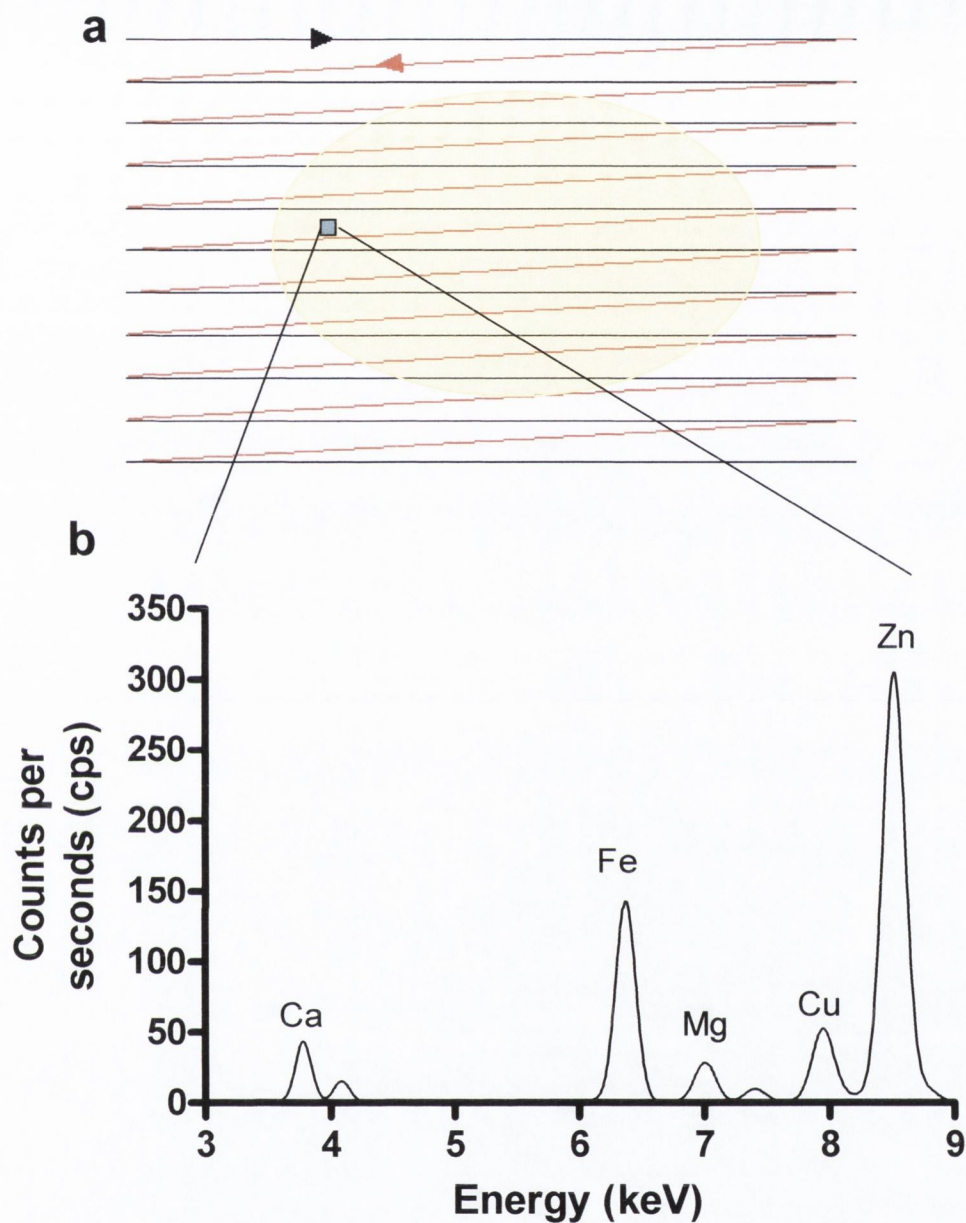


Figure 1.6. XRF map creation.

A schematic of the XRF sample raster (a) and a single pixel XRF spectra (b). Peak analysis of the XRF spectra enables element specific XRF maps to be generated.

Chapter 2

Methods

2.1 Materials

2.1.1 Synthesis and advanced characterisation of nanostructures

Synthesis and advanced characterisation of nanostructures was carried out by Renata Tekoriute of Prof. Iouri Gung'ko's group in the Department of Chemistry (Trinity College, Dublin, Ireland).

2.1.2 Photoluminescence spectroscopy

Samples of nanostructures were diluted with Millipore water (Millipore, USA) to a known concentration and placed in a quartz cuvette (2 ml working volume, 10 mm path length). Absorption and emission measurements were performed at room temperature using a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, USA).

2.1.3 Nanostructure behaviour in a magnetic field

To examine the behaviour of the nanostructures in a large magnetic field, 30 μl of nanostructure suspension (2.31×10^{-4} , 4.62×10^{-4} , 9.34×10^{-4} g/ml Fe_3O_4) was pipetted onto poly-L-lysine-coated coverslips, placed in the bore of a 7T spectrometer (Bruker Biospin, Germany) and left overnight. Coverslips were washed twice with ice-cold phosphate-buffered saline (PBS), fixed with ice-cold methanol and washed 3 times (5 minutes/wash) with PHEM buffer. Coverslips were mounted onto microscope slides, using fluorescence mounting medium (Vectashield, Vector Labs, UK), for analysis by confocal microscopy (Axioplan 2, Zeiss, Germany).

For Transmission Electron Microscopy (TEM) analysis, 30 μL of the nanostructure suspension (2.31×10^{-4} g/ml Fe_3O_4) was pipetted onto a TEM copper imaging grid, placed in the bore of the 7T spectrometer and left overnight. TEM scanning was carried out on a Jeol 2100 Transmission Electron Microscope (Joel Ltd., Japan). Samples outlined for assessment of nanostructure size were prepared for TEM by ultrasonically dispersing powder samples in Millipore water. One drop of the solution (5 μL) was placed onto a formvar-coated copper TEM grid. The grid was then dried in air. The size, shape and arrangements of the particles were analyzed in TEM images, obtained on a Jeol 2100 Transmission Electron Microscope. Particle sizes were determined by manually counting over 100 particles, in addition x and y measurements were determined for each particle.

2.2 Animals

Specific pathogen-free (SPF) C57BL/6 mice were purchased from Harlan UK Ltd. (Bicester, UK). Transgenic animals from an existing colony of APP^{swe}/PS1^{de9} mice, purchased from The Jackson Laboratory (Maine, USA), were used to form breeding pairs with the C57BL/6 mice and bred in an SPF animal housing facility in the Bioresources Unit (Trinity College Dublin). Mice were maintained according to the regulations and guidelines provided by the Irish Department of Health. All animals were housed under a 12-hour light-dark cycle at an ambient temperature of 22°C - 23°C; animals were maintained under veterinary supervision throughout. Normal laboratory chow and water were available to all animals at all stages. Animal experimentation was performed under a licence granted by the Minister for Health and Children (Ireland) under the Cruelty to Animals Act 1876 and the European Community Directive, 86/609/EEC, and every effort was made to minimise animal stress.

2.2.1 APPswe/PS1dE9 Genotyping

2.2.1.1 Genomic DNA isolation

Ear punches were taken from all mice produced from the APPswe/PS1dE9 and C56BL/6 breeding pairs. Ear punches were stored at -20°C until required. All solutions and reagents used to isolate genomic DNA were taken from the Wizard Genomic DNA Purification Kit (Promega, Madison, USA). Digestion buffer was freshly prepared by adding 120 µl of 0.5M ethylenediaminetetraacetic acid (EDTA; pH 8.0) to 500 µl of Nuclei Lysis solution. Ear punches were incubated in 500 µl chilled digestion buffer (buffer was chilled until cloudy) and 4 µl of 20 mg/ml Proteinase K at 55°C for 3 hours. Samples were mixed gently once every hour by flicking the tube. Visual inspection of samples confirmed the digestion of ear punches. Protein was precipitated from the sample through addition of 200 µl of Protein Precipitation Solution and vigorous vortexing for 20 seconds. The sample was chilled for 5 minutes and centrifuged for 4 minutes at 15,000 g. The precipitated protein formed a tight white pellet. The supernatant, containing the DNA, was removed and transferred to a clean 1.5 ml microfuge tube containing 600 µl of isopropanol at room temperature. A layer of supernatant coating the precipitated protein pellet was left, to avoid contaminating the DNA solution with protein. The isopropanol solution was mixed by inversion until white thread-like strands of DNA were visible. The solution was centrifuged, at room temperature, for 1 minute at 15,000 g. The DNA formed a small white pellet. The supernatant was carefully decanted and 600 µl of room temperature 70% ethanol was added. The pellet was washed by inverting the tube several times. The samples were centrifuged for 1 minute at 15,000 g. The majority of ethanol was removed and 100 µl of DNA Rehydration Solution was added. The DNA was incubated at 65°C for 1 hour, with the tubes open to allow any remaining ethanol to evaporate. To ensure DNA was solubilised the samples were stored overnight at 4°C.

2.2.1.2 Polymerase chain reaction for APPswe and PS1dE9 genes

The presence of the APPswe and PS1dE9 mutations in genomic DNA samples was assessed using polymerase chain reaction (PCR). Mastermix was prepared by the addition of DNase-free water (17.3 µl), 10X reaction buffer (2.5 µl; 500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100, pH 9, Promega, USA), magnesium chloride (1.5 µl; 25 mM, Promega, USA), nucleotide mix (0.2 µl; 25 mM, Biotium, UK), Jumpstart™ Taq DNA polymerase (0.5 µl; 100 µM, MWG Biotech, Germany) to a sterile tube (see Table 2.1 for primer sequences). The mastermix for the PS1dE9 gene contained sense and anti-sense primers for the PrP gene as an internal control. DNA (2 µl) and mastermix (23 µl) were added to fresh tubes, which were then 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 30 minutes, followed by 35 amplification 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. A final extension step of 72°C for 10 minutes was completed after the final amplification cycle to ensure complete extension of all PCR products.

Equal volumes from each sample (10 µl) and a 100 base pair ladder (Promega, USA) were mixed with loading buffer (2 µl; Promega, USA), and loaded onto a 1% (w/v) agarose gel containing gel red (1:10,000; Biotium, USA). Samples were separated by application of 90 volts for 120 minutes. PCR products were visualized under an ultraviolet light and photographed using an ultraviolet transilluminator (Labworks, Ultra Violet, Bioimaging Systems, USA).

Target gene	Primer Sequence
PrP (control)	fwd 5'-CCTCTTTGTGACTATGTGGACTGATGTCGG-3' rev 5'-GTGGATAACCCCTCCCCCAGCCTAGACC-3'
APPswe	fwd 5'-GACTGACCACTCGACCAGGTTCTG-3' rev 5'-CTTGTAAGTTGGATTCTCATATCCG-3'
PS1dE9	fwd 5'-CCTCTTTGTGACTATGTGGACTGATGTCGG-3' rev 5'-GTGGATAACCCCTCCCCCAGCCTAGACC-3'

Table 2.1 Primers used for DNA amplification

2.2.2 Animal Treatments

2.2.2.1 Intravenous injection

Mice were exposed to a heat-lamp for 5-7 minutes and individual mice inserted head-first into a modified 50ml falcon tube. The tube was shortened such that the overall length was similar to that of a mouse and airholes were made at the anterior end of the tube. At the posterior end a rubber cork, with a groove cut along its length was inserted to restrict movement of the mouse and enable a fibre-optic microscopic light source to be placed underneath the tail to allow visualization of the lateral caudal veins, when other light sources were dimmed. Magnifying glasses were used to aid visualization when necessary.

2.2.2.2 Induction of EAE in C57BL/6 mice

A mouse model of multiple sclerosis (MS), experimental autoimmune encephalitis (EAE), was used to model cell infiltration from the periphery to the CNS. To induce EAE, 10-18 week old C57BL/6 were injected subcutaneously with 100 µg Myelin Oligodendrocyte Glycoprotein (MOG) peptide (Cambridge Biosciences, UK) in Complete Freund's Adjuvant (CFA; DIFCO, UK) supplemented with 5mg/ml H37RA (*Mycobacteria tuberculosis*, DIFCO, UK). The mice were also injected intraperitoneally (i.p.) with 500 ng pertussis toxin (Tocris Biosciences, USA) on day 0 and day 2 post immunisation. EAE was scored according on a 0-5 scale as follows: limp tail, 1; wobbly gait, 2; hind limb weakness, 3; hind limb paralysis, 4; tetra paralysis, 5.

2.3 Behavioural

Prior to behavioural assessment all animals had muscle strength and co-ordination assessed using the hang-wire and the inverted screen test.

2.3.1 Hang-wire

Testing took place on a horizontal steel wire (30 cm long) suspended between 2 wooden poles (20 cm high) above a padded surface. Two 60 second trials were performed with an inter-trial interval of 28 minutes. In the first trial animals were suspended from the wire by all 4 paws, and the length of time a grip was maintained on the wire was recorded. Only the front 2 paws of the animal were placed on the wire for the second trial. The trial lasted until the mouse fell from the wire or reached the 60 seconds cut-off time. Any mice that crossed the wire to the poles at either end were given a score of 60 seconds.

2.3.2 Inverted screen

Mice were placed on a wire-mesh screen which was slowly inverted and held above a padded surface. The length of time the mouse maintained a grip on the screen was recorded, the trial lasted until the mouse fell or the 60 seconds cut-off time was reached. Each mouse was assessed twice with an inter-trial interval of 28 minutes.

2.3.3 Morris water maze

2.3.3.1 Morris water maze apparatus

The MWM apparatus was assembled in a well-lit room with the temperature maintained at 22-23°C. It consisted of a circular tank, 1.2 m in diameter and 0.6 m in height. It was filled with water to a depth of 0.24 m. A perspex platform, 0.15 m in diameter and 0.23 m in height, was positioned in the northwest quadrant of the pool, 0.13 m from the edge of the pool (Figure 2.1). The accuracy of the positioning was confirmed by visually inspecting the digital overlay from the MWM software (Water 2020, HVS Image, UK) on images from an overhead camera used for tracking the mice. Distinct visual cues were placed on each of the 4 walls surrounding the tank at a height of 1 metre.

2.3.3.2 Habituation

A single day of task habitation was carried out prior to data being acquired. The water was clear and the platform clearly labelled with a large flag. No visual cues were used during habituation. The animal was placed into the pool in the southeast quadrant facing the wall and guided to the platform by the experimenter using a plastic rod. All mice were brought to the platform within 60 seconds and left to remain on the platform for 45 seconds. Animals completed a single trial.

2.3.3.3 Training & reversal assessment

Training commenced the day following habitation and continued for 4 consecutive days, at the same time each day. The water was made opaque through the addition of white tempura paint powder (Crafty Devils, UK) and visual cues were placed upon the walls. Mice carried out 4 one-minute trials with an inter-trial interval of 5 minutes, during which the animal was returned to a home-cage warmed by a hot water bottle placed underneath. At the start of each trial, mice were lowered into the water while holding the tail and facing the mouse towards wall of the tank. During the trials, mice were placed into the pool at four separate points (north, south, east and west) with each point being used once. The order of these points was randomly generated for each day. The order mice were assessed on each day was also random. The MWM software tracked the animal and upon reaching the platform within 60 seconds the trial was stopped. Unsuccessful mice were shown to the platform after the 60 seconds cut-off time, and all animals remained on the platform for 20 seconds at the end of each trial.

Animals were trained until they could be shown to have learned the task via statistical analysis of the time taken and pathlength to reach the submerged platform. Following this the position of the submerged platform was moved diagonally and the animals assessed daily for 4 days as previously described.

2.4 MRI Scanner

MRI was carried out on a dedicated small rodent Bruker Biospec (Bruker Biospin, Germany) system with a 7 Tesla magnet and a 30 cm core.

2.4.1 MRI Animal Setup & maintenance

Mice were anaesthetized using isoflurane and placed into an MR-compatible mouse cradle. The animal's upper teeth were placed in a bite bar and supports inserted into the ear to ensure optimal positioning and minimal movement. The mice were maintained under anaesthetic during scans using a mixture of isoflurane and oxygen administered via a gas facemask (1.5 – 2 % at 1 litre/minute of 100% oxygen). The animals respiration was monitored using custom-built hardware and software (SA Instruments Inc., Stony Brook, NY, USA). The temperature of the animal was maintained by pumping water from a temperature controlled water bath (SA Instruments Inc.) through tubes lying underneath the animal. A mechanical ventilator (Ugo Basile, Comerio, VA, Italy) was used to ensure appropriate delivery of gas to the facemask. The cradle was then inserted into the MRI scanner. Accurate positioning was ensured by acquiring an initial rapid pilot image. To maintain consistent positioning throughout all experiments the animal was repositioned and scanned until the rhinal fissure was 5 mm anterior to the isocentre of the B_0 magnetic field. All subsequent images were run after a magnetic field shim sequence and gain adjustment procedure was carried out.

2.4.2 MRI protocols

2.4.2.1 Relaxometry scans

A single slice RAREVTR sequence, positioned at the isocentre, was used to acquire images enabling calculation of T1 relaxation times. The parameters used were; TE = 12.637 ms, TR = 300, 589.116, 942.255, 1396.084, 2031.981, 3103.081 and 8000 ms, RARE factor = 4, field of view = 1.8 x 1.8 cm, slice thickness = 1 mm, matrix = 128

x 128, pixel dimensions = 141 x 141 μm . Scanning time was 8 minutes and 43 seconds.

A MultiSpin MultiEcho (MSME) sequence was used to acquire images enabling calculation of T2 relaxation times, 12 echo times starting at, and spaced, 8.06 ms apart were acquired with TR = 2000 ms. Other sequence parameters were; field of view = 1.8 x 1.8 cm, slice thickness = 1 mm, number of slices = 5, interslice distance = 1.10 mm, matrix = 128 x 128, pixel dimensions = 141 x 141 μm . Scanning time was 4 minutes and 16 seconds. Only the centre slice was used to determine T2 relaxation times as refocusing artefacts were present in the outer slices.

2.4.2.2 High-Resolution T2-weighted scan

T2-weighted images were collected using a RARE T2-weighted sequence with the following parameters; TE = 14.074 ms, TE_{effective} = 42.22 ms, RARE factor = 8, TR = 7478 ms, field of view = 1.8 x 1.8 cm, slice thickness = 0.35 mm, number of slices = 54, matrix = 128 x 128, pixel dimensions = 141 x 141 μm . The scan time was 1 minute 56 seconds. The scan was repeated 20 times and individual scans were visually inspected for movement artifact, and removed if necessary, prior to averaging using FSL. The overall scanning time was 50 minutes.

2.4.2.3 Arterial Spin Labelling (ASL) scan

A theoretical model to facilitate the quantification of cerebral perfusion with ASL has been described in detail previously (Kelly et al., 2009) and is outlined in Appendix I. The model uses a bolus-tracking ASL (btASL) sequence to provide signal-time curves of the passage of a 3 second bolus through a user-defined ROI. The sequence consists of a 5 second preparation interval containing the inversion pulse, followed by snapshot fast low angle shot (FLASH) image acquisition (Kerskens et al., 1996). Flow-induced fast adiabatic passage of inflowing inverted arterial spins was performed using a rectangular pulse with a maximum B₁ of 120 mG, thus providing inverted arterial spins to the imaging location (Dixon et al., 1986). The inversion pulse radiofrequency (RF) power was set to achieve inversion at the desired location,

and had a bandwidth of 2.8 kHz. The gradient strength was set to 14 mT m^{-1} to provide an inversion region thickness of 4.7 mm. The pulse frequency was offset by -12 kHz, resulting in a tagging location 2 cm proximal to the imaging slice. A control image with the offset frequency reversed (+12 kHz) was also acquired, in which inflowing spins were left undisturbed. Corresponding pairs of labelled and control images were subtracted to provide perfusion-weighted maps.

The preparation interval contained the inversion pulse of duration, τ , and two variable delays, D_1 and D_2 (Figure 2.2a). The delays and the inversion pulse duration were varied, as shown in Table 2.2, to allow the full signal-time curve to be plotted (Figure 2.2b). The snapshot FLASH imaging was acquired with centric phase encoding to enhance sensitivity to contrast provided by inflowing labelled magnetization (Holsinger and Riederer, 1990). The following parameters were used: slice thickness = 2 mm, TR = 6.66 ms, TE = 2.99 ms, RF flip angle = 30° , field of view = $3.0 \times 3.0 \text{ cm}$, image matrix = 128×64 , receiver bandwidth = 50 kHz. The total time for the ASL preparation and image acquisition for a single measurement point was 5.426 seconds.

Time-Point	<u>3.0 s Bolus</u>		
	D_1	τ	D_2
1	4.9	0.1	0.0
2	4.5	0.5	0.0
3	4.0	1.0	0.0
4	3.5	1.5	0.0
5	3.0	2.0	0.0
6	2.5	2.5	0.0
7	2.0	3.0	0.0
8	1.5	3.0	0.5
9	1.0	3.0	1.0
10	0.5	3.0	1.5
11	0.0	3.0	2.0

Table 2.2 Variations of D_1 , D_2 and τ for 1.5 , 2.0 and 3.0 second bolus

2.4.3 MRI analysis

2.4.3.1 Relaxometry

T1 and T2 relaxation times were calculated using the Image Sequence Analysis (ISA) software in the Bruker software, Topspin. T1 and T2 relaxation times were fitted to mono-exponential rise and decay functions, respectively. Regions of interest (ROI) were drawn with reference to a mouse brain atlas (Paxinos & Franklin, 1995). ROIs were drawn on the centre slice of the T2 relaxation scan. These ROI were also used to assess T1 relaxation times, as the centre slice of the T2 scans were geometrically identical to the T1 scan. The cortex, hippocampus, thalamus and corpus callosum were mapped. A representative T2 scan (TE = 16.16 ms) with ROI shown is presented (Figure 2.3)

2.4.3.2 Volumetric Based Morphometry

High-resolution T2-weighted scans were converted from the Bruker file format to the analyze file format using the `pvconv.pl` conversion script (<http://pvconv.sourceforge.net>). Brain extraction was performed in the MIPAV software using the brain extraction algorithm. The MIPAV paint tool was used to complete inadequate results from the brain extraction software, an example is shown (Figure 2.4a,b). Images were reoriented to replicate human MRI experimentation orientation and voxel sizes enlarged by a factor of 10 in MIPAV. These changes enabled usage of the FSL software library (Smith et al., 2004), written primarily for use on human MRI data, with minimal changes in performance. Gray matter segmentation was performed using the *fast* segmentation tool provided as part of the FSL software library (Figure 2.4c). Gray matter segmented images were nonlinearly registered to a C57 gray matter template (Ma et al., 2005) using *fslreg*. Study-specific gray matter templates were created by averaging registered images associated with each experimental comparison. Following this, C57 template-registered images were nonlinearly registered, using an initial estimate by *flirt* to ensure adequate results by

fnirt, to the study specific template. The study-specific registered image was compensated for areas of contraction/enlargement due to the non-linear component of the transformation. Each voxel of each registered grey matter image was divided by the Jacobian of the warp field for that image. A general linear model designed using *Glm* was used to analyse gray matter volumetric differences between experimental groups in a specific study, using *randomise*, a permutation-based inference tool for nonparametric statistical thresholding. Images were smoothed with a 1mm kernel prior to analysis with *randomise*. The output of *randomise* is a statistical parameter map which can be overlaid onto the study specific C57 gray matter template to indicate areas of significant difference between groups, the threshold for the map was set at $p < 0.001$ for all comparisons performed.

2.4.3.3 ASL

Cortical, hippocampal and whole brain ROI were drawn on perfusion-weighted images and the average for each ROI plotted against acquisition timepoint in the btASL sequence (Table 2.2). This curve was fitted to the solution of the non-compartmental ASL theoretical model found by Kelly and colleagues, 2009, enabling the quantification of mean transit time (MTT) and capillary transit time (CTT). The curve-fitting routine in Mathematica (Wolfram Research Inc., Version 5.1, Champaign, IL, USA) was used to find the least squares fit to the experimental concentration-time curves by the Levenberg-Marquardt method and excellent agreement between ASL signal-time curve and the fit of the theoretical model was achieved (Figure 2.4b). MTT is defined as the average time it takes a particle to traverse the vasculature (Meier and Zierler, 1954). The CTT variable has been interpreted as the time taken for labeled arterial water to be distributed at the ROI (Kelly *et al*, 2009). Furthermore, estimation of cerebral blood volume (CBV), from analysis of fitting parameters, is possible and is reported as CBV_{lv} , as it is composed of contributions from both the intra- and extra-vascular spaces (Silva *et al*., 1997). The amplitude of the fitted curve in Figure 2.4b, is directly proportional to the area

under the curve and was used to estimate the $rCBV_{lw}$ in the cortical, hippocampal and whole brain ROI. It should be noted that the subscript lw is used here to signify that the ASL signal is composed of contributions from both the intra- and extravascular spaces (Silva et al., 1997) and as a result, the volume estimated from the area under the ASL signal-time curve is not purely intravascular in nature. Finally, the division of $rCBV_{lw}$ by MTT represents the division of volume by a measure of the time taken for this volume to flow and can be considered an approximation of cerebral blood flow (rCBF).

2.5 Tissue Preparation

2.5.1 *In vivo*

2.5.1.1 Cervical dislocation and brain dissection

Mice were decapitated following cervical dislocation and the brain was dissected after careful removal of scalp tissue and skull using curved forceps and fine surgical scissors. The brain was placed onto an ice-chilled glass surface. A cryostat blade was used to cut coronally at approximately 4-5 mm anterior to the cerebellum. The brain portion containing the cerebellum was then coated in optimal cutting temperature (OCT) compound (Tissue-Tek, USA) and placed cutting surface down onto a pre-labelled cork disc. This was then snap-frozen in dry-ice chilled isopentane (Sigma-Aldrich Inc.) and stored at -80 °C until sectioning.

2.5.1.2 Surgical equipment and surface trace-metal decontamination

Ceramic forceps, ceramic tweezers and a sapphire surgical blade (Electron Microscopy Services, USA) were acid-washed (3% HCl) and rinsed in deionized water. All surfaces were plastic and acid-washed and rinsed in deionized water prior to use. All containers with reagents were placed on NdFeB bar magnets, and reagent taken from the top of the container for use in the procedure to remove magnetic contaminants.

2.5.1.3 Intracardial perfusion

All equipment and surfaces used in this procedure were treated as outlined in Section 2.5.1.2. Wild-type control and APP^{swe}/PS1^{dE9} transgenic mice were sedated with an i.p. injection of sodium pentobarbital (30 μ l, Euthanol, Merial Animal Health, UK). Sedation was deemed adequate on the absence of the toe-pinch reflex. The animal was placed on a polystyrene surface. Ethanol was used to wet the abdomen of the animal and all four paws were secured with 25G needles (BD, UK) into the polystyrene. The skin was cut at the level of the liver and cut towards the head, through the rib cage until the heart was exposed. This area of skin was secured to the polystyrene using a small needle. A 25G needle was placed into the left ventricle and perfusant pumped via a syringe pump (Razel Scientific Instruments, UK) at a rate less than 0.5 ml/minute. The right atrium was cut immediately following pump turn-on. Animals were perfused with 0.1 M sodium cacodylate (Sigma-Aldrich, UK) made up with ultrapure water (Sigma-Aldrich, UK). The animals were perfused for 20 minutes or until the liver turned a light coffee colour. The head was cut at the base of the head and the skin cut away from the skull. The skull was cracked using ceramic tweezers and removed using ceramic forceps. The brain was transferred to a plastic surface where the tissue was preserved as required.

2.5.1.4 Cryosectioning

Gelatin-subbed microscope slides (25 mm x 75 mm x 1 mm, Superfrost Plus, ThermoFisher Scientific, UK) were labelled prior to cryosectioning. Brain tissue was placed in the interior of the cryostat (Leica CM1900, Leica Microsystems Inc, USA) and allowed to equilibrate to -20°C, the temperature at which sectioning was done. Sections were cut at 10 μ m thickness. Anatomical position was assessed by light microscopy in Toluidine Blue-stained sections by reference to a Mouse Brain Atlas (Paxinos and Franklin, 1995). Sections from the interior, middle and exterior portion of the sectioned hippocampus were placed on each slide. Retina cryosectioning was

performed as outlined for brain tissue. Sections were air-dried for 30 minutes and stored at -20°C until required.

2.5.1.5 Protein quantification

The protein concentrations in tissue samples were assessed using the bicinchoninic acid (BCA) protein assay kit (Pierce, The Netherlands). A standard curve was prepared (0 – 2000 µg/ml) by diluting concentrations of bovine serum albumin (BSA) in working reagent (1:50, Reagent B to Reagent A). Aliquots (25 µl) of samples and standards were added in triplicate to a 96-well plate and incubated for 30 minutes at 37°C. The optical density values were determined by measuring the absorbance at 590 nm using a microtitre plate reader (Multiskan RC, Labsystems, Finland). Protein concentrations in test samples were calculated with reference to the standard curve.

2.5.2 *In vitro*

2.5.2.1 Primary mouse mixed glia preparation

Primary cortical glial cells were prepared from the brains of 1-day old C57 mice (BioResources Unit, Trinity College, Dublin, Ireland). The mice were decapitated, the cerebral cortices dissected and the meninges were removed. Tissue was bi-directionally chopped using a sterile scalpel and placed in 15 ml falcon tubes (Sarstedt, Ireland) containing warm filter-sterilized DMEM. The tissue was incubated for 15 minutes (37°C; 5% CO₂:95% air; Nuaire Flow CO₂ incubator; Jencons, UK), triturated, filtered through a sterile mesh filter (40 µm; Becton Dickinson Labware, France), centrifuged at 2000 rpm for 3 minutes at 20°C and the pellet resuspended in warmed DMEM. Equal numbers of resuspended cells were plated in 24-well plates at a density of 1.0×10^6 cells/ml and incubated for 2 hours before addition of warmed DMEM. Cells intended for confocal and light microscopy analysis were plated onto poly-L-lysine-coated coverslips and grown at 37°C in a humidified 5% CO₂, 95% air environment for 12-14 days (with 3 intermediate changes of medium) until confluent.

2.5.2.2 Blood brain barrier cell model

The blood brain barrier (BBB) was modeled using 24-well plate transwell inserts on which brain microvessel endothelial cells (BMEC) were grown (Montesano et al., 1990). The inserts contained 8 μm porous membranes (Greiner Bio-one, Germany) and cells were grown until confluence was reached, with intermediate changes of growth media every 3-4 days. A fluorescein permeation test was used to investigate BMEC layer tightness in a parallel batch. Fluorescein (2 $\mu\text{g/ml}$) was added to the apical compartment and after 1 hour of incubation, the amount of fluorescein in the basolateral compartment was calculated by measuring the fluorescence signal using a spectrofluorometer. If the fluorescence signal was comparable to the signal from pure DMEM, the BMEC layer was regarded as confluent.

2.6 Nanostructure cellular interaction methods

Cells were incubated in the presence of a known concentration nanostructures for different time periods and prepared for analysis as detailed in the following sections.

2.6.1 Confocal microscopy

Cells were washed twice with ice-cold PBS buffer, fixed in ice-cold methanol and washed 3 times with PHEM buffer. The coverslips were mounted onto microscope slides, using mounting medium containing DAPI (Vectashield, Vector Labs, USA), for analysis by confocal microscopy (Axioplan 2, Zeiss, Germany).

2.6.2 Light microscopy

Cells used in the confocal microscopy experiments were also assessed using light microscopy. Cells were washed twice with ice-cold PBS buffer, fixed in ice-cold methanol and washed 3 times with PHEM buffer. Cells were incubated in the

presence of Prussian blue reagent for 20 minutes, washed in deionized water, dehydrated through alcohol, cleared in xylene and mounted using DPX. Light microscopy was carried out on an Olympus IX51 microscope (Japan).

2.6.3 Live cell imaging

Freshly-dissected and resuspended mouse mixed glial cells were plated onto glass bottom dishes (MatTek Corporation, MA, USA) and grown for 13 – 14 days, with 3 – 4 intermediate changes of media. Cells were assessed using a live cell imaging protocol enabling serial acquisition of confocal micrographs for 30 minutes. Phase contrast and fluorescent images were acquired following the addition of nanostructure solution to the glass bottom dishes. Confocal images were obtained using an Axioplan 2 (Zeiss, Germany).

2.6.4 Flow Cytometry Analysis

Cells were washed twice with ice-cold PBS following treatment and incubated at 37°C for 3 – 5 minutes in fresh PBS-containing trypsin (30 µl). DMEM (200 µl) was added and samples were centrifuged at 2000 rpm for 3 minutes. The pellet was resuspended in FACS buffer and transferred to FACS tubes. Measurements of the cell-associated fluorescence of non-treated, and nanostructure-treated, cells was completed on a DAKO CyAN_{ADP} flow cytometer calibrated using Flow-Check Fluorospheres (Beckman Coulter, Ireland).

2.6.5 Cytotoxicity

The cytotoxicity of the nanostructures was evaluated using the MTS assay (Cell Proliferation Assay, Promega, USA). Cells were incubated for 2, 24 and 48 hours in the presence of nanostructures (0.46×10^{-4} , 2.31×10^{-4} , 4.62×10^{-4} , 9.24×10^{-4} g/ml of Fe₃O₄). Cells were incubated for 3-4 hours in the presence of the MTS solution (20 µl of MTS solution per 100 µl of DMEM). Media (100 µl) was placed into the wells of a 96-well plate and the absorbance at 490 nm read by a spectrophotometer, 3 samples (100 µl) were taken from each well and the experiment was carried out in triplicate. Cell viability was assessed by calculating the ratio of the number of viable

cells in the nanostructure-treated culture to that in the non-treated culture. To investigate the cytotoxicity of the nanostructure following polyelectrolyte layer degradation, a volume of nanostructure was assessed for cytotoxicity 5 days after nanostructure fabrication. Vigorous vortexing was carried out to ensure the ratio of polyelectrolyte to magnetite added to cell cultures was comparable to that added when intact nanostructure was added. The cytotoxicity of degraded nanostructure solution (0.46×10^{-4} , 2.31×10^{-4} , 4.62×10^{-4} , 9.24×10^{-4} g/ml of Fe_3O_4) was assessed following incubation for 2, 24 and 48 hours with mouse mixed glial cultures.

2.6.6 Analysis of cytokine expression

Primary mouse mixed glial cultures were incubated with nanostructures (0.46×10^{-4} , 2.31×10^{-4} , 4.62×10^{-4} , 9.24×10^{-4} g/ml of Fe_3O_4) for 2 hours. Supernatant was collected and stored at -80°C until analysis. The concentrations of IL-1 β , IL-6 and TNF- α in the supernatant and was assessed by ELISA (R&D Systems, UK) as detailed in section 2.8.2.

2.6.7 MRI phantoms and measurements

A nanostructure-treated cell preparation was prepared and washed 3 times with PBS to ensure the removal of non-internalized nanostructures. Six cell phantoms were prepared in 2 ml cylindrical Eppendorf tubes, each containing 1 ml of 0.5 % warm agarose gel and 1 ml of cell suspension containing either 2.0×10^6 , 1×10^6 , 0.5×10^6 , 0.25×10^6 , 0.05×10^6 or 0.01×10^6 cells/ml. Homogenous distribution of the cells was ensured by 30 seconds of trituration.

MRI was performed in a 7T Bruker Spectrometer (Bruker Biospin, Germany) with a small volume resonator used in transceiver mode. T2 relaxation times were calculated from a MSME sequence (TR = 2000 ms, TE = 10 ms, 30 echoes with 10 ms echo spacing). T2* relaxation times were calculated from a Multi-Gradient-Echo (MGE) sequence (TR = 1500 ms, TE = 2.52 ms, 30 echoes with 5 ms echo spacing). Both sequences shared the following parameters: field of view = 2.56 cm, slice thickness =

2 mm, matrix = 156x156, pixel dimensions = 131 x 131 μm . The overall scanning time for both scans was 12 minutes. T2 and T2* relaxation times were calculated using the Image Sequence Analysis (ISA) software in the Bruker software, Topspin. The same ROI was used to generate values for T2 and T2* relaxation time calculation. T2 and T2* relaxation times were fitted to a mono-exponential decay function. The field inhomogeneity, ΔB , due to the presence of the nanostructures was calculated using $\Delta B\gamma = (1/T2^*) - (1/T2)$, where γ is the gyromagnetic ratio for hydrogen (42.58 MHz/T). ΔB was calculated for each of the phantoms.

2.6.8 *In vitro* BBB model nanostructure interaction

Confluent layers of BMEC were incubated in the presence of nanostructures, 4.62×10^{-4} g/mL of Fe_3O_4 . Nanostructures were also added to the apical compartment of transwell inserts without any BMEC cells. Samples from the basolateral compartment were taken 0, 40, 80 and 120 minutes after addition of nanostructures and the fluorescence was measured. A calibration curve of fluorescence from known concentrations of nanostructures in DMEM was used to convert the fluorescent signal measured from basolateral layer samples to nanostructure concentrations. To assess the effect a magnetic field would have on the passage of nanostructures, a separate 120 minute timepoint experiment was replicated in the presence of a magnetic field ($\sim 0.1\text{T}$). Triplicate data were obtained for each timepoint.

2.6.9 Nanostructure intravenous administration

Nanostructures were placed in an ultrasound bath for 25-30 minutes and loaded in a tinfoil-covered syringe using a 25G needle, air bubbles were removed and a maximum volume of 300 μl was injected into the tail vein as detailed in section 2.2.2.1. Mice were monitored for 5 – 7 minutes after injection to ensure bleeding had ceased.

2.6.10 DAB-enhanced Prussian blue staining of brain sections

Sections were thawed and air-dried for 1 hour and encircled using a PAP-pen (Dako Pen, Dako, Denmark) and left to dry for a further 20 minutes. Sections were fixed in 100% acetone for 10 minutes at -20°C and rehydrated in PBS for 5 minutes. Endogenous peroxidase was blocked with 1% H₂O₂ (Hydrogen Peroxide) for 15 minutes. The sections were washed with running tap water for 15 minutes followed by 3 washes in distilled water (5 minutes/wash). The sections were incubated with Prussian blue reagent for 45 minutes in a humidified chamber, washed for 15 minutes in running tap water and 3 times in distilled water (5 minutes/wash). Sections were incubated in diaminobenzidine (DAB) for 20 minutes (1:10 solution from DAB-liquid. (Sigma-Aldrich, UK)), washed 3 times with distilled water, dehydrated, cleared in xylene and coverslipped.

2.6.11 Rhodamine detachment experiments

A sample of DMEM-containing prepared nanostructures was incubated in the dark at 37°C for 48 hours. Samples were taken after 0, 8, 16, 24, 36 and 48 hours. Magnetic separation of any magnetite-bound nanomaterial from these samples ensured only detached rhodamine was assessed and its concentration was measured using a spectrofluorometer. After 48 hours, magnetically-separated magnetite-bound nanomaterial was re-dispersed and examined using a spectrofluorometer.

2.7 Metal and Iron analysis techniques

2.7.1 SQUID magnetometry

2.7.1.1 Tissue preparation

All equipment and surfaces used in this procedure were treated as outlined in Section 2.5.1.2. Following brain dissection as detailed in Section 2.5.1.3, the intact brain was

placed on a plastic chopping board. A sapphire blade (Electron Microscopy Services, USA) was used to cut along the longitudinal fissure. The right hemisphere was used for SQUID magnetometry while the left hemisphere was retained for molecular analysis and immuno- histochemical and fluorescence analysis. The right hemisphere was placed in plastic specimen vials (Sarstedt, Ireland) and maintained at -80°C until required. The hemispheres were weighed (Ohaus Pioneer, Ohaus, USA) and freeze-dried (Edwards, UK) for 2-3 hours, to remove water and concentrate the fraction of iron species within the tissue solids, and weighed again. The samples were transferred to a plastic chopping board. A sapphire blade was used to section the brain tissue. The sectioned-brain tissue was then placed into the centre of a SQUID sample straw (Quantum Design, CA, USA) and pressed into a pellet of diameter 5mm and height 2-4 mm using custom-made non-magnetic presses. Samples were frozen and maintained at -80° C until SQUID magnetometry measurements took place.

2.7.1.2 SQUID magnetometry experimental procedure

Smaller straws were placed into the sample straw from both ends to ensure the pellet remained intact throughout the experiment (Figure 2.5). Samples were mounted onto the end of the SQUID sample rod and kapton tape (Electron Microscopy Services, USA) was used to seal the bottom of the sample straw and ensure adequate adhesion to the SQUID sample rod. Kapton tape was used as it's adhesive characteristics remain stable at very low temperatures. The sample was inserted into the evacuation chamber of the MPMS 7 SQUID magnetometer (Quantum Design, CA, USA) and the chamber was air-evacuated. Several small holes had been made in the sample straw to ensure air was able to escape. Upon successful evacuation of the sample chamber, the sample rod was lowered into the measurement area. The temperature throughout this setup phase was 300 K. The sample space was air-evacuated and IRM was measured at 5 K and 150 K following a 5 minute exposure to a field of 10,000 Oersted, following a sample degaussing sequence carried out at 300 K. The sequence files were run on the MPMS MultiVu software (Quantum Design, CA, USA) and

controlled all aspects of the SQUID measurement device. A negative control sample containing no sample was prepared and used as the blank.

2.7.2 ICP-MS

2.7.2.1 Tissue preparation

Freeze-dried samples analysed using SQUID magnetometry were assessed for metal content using a Varian 820 ICP-MS (Varian, USA). A known weight of each sample was placed in separate digestion vessels and 7 ml of 69% HNO₃ and 1 ml of 35% H₂O₂ added. The vessels were sealed and placed in an Ethos EZ microwave digester (Milestone Scientific, USA) and heated to 200°C at 1000W for 20 minutes. Samples were brought to a volume of 50 ml with ultrapure water (Sigma-Aldrich). Certified calibration standards (Carl Stuart, Ireland) were used to calibrate the ICP-MS and a quality control standard run to ensure a tolerance of $\pm 10\%$. The samples were diluted where necessary to bring them into the range of the calibration curve. A blank containing only the reagents was also prepared and digested and used for blank subtraction purposes.

2.7.2.2 ICP-MS experimental procedure

ICP-MS measurements were acquired in peak-hopping mode with coarse spacing, 1 point per point, 15 scans per replicate and 3 non-consecutive replicates per samples. Plasma flow was 17.50 litre/minute with an auxiliary flow of 1.65 litre/minute. Each sample was introduced using a glass nebulizer at a flow of 0.97 litre/minute.

2.7.3 X-Ray analysis

2.7.3.1 Tissue preparation

All equipment and surfaces used in this procedure were treated as outlined in Section 2.5.1.2. Following brain dissection as detailed in Section 2.5.1.3, an intact brain from a female transgenic APP/PS1, male transgenic APP/PS1 and female wildtype mouse

was placed on a plastic chopping board. The brain was transferred into a 50 ml plastic specimen container (Sarstedt, Ireland) and stored at -80°C until required. Care was taken to ensure that only the brainstem portion of the brain was in contact with the plastic. The brain was cryosectioned using a sapphire cryostat blade (Electron Microscopy Services, USA). The cryostat (Leica CM1900, Leica Microsystems Inc, USA) was not used for 2 days prior to sectioning in an effort to minimise air-borne dust particles from previous work. The brains were placed in the cryostat and left for 20 minutes to equilibrate to the cryostat temperature, -20°C. The brains were adhered to cryostat chucks using water and orientated such that coronal sections could be taken. Anatomical position was assessed by light microscopy in 10 µm Toluidine Blue-stained sections by reference to a mouse brain atlas (Paxinos and Franklin, 1995). Upon identification of the hippocampus, 50 µm sections were taken. A single section, free from tears and physical deformities, as assessed by visual inspection, from each animal was placed onto a single high quality quartz microscope slide (75mm x 25mm x 1mm; H Baumbach & Co. Ltd., UK). Slides were placed into slide mail box, sealed with parafilm and stored at -80°C until required.

2.7.3.2 Synchrotron hutch setup

A layer of kapton (Electron Microscopy Services, USA) was used to cover the tissue sections on the quartz microscope slide during synchrotron measurements. Kapton was used as it displays high transmittance to X-rays. This protected the tissue sections from air-borne dust particle contamination. A PAP-pen (Dako Pen, Dako, Denmark) was used to trace the perimeter of the microscope slides, this trace was used to stick the kapton to the slide, due to the adhesive nature of the PAP liquid. TAAB epoxy resin (TAAB Ltd., UK) was used to glue the kapton to the microscope slide along the perimeter of the kapton. Kapton, PAP-pen and TAAB epoxy resin were all utilised due to the very low iron content and previous XRF work has indicated the trace metal content of these materials to be below the detectable limits of the Diamond Light Source. The microscope slide was inserted into a custom-built aluminium holder that attaches to the sample stage by 3 magnetic contacts ensuring accurate positioning and restriction of sample holder movement during stage movement.

2.7.3.3 XRF acquisition and analysis

High resolution XRF mapping was carried out on the I18 beamline at Diamond Light Source (www.diamond.ac.uk). Beamline control and data acquisition was controlled through the Generic Data Acquisition (GDA) software, developed at Diamond. Sections were screened for metal content by measuring XRF intensity above the K-edge for iron (7112 keV) at 10 keV. XRF maps were obtained by rastering the sample on an x-y stage capable of 1 μm positioning resolution. A 60 μm beam was used to obtain XRF maps of a hemisphere of the tissue section under inspection. Microresolution maps of smaller areas of interest were examined with a beam diameter of 5 μm . This was achieved by microfocussing with mirrors in the Kirkpatrick-Baez configuration (Figure 2.6a). X-ray fluorescence was detected using a high rate fluorescence 9 element solid state detector system. The detection system collected 1 second of fluorescence for both the 60 μm and 5 μm beam sizes during XRF map acquisition. The sample was placed at 45° to the oncoming beam and the detector was positioned perpendicular to the beam (Figure 2.6b). Off-site analysis was carried out in the PyMCA software program developed at the European Synchrotron Radiation Facility (ESRF) (Solé et al., 2007). Data was normalized to incident beamline energy and XRF maps created in PyMCA. XRF spectra were normalized to pixel number in PyMCA, peak analysis was carried out using the OriginLab statistical package (OriginLab Corporation, MA, USA).

2.7.3.4 XANES acquisition and analysis

Areas exhibiting high iron fluorescence were chosen for XANES analysis of iron valence. XRF spectra from chosen areas were inspected to confirm absence of non-biogenic metals such as nickel or chromium. XANES was obtained at the K-edge over the energy range of 6960 to 7450 keV. Steps of 5.5 keV were taken from 6960 to 7090 keV, steps of 0.5 keV were taken from 7090 to 7145 and steps increasing

from 1.0 to 3.0 keV were taken from 7145 to 7450 keV. XANES data was analysed using the Athena software package and was compared to XANES spectra of standards using linear combination fit analysis, as provided by Athena (Ravel and Newville, 2005).

2.8 Molecular analysis techniques

2.8.1 Analysis of soluble and insoluble A β concentration

2.8.1.1 Extraction of insoluble and soluble A β

Soluble and insoluble A β were extracted from snap-frozen brain tissue dissected from a single hemisphere of wildtype and APP/PS1 transgenic mice, following intracardial perfusion (Section 2.5.1.3). The tissue was homogenised using a polytron homogeniser (Kinematica, Switzerland) in 5 volumes (w/v) of homogenising buffer. The samples were centrifuged at 15,000 rpm for 40 minutes at 4°C. The supernatant was used to extract SDS-soluble A β and the pellets for isolation of insoluble A β . Protein concentrations in the supernatants were equalised to 1.5 mg/ml with homogenisation buffer, following assessment of protein concentration using the BCA protein assay (see section 2.5.1.5). Equalized supernatants were neutralized by the addition of 10% (v/v) 0.5 M Tris-HCl (pH 6.8) and stored at -20°C until assessed for soluble A β concentrations. Pellets were disrupted by ultrasonication at 23 kHz for 2 x 5 minutes in guanidine buffer and incubated on ice for 4 hours. Following 30 minutes of centrifugation at 15,000 rpm, samples were equalized to 0.4 mg/ml with guanidine buffer, aliquoted and stored at -20°C until assessed for insoluble A β concentrations.

2.8.1.2 Detection of SDS-soluble and insoluble A β by multi-spot ELISA

Concentrations of A β were determined using “MSD 96-well multi-spot 4G8 A β triple ultra-sensitive assay” kits (MesoScale Discovery, USA). Multi-spot A β 3-plex plate

wells were blocked in 150 μ l of 1% Blocker A for 1 hour at room temperature. Plates were washed 3 times with 1X Tris wash buffer (TWB) supplied with the plate. The detection antibody was added to each well (25 μ l per well). SDS-solubilised samples were diluted to 200 μ g/ml in MSD kit buffer and 25 μ l of sample was added in duplicate to the appropriate wells of the 96-well plate. Guanidine samples were diluted to 0.4 μ g/ml in TWB and 25 μ l of sample was added in duplicate to the appropriate wells. Serially diluted recombinant human A β ₃₈ (0 – 3,000 pg/ml), A β ₄₀ (0 – 10,000 pg/ml) and A β ₄₂ (0 – 3,000 pg/ml) in 1% Blocker A solution were added to the appropriate wells. The plate was incubated at room temperature for 2 hours, washed in TWB and 150 μ l of 2X MSD read buffer was added to each well. The plate was read using a Sector Imager plate reader and A β samples were evaluated with reference to the standard curve prepared using the recombinant A β ₃₈, A β ₄₀ and A β ₄₂.

2.8.2 ELISA

2.8.2.1 Determination of cytokine concentrations by ELISA

Tissue, flash frozen in Krebs was homogenized using a polytron homogenizer (Kinematica, Switzerland) and equalized using Krebs following a BCA protein assay (section 2.5.1.5). Antibody-coated plates (goat anti-mouse antibodies at a concentration 1 μ g/ml in PBS) were incubated overnight at room temperature. The plates were washed 3 times with ELISA wash buffer and incubated with blocking buffer (0.5% BSA in PBS) for 1 hour at room temperature. Plates were washed 3 times with wash buffer and triplicate standards (0-2000 pg/ml for IL-1 β , 0-4000 pg/ml for TNF α , 0-4000 pg/ml for IL-6) or samples (100 μ l) were added to wells and incubated for 2 hours. The wells were washed 3 times, detection antibody (350 ng/ml biotinylated goat anti-mouse antibody for IL-1 β , 50 ng/ml biotinylated goat anti-mouse antibody for IL-6 and TNF α ; diluted in PBS containing 1% BSA and 2% normal goat serum) was added and incubation continued for 2 hours at room temperature. The wells were washed and incubated in horseradish peroxidase-conjugated streptavidin (1:200 dilution in PBS containing 1% BSA) for 20 minutes at room temperature in the dark, washed again and substrate solution (R&D Systems, UK; 1:1 ratio) was added and samples were incubated in the dark at room

temperature for 30 minutes. The reaction was stopped using 1M H₂SO₄ and absorbance was read at 450 nm. IL-1 β , TNF α and IL-6 concentrations were measured in pg/ml.

2.8.3 Immunofluorescence staining for A β and MHCII

Sections prepared from the brains of wildtype and APP/PS1 transgenic mice were thawed and air-dried for one hour and encircled using a PAP-pen (Dako Pen, Dako, Denmark) and left to dry for 20 minutes. Sections were fixed in ice-cold methanol for 10 minutes and washed 3 times in PHEM (1 minute/wash). A 10 minute wash in 0.1% Triton (Sigma-Aldrich, UK) in PHEM to permeabilize the sections was followed by 3 washes in PHEM (1 minute/wash). Non-specific binding was blocked by incubating samples in 4% BSA containing 10% normal goat serum (NGS) for 2 hours at room temperature. This blocking solution was tipped off the sections after two hours and the primary antibody applied (1:1000, Pan-Amyloid antibody, Calbiochem, USA) in 2% BSA containing 5% NGS. The primary antibody was left overnight at 4°C. The following day sections were washed 3 times in PHEM (1 minute/wash) and the secondary (ALEXA 488, Invitrogen, USA) was applied (1:4000 in 2% BSA containing 5% NGS for 90 minutes at room temperature. The sections were washed every 6 minutes for 90 minutes and mounted mounted with DAPI-containing mounting medium (Vectashield, Vector Labs, USA) and left to dry for 24 hours before analysis using confocal microscopy (Axioplan 2, Zeiss, Germany). In instances of double-staining, A β stained sections were washed 6 times with PHEM (5 minutes/wash) and the staining process repeated with a primary antibody for MHC II used (1:1000, MHC II antibody, Calbiochem, USA). Following the repeated application of the steps outlined above, the sections were washed every 6 minutes for 90 minutes with PHEM, and mounted mounted with DAPI-containing mounting medium (Vectashield, Vector Labs, USA) and left to dry for 24 hours before analysis using confocal microscopy (Axioplan 2, Zeiss, Germany).

2.8.4 Immunohistochemistry

A congo red staining protocol was used to visualize amyloid plaques (Wilcock et al., 2006). A saturated NaCl solution was prepared the day before staining took place. On the day of staining, sections were thawed and air-dried for 30 minutes. Sections were fixed in ice-cold ethanol for 10 minutes and washed twice with PBS. During these PBS washes, 2 ml of NaOH solution was added to the saturated NaCl solution (200 ml) to produce an alkaline solution. Sections were incubated in this solution for 20 minutes at room temperature. NaOH solution (2 ml) was added to filtered congo red solution (200 ml) to produce an alkaline solution and the sections were incubated in this freshly-prepared solution for 30 minutes at room temperature. Sections were rinsed in ddH₂O for 30 seconds and counterstained for 1 minute in a 1% (w/v) methyl-green solution (Sigma-Aldrich, UK). Excess staining solution was removed by washing in ddH₂O for 1 minute and sections were dehydrated through an ethanol series. Sections were air-dried and placed in 3 separate xylene baths for 5 minutes each. Coverslips were placed onto DPX (Electron Microscopy Services, USA) covered sections and left to dry in a fume hood for 24 hours.

In instances of double staining, congo red stained sections, prior to dehydration steps, were washed 3 times with ddH₂O (5 minutes/wash) and subsequent immunohistochemical study of CD68 expression was performed using the avidin–biotin–peroxidase complex reaction in Tris-buffered saline (TBS, pH 7.5). Non-specific interactions were blocked by incubation in TBS (4% bovine serum albumin, BSA; Sigma, UK, 10% normal goat serum; Vector, UK), for 30min at RT. Sections were incubated overnight in rabbit anti-CD68 antibody solution (1/200 Santa Cruz Biotechnology, US; in 2% BSA in TBS). Negative controls were incubated in 2% BSA in TBS alone. Sections were rinsed 3 times in TBS, and incubated in biotin-conjugated goat anti-rabbit secondary antibody, (Vector, UK; 1/200 in 2% BSA in TBS) for 2 hours at RT. Sections were rinsed 3 times in TBS. Endogenous peroxidases were blocked by incubating the sections in 0.3% hydrogen peroxide in TBS for 15 minutes, and rinsing 3 times in TBS. Sections were incubated in a pre-made avidin solution, and rinsed 3 times in TBS. Diaminobenzidine (DAB solution;

Vector, UK, 0.06% H₂O₂) was applied to the sections and incubated for 10 minutes. The colour development was monitored and stopped with distilled water. The sections were dehydrated through a series of graded alcohols (75%;95%;100%; Sigma, UK), and cleared by immersion in xylene (Sigma, UK). Coverslips were applied using DPX (RA Lamb, UK) as the mount, and slides were left overnight to set in a fume hood.

2.8.4.1 CD68 expression quantification

Digital micrographs of brain sections double stained to visualize amyloid plaques and CD68 expression were taken under the same light and magnification. CD68 expression was calculated as the reciprocal of the RGB image pixel values. To examine CD68 expression levels proximal to amyloid plaques, an area around the plaque, but excluding the plaque itself, was compared with CD68 expression determined from other areas of the brain section.

2.8.5 PCR

2.8.5.1 Isolation of RNA from brain tissue

Messenger RNA (mRNA) was extracted from snap-frozen CNS tissue stored at -80°C in RNAlater (Ambion Inc., Applied Biosystems, UK). Tissue was washed 3 times in Krebs buffer and homogenised, using a polytron homogeniser (Kinematica, Switzerland), in 350 µl of cell lysis buffer (NucleoSpin RNA II, Macherey-Nagel, Germany) for RNA extraction. Lysate was added to the membranes of NucleoSpin Filters and filtered by centrifugation at 11,000 g for 1 minute. Filters were discarded and 350 µl of 70% ethanol was added to the filtrate. The filtrate was mixed and loaded onto the membrane of a NucleoSpin RNA II column and centrifuged at 8,000 g for 30 seconds allowing the RNA to bind to the membrane. The silica membrane was desalted by adding 350 µl of membrane desalting buffer to the membrane and samples were centrifuged at 11,000 g for 1 minute to dry the membrane.

DNA was digested by adding 95 µl of DNase reaction mixture directly onto the centre of the NucleoSpin RNA II column and incubating the tubes at room temperature for 15 minutes. The DNase was inactivated by washing samples in 200 µl of RA2 buffer and tubes were centrifuged at 11,000 g for 30 seconds to dry the membrane. The silica membrane was washed with 600 µl RA3 buffer and centrifuged for 30 seconds. A further wash with 250 µl of RA3 buffer was carried out. The membranes were dried by centrifugation at 11,000 g for 2 minutes. Filters were placed in nuclease-free collecting tubes and 40 µl of RNase free H₂O was added to the membrane. RNA was eluted by centrifuging samples at 11,000 g for 1 minute and RNA concentration was quantified using a NanoDrop Spectrophotometer (ND-1000 v3.5, NanoDrop Technologies Inc., USA).

2.8.5.2 Reverse transcription for cDNA synthesis

Total mRNA was reverse-transcribed into complementary DNA (cDNA) using a high-capacity cDNA archive kit (Applied Biosystems, Germany) according to the manufacturer's protocol. RNA (0.5 µg) was added to fresh tubes and brought to a final volume of 10 µl using nuclease-free H₂O. Mastermix was prepared using 10X RT Buffer, 25X dNTPs, and 10X random primer multiscribe reverse transcriptase (50 U/ µl). The mastermix (10 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 (PTC-200, Peltier Thermal Cycler, MJ Research, Biosciences, Ireland).

2.8.5.3 Real time PCR

Real-time PCR dye-labelled primers were delivered as Taqman Gene Expression Assays for the mouse genes listed in Table 2.3. cDNA was diluted 1:5 with RNase free H₂O and 4.5 µl of sample was added to each well. This was mixed with 4.5 µl of TaqMan Universal PCR Fast Mastermix (Applied Biosystems, Germany) and 0.5 µl each of the target primer and the endogenous control primer, for a total volume of 10 µl per well. Human 18S RNA (Applied Biosystems, Germany) was used as the

endogenous control. Real-time PCR was performed using Applied Biosystems ABI Prism 7300 Fast Track Sequence Detection System v1.4.1 in 96-well format with a 10 µl reaction volume per well. Each sample was measured in duplicate in a single real-time PCR run. Samples were initially brought to 95°C for 20 seconds before being run for 40 cycles of 95°C for 3 seconds followed by 60°C for 30 seconds.

2.8.5.4 *PCR quantification*

The expression of each target gene was determined using the efficiency-corrected comparative method. Target genes in different samples were compared to a reference gene (18S RNA). These values were normalised to the control sample and the relative differences in expression of the target gene between the samples were expressed as a ratio.

Gene Name	Gene Description	TaqMan Gene Expression Assay Number
CD11b	Integrin alpha M	Mm01271263_m1
GFAP	Glial Fibrillary Acidic Protein	Mm01253033_m1
CD68	CD68	Mm03047343_m1
IL-1β	Interleukin-1 beta	Mm00434288_m1

Table 2.3 Gene expression assay numbers of PCR primers

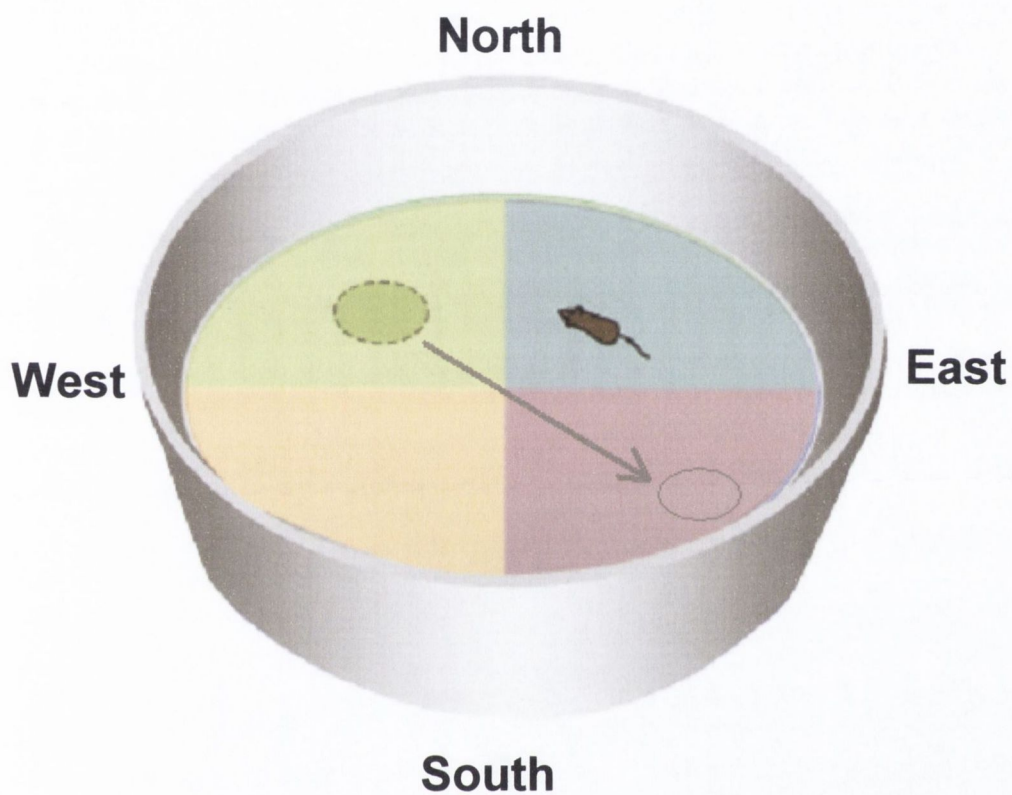


Figure 2.1. Schematic illustration of Morris water maze apparatus.

Each quadrant in the tank is denoted with a colour and trials start from each quadrant. The repositioning of the platform from the northwest to southeast quadrant for the reversal phase of the task, is illustrated by the grey arrow.

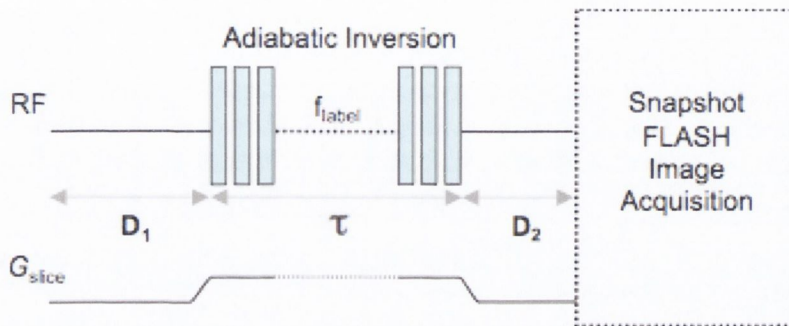
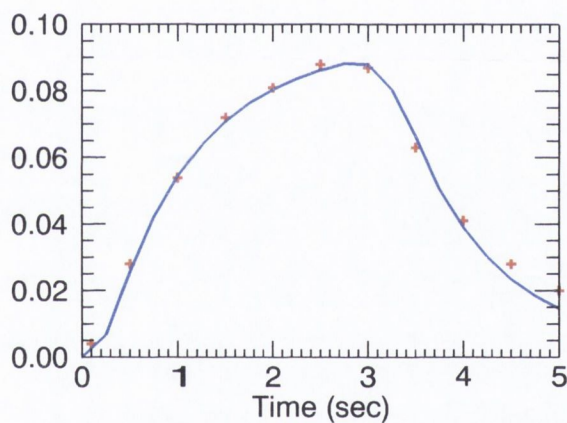
a**b**

Figure 2.2. ASL pulse sequence schematic and representative fit of the non-compartmental model of cerebral perfusion.

Bolus-tracking ASL sequence schematic (a) and a representative fit of the non-compartmental model of cerebral perfusion (b) are presented. The preparation interval containing the inversion pulse of duration τ and the variable delays D_1 and D_2 was followed by snapshot FLASH image acquisition. A representative least squares fit of the bolus-tracking ASL data to the non-compartmental model of cerebral perfusion is presented (b). The model is represented by the blue solid line and the experimental data by the red asterisks.

Adapted with permission from Kelly and colleagues, 2009.

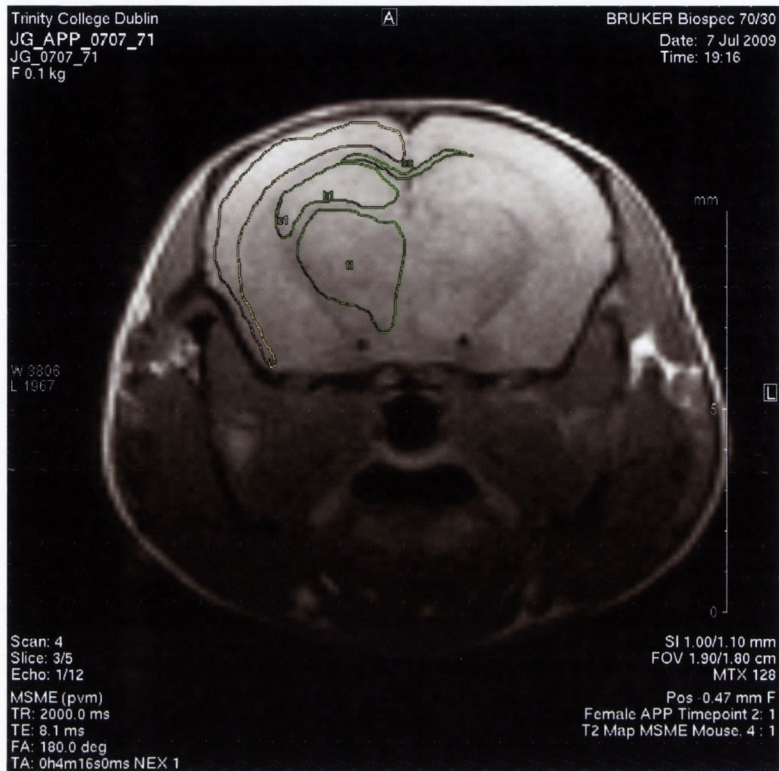


Figure 2.3. Representative ROIs drawn on a T2-weighted image.

ROIs were drawn for the cortex, hippocampus, thalamus and corpus callosum. Separate ROIs were drawn on each hemisphere in all cases except the corpus callosum.

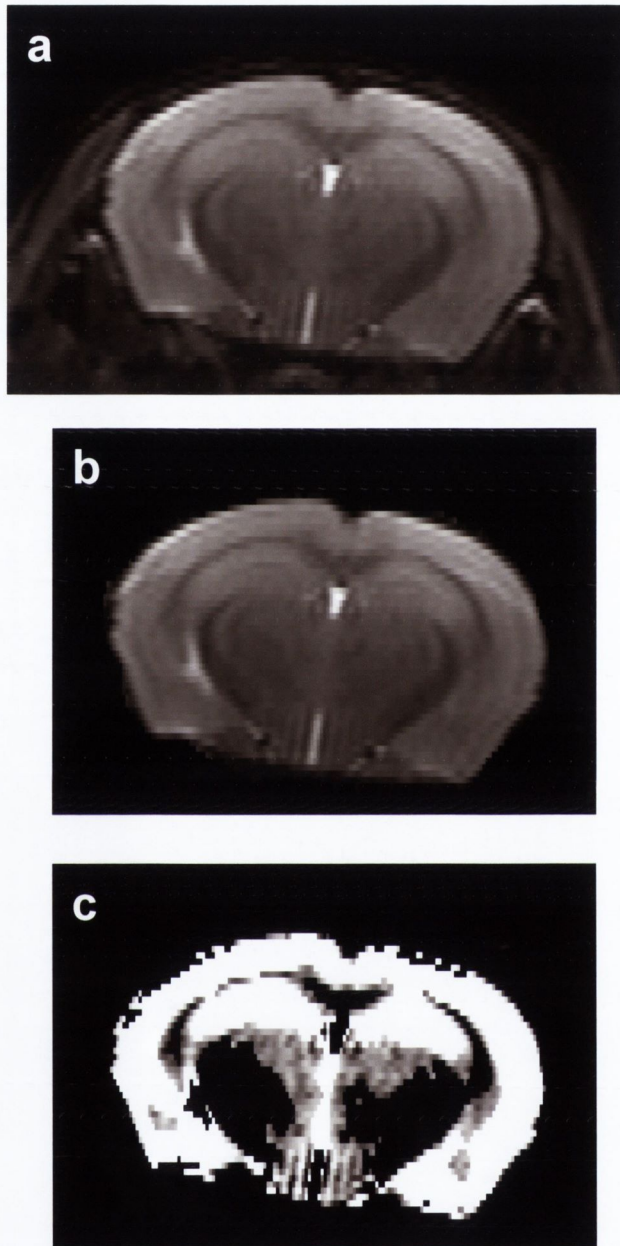


Figure 2.4. Brain extraction and gray matter segmentation.

A representative T2-weighted high resolution image (a) and associated brain extraction (b) and gray matter segmentation (c) are presented

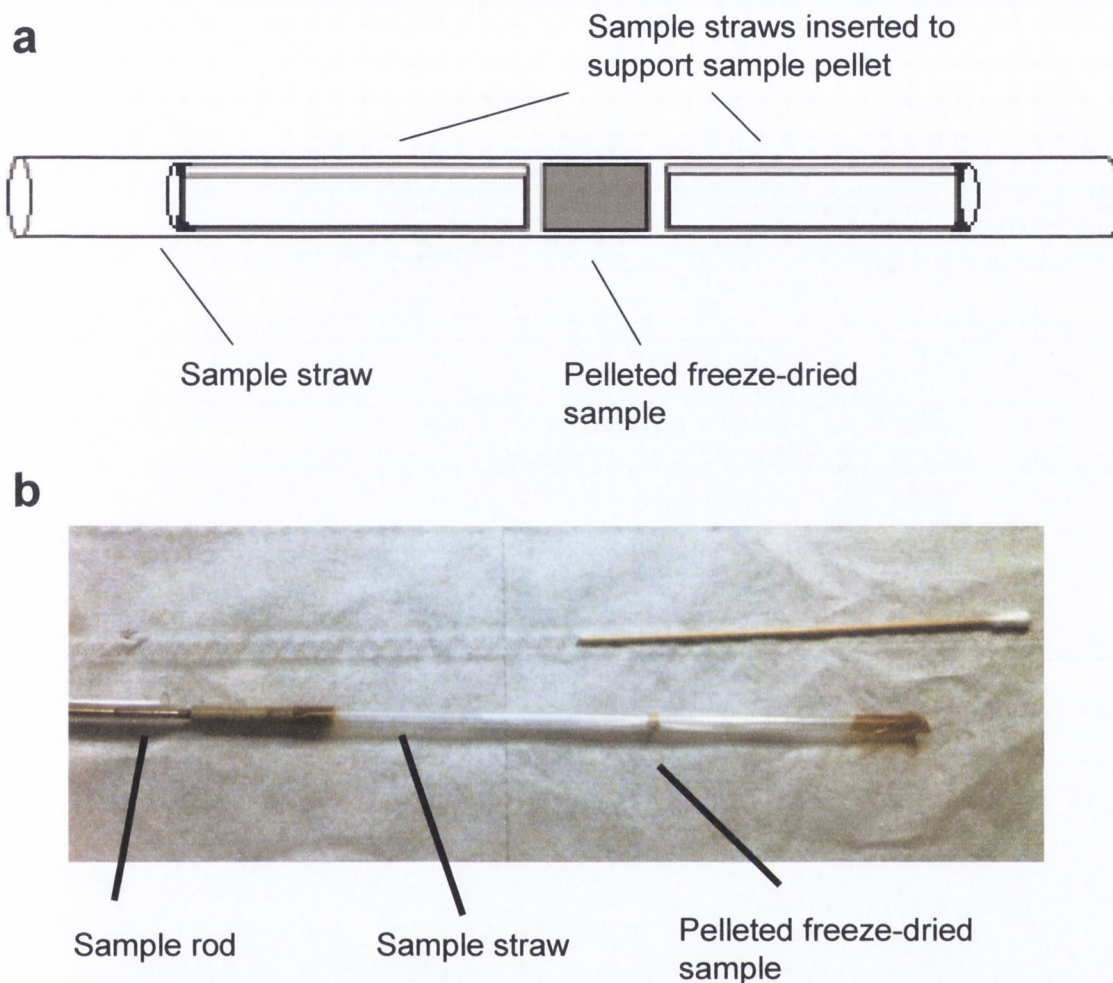


Figure 2.5. Schematic illustration and photograph of SQUID magnetometry sample setup.

The sample is pressed into a pellet using non-magnetic presses. This pellet is supported from both sides by cut samples straws (a) inserted until they are touching the sample. One end of the sample straw is then taped with kapton and the other attached to the SQUID sample rod as shown in (b).

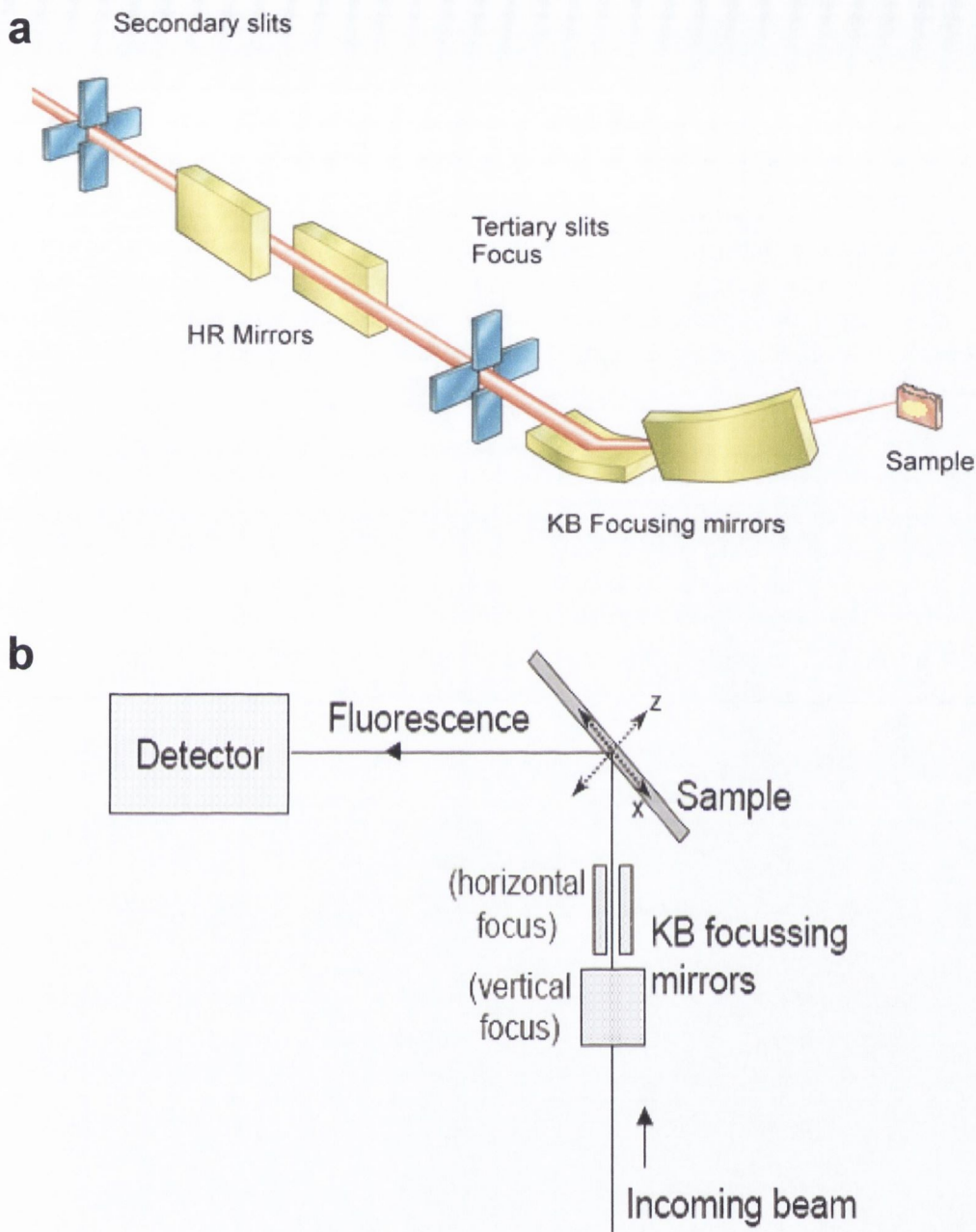


Figure 2.6. Schematic of I18 beamline and sample placement.

A schematic illustration (a) of the technical hardware required to modulate the main beamline energy, control the beam cross-sectional area and position relative to the sample (Adapted with permission from Diamond Light Source website, www.diamond.ac.uk). A schematic of the sample placement relative to the beam (b) shows the 45° placement of the sample relative to the beam and the detector position, perpendicular to the beam (adapted with permission from Collingwood et al, 2005).

Chapter 3

Results

3.1 Introduction

The ability to track cells using multiple contrast-dependant imaging modalities is an attractive technique, especially in the neurosciences where cell migration from the PNS to the CNS is considered to be an important event in many CNS disorders. In this context, a bi-modal contrast agent, capable of generating T2-weighted MRI contrast *in vivo* and confocal microscopy contrast *ex vivo* would be extremely useful. Similarly, the delivery of therapeutic agents to the CNS is an important step in developing treatments of CNS disorders. Much of the focus in the field has been on developing nanotechnology-driven drug delivery platforms, which display efficacy in entering the CNS, through interaction with the endothelial cells of the BBB (Kreuter, 2004). However, little is known about the potentially deleterious effects the nano-sized constituents of these drug delivery systems may have on CNS tissue cells. Inflammation, a response largely controlled by microglia cells, is a hallmark of many neuropathologic disorders and appears to contribute to many of the deficits associated with these disorders. It is important therefore that the interaction of nanostructures with glial cells is investigated.

Thus the central aim in this study was to assess the interaction of a nanostructure, designed with consideration to CNS drug delivery and bimodal contrast generation in cell tracking experiments, with primary mixed glial cell cultures, while validating the potential of this nanostructure to be used as a bimodal contrast agent. The interaction of this nanostructure with BBB endothelial cells was assessed *in vitro* and *in vivo* in a cell tracking experiment using EAE as the mouse model.

3.2 Results

Basic characterization of nanostructures and nanostructure behaviour in a magnetic field.

A fabrication schematic illustrating the nanostructure is presented (Figure 3.1a). The emission and excitation spectra of the prepared nanostructure are comparable to a rhodamine standard and confirm that the fabrication method prevents paramagnetic quenching of the fluorescent dye (Figure 3.1b, 3.1c). Analysis of TEM images of prepared nanostructures indicated an average size of 232 nm (Figure 3.1d). The data shown in Figures 3.2 – 3.4 demonstrate the effect of a magnetic field on the nanostructures. The nanostructures appear aggregated in a linear necklace- or chain-like structure following placement in a magnetic field. Data presented in Figure 3.2 highlight the bimodal nature of the nanostructure with visualization enabled by confocal microscopy and control exerted over the nanostructure by magnetic stimulation of the iron oxide core.

Cellular interaction of nanostructures.

Mixed glia were incubated in the presence of nanostructures and confocal microscopy, and light microscopy staining for iron, confirmed the cellular internalization of the nanostructures (Figures 3.5 – 3.8). Importantly, the contrast-generating agents, rhodamine and iron, show simultaneous cellular internalization. Data presented in Figures 3.6 and 3.8 indicate that increasing the concentration of nanostructures increases the cellular uptake. Cell nuclei are clearly delineated through the absence of fluorescence and the z-sectioning data presented provides clear visualization of the nuclei in different planes and at different cross-sectional depths through the cell body (Figure 3.9).

Nanostructure internalization method.

These data confirmed cellular internalization (Figures 3.5 – 3.8) however some level of membrane localization of nanostructure was evident (Figure 3.10). Membrane localization may be a stage in the process of endocytosis, which astrocytes, or perhaps microglia use to internalize the nanostructure but it could also reflect the phagocytic activity of microglial cells. In order to investigate the method of internalization, live cell imaging and flow cytometric analysis of nanostructure-treated mixed glial cells was performed.

The data presented in Figure 3.11 provides evidence that glia phagocytose nanostructures. Cellular processes from cell A can be seen to phagocytose nanostructures for cellular internalization. These data also provide support for endocytosis as an internalization method with evidence of partial movement of a nanostructure across the cellular membrane of cell B.

Data from flow cytometric analysis are presented in Figure 3.12. The mean peak fluorescence increased from 1.34 in non-treated cells (R3), to 7.84 in nanostructure-treated cells (R4). This correlates with an increase in the fluorescence of cells associated with the nanostructure to 83%. Approximately, 30% of a mixed glial cell population are microglia, capable of phagocytosing the nanostructure. However, a greater percentage of the nanostructure-treated cell sample is associated with nanostructure fluorescence suggesting endocytosis of the nanostructure by astrocytes, which comprise approximately 70% of the cells.

Nanostructure cytotoxicity and cytokine profile alteration.

The data presented suggest both endocytosis and phagocytosis are involved in nanostructure internalization. Both of these processes terminate with lysosomal degradation of the material internalized into the cells. In the case of the nanostructures this would involve degradation into constituent parts; rhodamine, polyelectrolyte material and magnetite. It is conceivable that this material may be exocytosed by cells in degraded form. Thus the cytotoxicity of intact and degraded nanostructures was assessed, with both forms displaying no effect on cell viability

following incubation at different concentrations (0.462×10^{-4} , 2.31×10^{-4} , 4.62×10^{-4} , 9.34×10^{-4} g/ml Fe_3O_4) for 2, 24 and 48 hours (Figure 3.13).

Although cell viability was unaffected the alteration in cytokine profile of mixed glial cells incubated in the presence of nanostructures (0.462×10^{-4} , 2.31×10^{-4} , 4.62×10^{-4} , 9.34×10^{-4} g/ml Fe_3O_4) was assessed (Figure 3.14)

LPS (10 ng/ml) induced significant increases in the supernatant concentration of TNF- α and IL-6 ($p < 0.001$, ANOVA) but no changes in IL-1 β concentration compared with all other treatments. Any changes induced by the nanostructures were negligible in comparison. The only exception was the modest, though statistically significant, ($p < 0.001$; ANOVA) increase induced by 9.34×10^{-4} g/ml Fe_3O_4 on TNF- α concentration compared with nanostructure treatments of lower concentrations.

Nanostructure MRI contrast generation.

The MR data presented in Figure 3.15 confirm the potential of this nanostructure to act as a MRI contrast agent. Alterations in the T2 relaxation time of agarose phantoms can be determined visually from Figure 3.15a, while Figure 3.15b presents the changes in T2 relaxation time in a parametric colour map. The field inhomogeneity of MR phantoms versus the number of nanostructure-labelled cells in each phantom is presented and is linear ($p < 0.001$; $R^2 = 0.9967$; Pearson correlation). The offset from the origin is due to the inhomogeneity present in the main static field.

Magnetic stimulation of nanostructures incubated with an in vitro model of the BBB.

Previous experiments confirm the bimodal contrast generation potential of the nanostructure. In order to assess the potential interaction nanostructures may have *in vivo*, assessment of nanostructure behaviour in an *in vitro* model of the BBB was assessed (Figure 3.16).

These data show that in the absence of a magnetic field nanostructures did not cross the endothelial cell layer over the 120 minute duration of the experiment. In the absence of the endothelial cell layer (diff control), nanostructure concentration in the basolateral compartment increases in a time-related manner to reach a plateau at 60 minutes. Application of a magnetic field significantly increased the ability of nanostructures to cross the endothelial cell layer from 0.0145 ± 0.007 g/ml Fe_3O_4 , in the absence of the magnetic field, to 0.0355 ± 0.0067 g/ml Fe_3O_4 in its presence ($p < 0.05$; Student's t-test for independent means; Figure 3.16b).

In vivo assessment of nanostructures.

A mouse model of multiple sclerosis (MS), experimental autoimmune encephalitis (EAE), was used to model cell infiltration from the periphery to the CNS. MOG-treated mice show clinical signs of EAE 10 days after treatment and this is associated with the presence of macrophages in brain tissue. It was hypothesized that intravenous injection of the nanostructure into mice at this time would result in the phagocytosis of the nanostructures by macrophages, enabling their subsequent bimodal visualization. Two experimental groups were assessed; sham-treated control mice, and nanostructure-treated mice 10 days after MOG injection. The concentration of nanostructure administered, 90 μM Fe/kg mouse body weight, was less than that reported in the literature (Baeten et al., 2008). This concentration was the maximum achievable as the fabrication of higher concentrations of the nanostructure resulted in precipitation of the nanostructure out of solution and the formation of aggregates.

Data presented in Figure 3.17 confirm the presence of nanostructure-associated fluorescence in the brain of mice with EAE. The nanostructure-associated fluorescence displays no spatial patterning that would suggest EAE lesion-specific aggregation or aggregation in the microvasculature. The absence of MRI contrast generation (Figure 3.18) may be due to the low levels of nanostructure administered. Confirmation using light microscopy of the presence of negligible deposits of exogenous iron, suggests the detachment of rhodamine molecules from the nanostructure.

To further assess this, experiments were carried out to investigate the putative detachment of rhodamine from the nanostructure-surface. Incubation of nanostructures in DMEM at 37°C, led to detachment of rhodamine after 8 hours which continued until the majority of rhodamine was detached after 48 hours (Figure 3.20).

In vivo assessment of non-fluorescent magnetic-nanostructures.

The detachment of rhodamine from the nanostructure suggests that *in vivo* application of the nanostructure as a bimodal contrast agent is not useful. In addition, the low concentration of the final nanostructure solution is a further limitation to effective *in vivo* usage. In order to assess the potential of the nanostructure, without rhodamine, as an MRI contrast agent, the fabrication was altered such that a concentration of 300 µM Fe/Kg mouse body weight could be achieved.

This preparation was then injected intravenously into control mice and mice which had received MOG 10 days previously. It was hypothesized that intravenous injection of the nanostructure into mice with EAE, in which macrophage infiltration into the brain tissue has been confirmed, would enable visualization of nanostructure-labelled macrophages in brain tissue.

The data presented indicate that the nanostructure did not label or track a specific cell population in either control or MOG-treated mice since T2-signal voids were observed in both treatment groups (Figure 3.21). The position and extent of these voids varied from slice to slice within animals, between animals of the same experimental group and between animals of different experimental groups. These data suggest the nanostructure does not specifically label infiltrating macrophages in EAE mice, but may have some interaction with the endothelial cells of the BBB.

DAB-enhanced Prussian blue staining of brain sections prepared from control- and MOG-treated mice identified iron deposits throughout brain tissue. No DAB-

enhanced Prussian blue deposits were identified in brain sections prepared from mice from the sham-injected group. Representative micrographs are presented in Figure 3.22. These data provide further evidence that nanostructures do not specifically label infiltrating macrophages.

3.3 Discussion

The aim of this study was to investigate the *in vitro* interaction of a nanostructure, designed with consideration to CNS drug delivery and bi-modal cell tracking experiments, with primary mixed glial cells. The data show the internalization of the nanostructures by primary mixed glial cells with no cytotoxic effects and no ability to modulate cytokine concentration. These data confirm the potential of this nanostructure for biomedical applications using the combined presence of magnetism, enabling MRI contrast generation, and fluorescence, enabling confocal microscopy and flow cytometric analysis.

The internalization of the nanostructure by astrocytes and microglia confirms the potential use of these nanostructures in drug delivery or cell tracking experiments. These data are supported by work indicating the *in vivo* internalization of similarly fluorescent nanoparticles by microglia in a mouse model of glioblastoma (Trehin et al., 2006). The nanostructures used in this study were capable of inducing phagocytosis in microglia that did not result in the production of proinflammatory cytokines. This is in direct contrast to work indicating nanoparticles of titanium dioxide induce the production of reactive oxygen species in microglia cells, as well as apoptosis (Long et al., 2006, Long et al., 2007). One possible explanation for the difference in findings is that in the present study the nanostructures contained a coating of polyelectrolyte layers and it might be suggested that this coating does not result in microglial activation. Data from the present study indicates the nanostructure is not cytotoxic to mixed glial cells. Experiments investigating cytokine concentrations levels after a 2 hour incubation with the nanostructures indicated there was no significant alteration. However, an increase in TNF- α was seen in mixed glia at the highest concentration of nanostructures, the level of cytokine produced was

minimal when compared with LPS-induced TNF- α production. These data highlight the potential of this nanostructure as a potential drug delivery agent by replacement of the iron-oxide core with a therapeutic compound.

The astrocytic internalization of the nanostructures in this study is comparable to a report indicating astrocyte internalization of zinc-sulphide based nanoparticles (Behrendt et al., 2009). The data indicated that zinc-sulphide based nanoparticles had a deleterious effect contrasting with the well-tolerated nature of the nanostructures used in the present study. Work investigating astrocyte uptake of magnetic fluorescent nanoparticles in a mouse model of glioblastoma reported poor uptake by astrocytes (Trehin et al., 2006). The nanostructure used in the present study is prepared such that the final PSS coating is positively-charged. This encourages adhesion to negatively-charged cell membranes and promotes endocytosis in non-phagocytic cells.

Confocal microscopy carried out in this study indicated nanostructures were confined to the cytosol and were not present in the cell nucleus. This finding concurs with studies which investigated the subcellular distribution of nanoparticles of different sizes. The nanostructures used here were approximately 100 nm in size and it has been shown that similar magnetic fluorescent particles of this size are confined to the cytosol in osteoblasts (Corr et al., 2006). TEM and confocal microscopy show that the nanostructures adopt a linear chain-like assembly, following placement in a magnetic field, and this supports reports of similar behaviour of nanostructures fabricated using similar material (Corr et al., 2008). This behaviour may be useful in developing techniques to disassociate cells labelled with nanoparticles displaying this tendency and nanoparticles that do not.

Recent studies have reported the application of positive-contrast techniques to generate bright T2-weighted contrast from magnetic nanoparticles (Cunningham et al., 2005, Zhang et al., 2009c). One of these techniques relies on an off-resonance saturation method that measures only the NMR signal of protons proximal to the

nanoparticles. The super paramagnetic behaviour of nanoparticles, used in MRI contrast experiments, results in an increase in the magnetic field close to the nanoparticles. The protons resident in the increased magnetic field can be selectively perturbed with off-resonance RF saturation (Cunningham et al., 2005). The extent of the magnetic field generated by the nanoparticles, and thus the contrast generated using positive contrast MRI, is dependant on the size, concentration and geometry of the nanoparticles. Similar concentrations of nanoparticles exhibiting different geometrical layouts, due to alignment with the magnetic field, would generate different-sized and shaped positive contrast T2 hyperintensities. The linear contrast generation of the nanostructure is similar to data presented indicating USPIO labelled cells MRI contrast alterations may be used to approximate labelled cell numbers (Cheung et al., 2006).

Evidence indicates that the application of magnetic field increases transcytosis of the nanostructures across an *in vitro* model of the BBB. This suggests some control on the BBB's interaction with magnetic nanoparticles can be exerted. The presence of the fluorescent component, of the nanostructure, in the absence of confirmation of the magnetic agent in the basal layer is a limitation of this study. It does not however, negate the possibility of increased endothelial cell internalization of the nanostructures, and subsequent transcytosis of the fluorescent agent only. Increased contact between the nanostructure and the endothelial cell may occur due to the application of the magnetic field. The nanostructures solution is colloidal and although precipitation is possible, it is unlikely to occur within 2 hours.

Experiments investigating the detachment of rhodamine from the surface of the nanostructure indicated this occurred within 48 hours. Experiments investigating the potential of the nanostructure to endogenously label macrophages, through phagocytosis in the bloodstream, were carried out in EAE mice 10 days post immunisation. The data indicate minimal nanostructure presence was found in the CNS of the EAE mice, even though macrophage infiltration of the CNS is ongoing at this time (Stromnes and Goverman, 2006). This suggests rapid clearance of the nanostructure from the bloodstream. The concentration of nanostructure administered

in these experiments was low in comparison with the literature (Baeten et al., 2008). The BBB of EAE mice 10 days post immunisation has been reported to be compromised and one might expect some level of passive migration of nanostructures into the CNS tissue due to this, however, one explanation for their absence could be rapid clearance from the bloodstream. The presence of fluorescence in CNS tissue, in the absence of iron-oxide staining, suggests the detachment of the fluorescent component from the nanostructure and the passive migration of the possibly detached fluorescent component to the CNS. Similar experiments carried out to assess if administration of higher concentrations of nanostructures, without rhodamine attached, would result in greater endogenous macrophage uptake found results similar to previous *in vivo* experiments. Data from experiments characterizing the fluorescent agent detachment from the nanostructure, coupled with the *in vivo* experiment results, confirm the unsuitability of the nanostructure, with rhodamine attached, for *in vivo* usage to label and track cells.

The presence of the nanostructure in brain sections in wildtype and EAE mice when administered at higher concentrations, 24 hours after administration, suggests some mechanism by which the nanostructures are not cleared from the bloodstream. Nanostructure presence in CNS tissue does not follow any pattern and predominantly occurs in large circular deposits. The nanostructure does not appear in small intracellular deposits suggesting that it either aggregates *in vivo* or is aggregated upon administration. It should be noted that samples were vortexed and sonicated prior to administration. It is possible that aggregates were formed before or during injection. These aggregates could remain intact and become lodged in small blood vessels or areas of great tortuosity. The lack of dispersion of the nanostructures into CNS tissue supports the proposal that they are present in blood vessels and aggregated. The size and location of many of the aggregates are consistent with this. These observations indicate that the nanostructures, increased in concentration and in the absence of the fluorescent component, are not viable for use in cell tracking experiments involving endogenous labelling.

Although many studies utilise the endogenous uptake of MRI contrast agents by macrophages to examine their CNS infiltration in a variety of disorders (Baeten et al., 2008, Dousset et al., 2006, Berger et al., 2006, Rausch et al., 2004, Rausch et al., 2003, Desestret et al., 2009, Stoll and Bendszus, 2009), studies investigating the migration of cells following administration to animals are also of great interest, most notably, stem cell migration studies (Schafer et al., 2007, Chen et al., 2007, Kustermann et al., 2005) and T-cell migration in EAE (Anderson et al., 2004). More recent work investigating the role played by different T cell phenotypes in disease progression (Sutton et al., 2006) and putative roles in A β immunotherapy studies (Cao et al., 2009, McQuillan et al., 2009) could benefit greatly from application of such techniques. Subsequent analysis of the cells tracked using MRI is currently difficult to achieve and is hampered by a need to confirm the co-localization of the MR contrast agent, commonly through Prussian blue staining. The use of a bimodal magnetic fluorescent contrast agent removes the staining step for iron and enables confocal microscopy to be utilised for MR correlation to preserve spatial information, or by flow cytometric analysis for macroscopic evaluation.

This study set out to investigate the *in vitro* interaction of a magnetic fluorescent nanostructure with primary mixed glial cells. The evidence validates their potential as bimodal contrast agents and, the *in vivo* data gathered recommends, their use exclusively in cell tracking experiments that involve administration of pre-labelled cells. The data also identify the polyelectrolyte coating of the nanostructures as potentially useful in CNS drug delivery agents, as evidenced by nanostructure *in vitro* internalization in the absence of an inflammatory response.

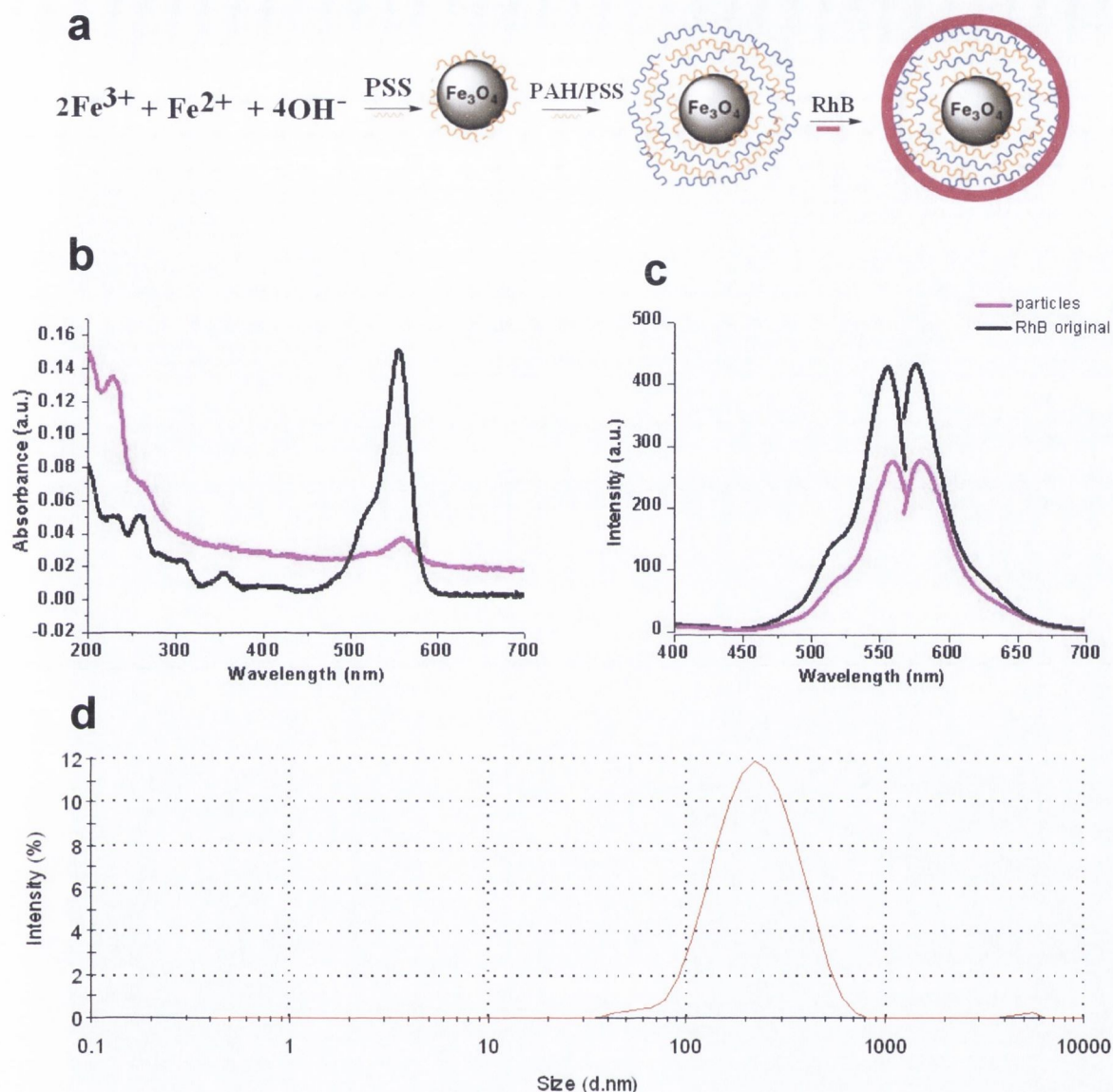


Figure 3.1. Schematic illustration of fabrication and characterization of fluorescent magnetic nanostructures.

Magnetite particles are coated in alternating layers of polypoly(sodium 4-styrenesulfonate) (PSSS) and poly(allylamine hydrochloride) (PAH) using the LbL technique. Rhodamine B is attached to the nanostructure core through electrostatic interactions (a). Absorbance spectra (b) and luminescence and emission spectra (c) of nanostructures (pink) and Rhodamine B standard (black). Absorbance (λ_{ex}) is displayed at 555 nm and emission (λ_{em}) at 578 nm. Analysis of TEM images of fabricated nanostructures indicated a wide range of nanostructure size with an average of 232 nm (d).

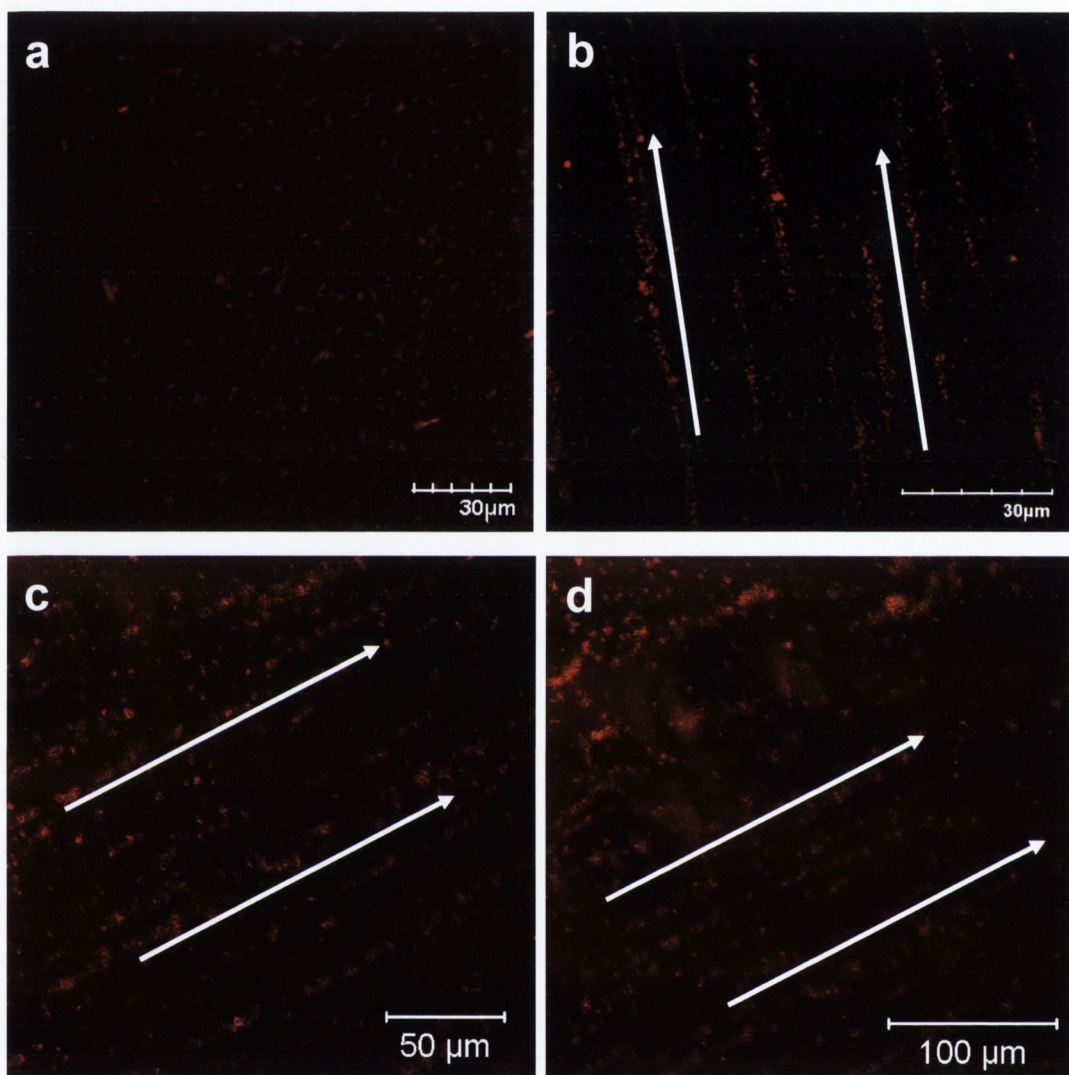


Figure 3.2. Effect of magnetic field on the orientation of nanostructures.

A representative confocal micrograph of nanostructures (2.31×10^{-4} Fe_3O_4 g/ml) following overnight placement in the earth's magnetic field is shown (a). Confocal micrographs of nanostructures (2.31×10^{-4} , 4.62×10^{-4} , 9.34×10^{-4} g/ml Fe_3O_4 ; (b, c and d) respectively) following overnight placement in a 7T field are also presented. Arrows represent the direction of the magnetic field. All micrographs were taken at x40 magnification.

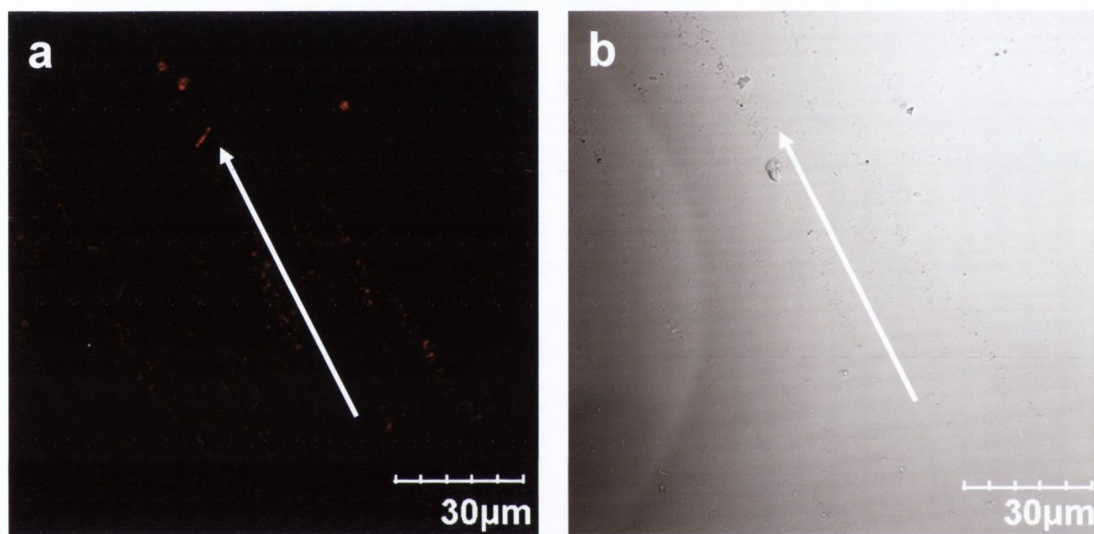


Figure 3.3. Effect of magnetic field on the orientation of nanostructures.

Confocal micrograph of nanostructures ($2.31 \times 10^{-4} \text{ Fe}_3\text{O}_4 \text{ g/ml}$) following overnight placement in a 7T field (a) and the corresponding phase contrast micrograph (b). Arrows represent the direction of the magnetic field in micrographs. All micrographs were taken at $\times 40$ magnification.

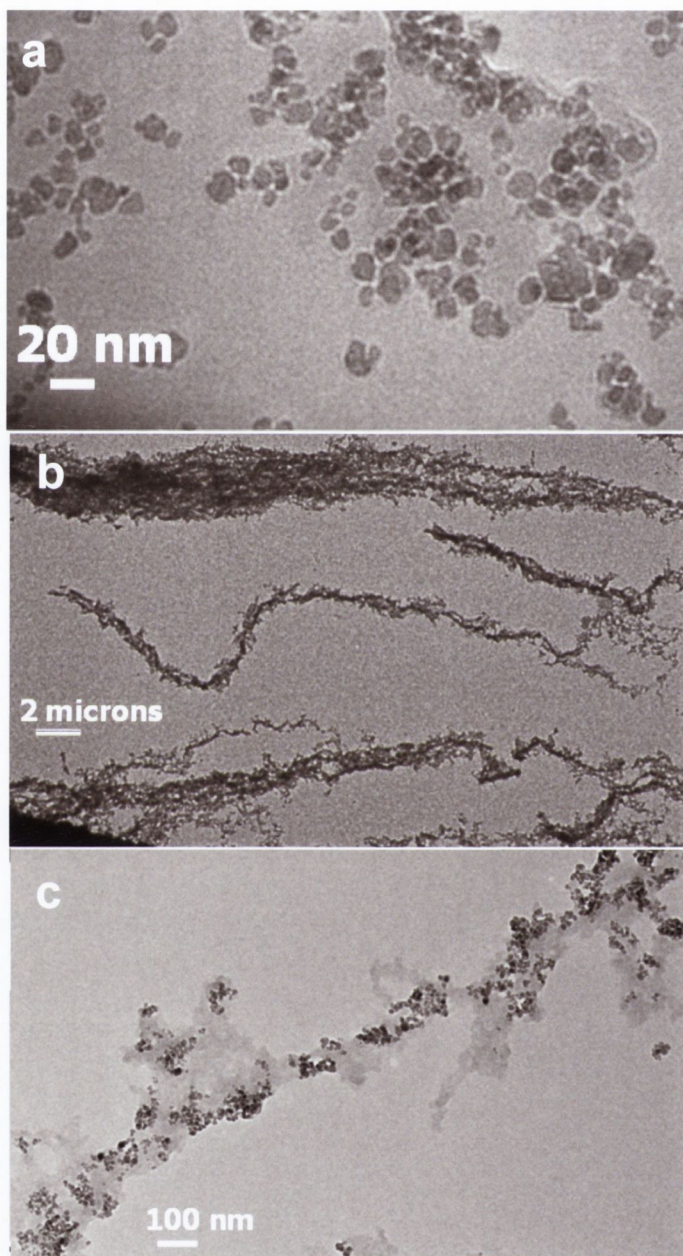


Figure 3.4. TEM images confirm the tendency of nanostructures to align with the direction of an applied magnetic field.

A representative TEM image of nanostructures ($2.31 \times 10^{-4} \text{ Fe}_3\text{O}_4 \text{ g/ml}$) following overnight placement in the earth's magnetic field (a) is shown. TEM image of nanostructures ($2.31 \times 10^{-4} \text{ Fe}_3\text{O}_4 \text{ g/ml}$) following overnight placement in a 7T magnetic field (b) and a higher magnification of aligned nanostructures (c) are also presented.

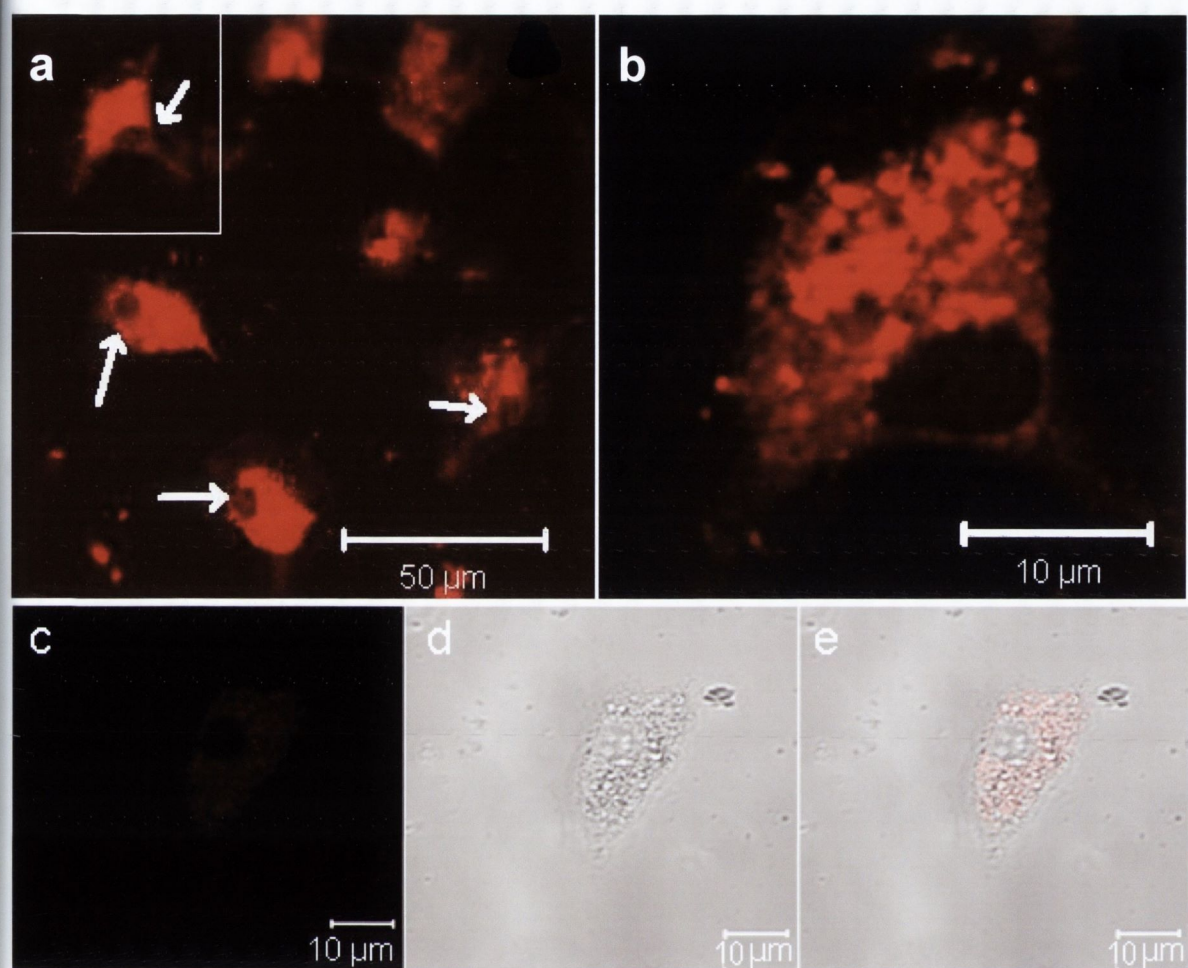


Figure 3.5. Sub-cellular distribution of nanostructures in mixed glial cells.

Internalization of nanostructures (2.31×10^{-4} g/ml Fe_3O_4) in mouse mixed glia occurs following 2 hour incubation (a). Arrows denote cell nuclei delineated by the absence of nanostructure fluorescence. Smaller field of view image focusing on boxed area in (a) demonstrates nanostructure distribution in a single cell (b). Single cell confocal micrograph following 2 hour incubation with nanostructures (c) and corresponding phase contrast (d) and overlay image (e). All micrographs were taken at x40 magnification.

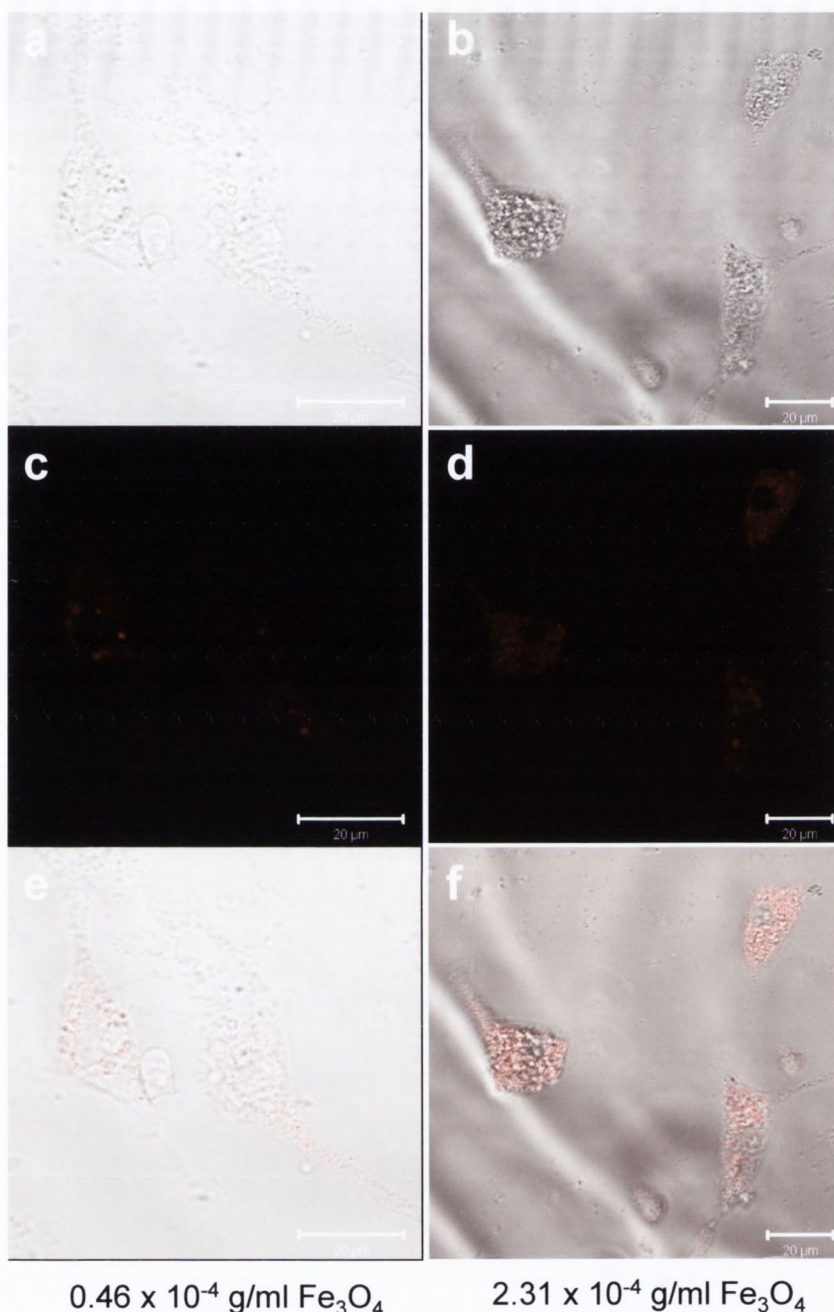
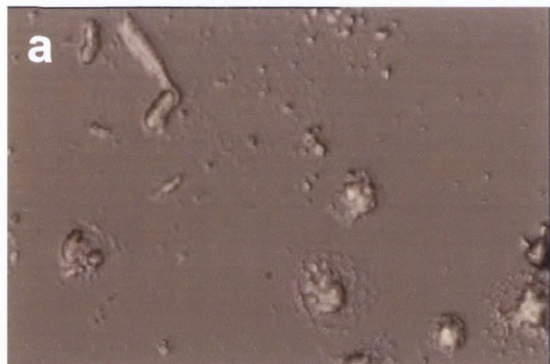


Figure 3.6. Nanostructure cellular uptake in mouse mixed glial culture is concentration dependant.

Confocal micrographs (a, b), corresponding phase contrast image (c, d) and overlap (e, f) of mouse mixed glial cells following 2 hour incubation in the presence of nanostructures at 0.46×10^{-4} or $2.31 \times 10^{-4} \text{ g/ml Fe}_3\text{O}_4$ are presented. Scale bar = 20 μm . All micrographs were taken at x40 magnification.

Magnification x40



Magnification x60

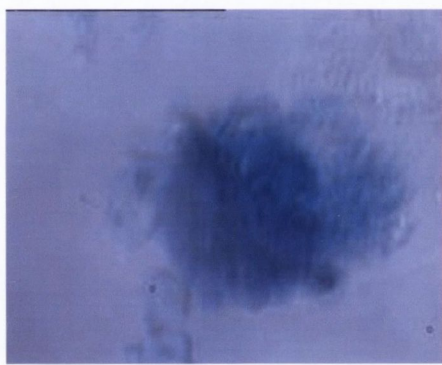


Figure 3.7. Light microscopy confirms cellular internalization of iron oxide core of nanostructure.

Light micrographs of mouse mixed glial cells following 2 hour incubation in the presence of a control solution (a) or nanostructures at 2.31×10^{-4} g/ml Fe_3O_4 (b) are shown. Prussian blue staining indicates the presence of iron. Light micrograph (a, b) were taken at different exposure settings than x60 magnification.

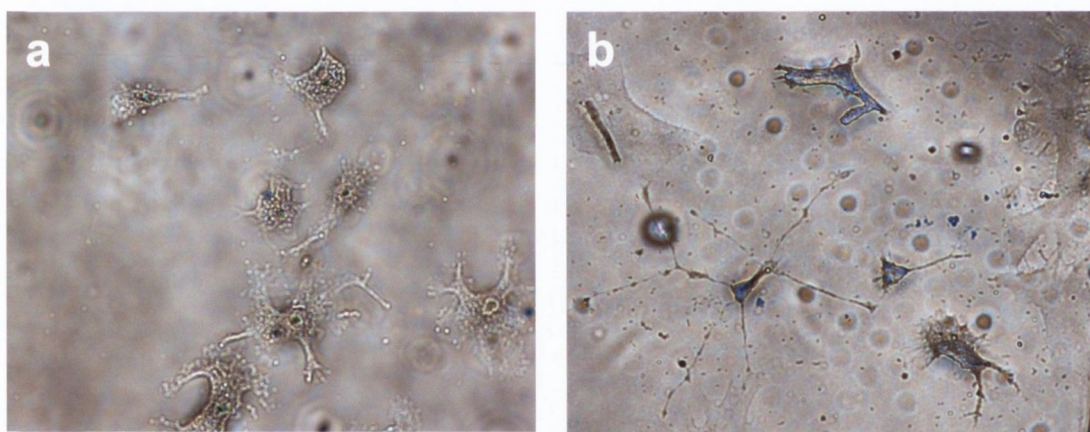


Figure 3.8. Nanostructure cellular uptake in mouse mixed glial culture is concentration dependant.

Light micrographs of mouse mixed glial cells following 2 hour incubation in the presence of nanostructures at 0.46×10^{-4} (a) or 2.31×10^{-4} g/ml Fe_3O_4 (b) are shown. Prussian blue staining indicates the presence of iron. All micrographs were taken at x40 magnification.

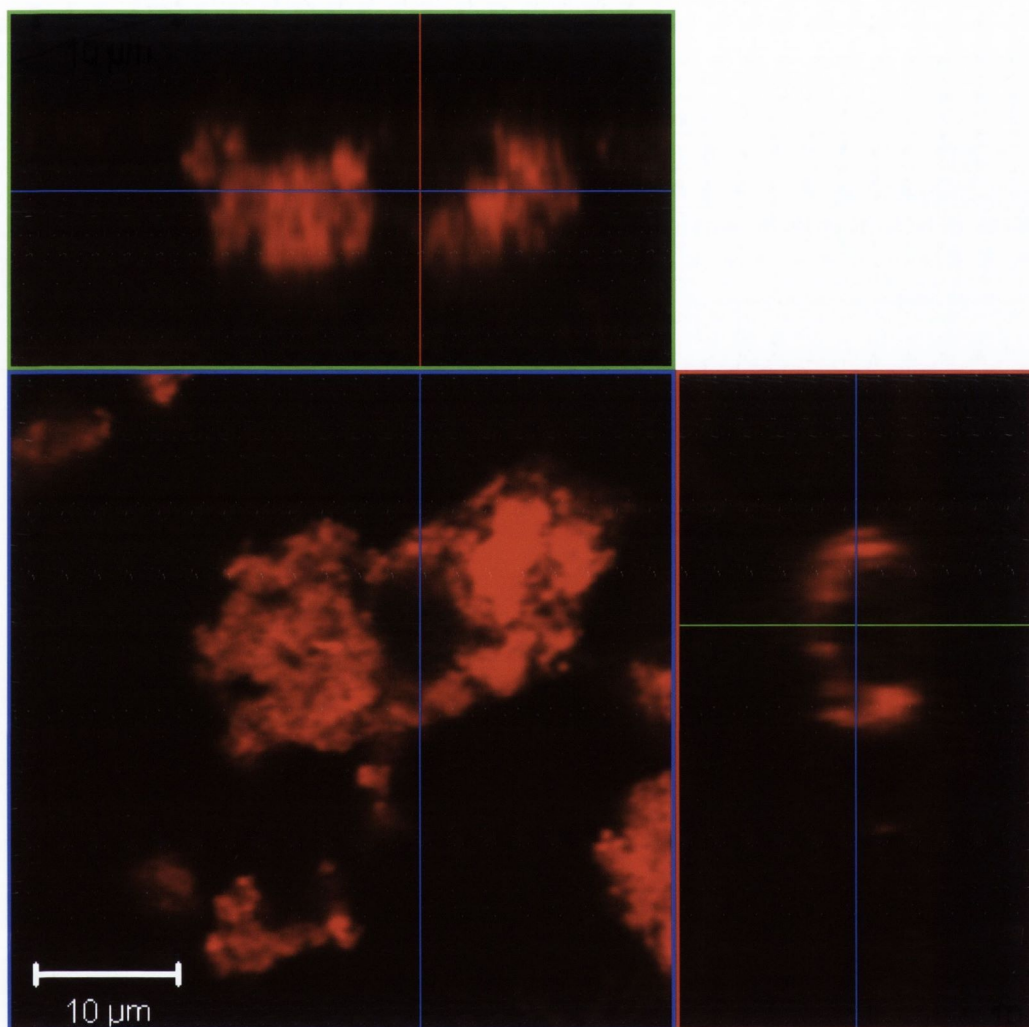


Figure 3.9. Z-stacking identifies the cell nucleus.

A confocal micrograph of a single mouse primary mixed glial cell following 2 hour incubation in the presence of nanostructures at 2.31×10^{-4} g/ml Fe_3O_4 is presented. Sample cross-sectional images reconstructed from $25 \times 1 \mu\text{m}$ micrographs indicate that the fluorescence was confined to the cell cytoplasm. All micrographs were taken at x40 magnification.

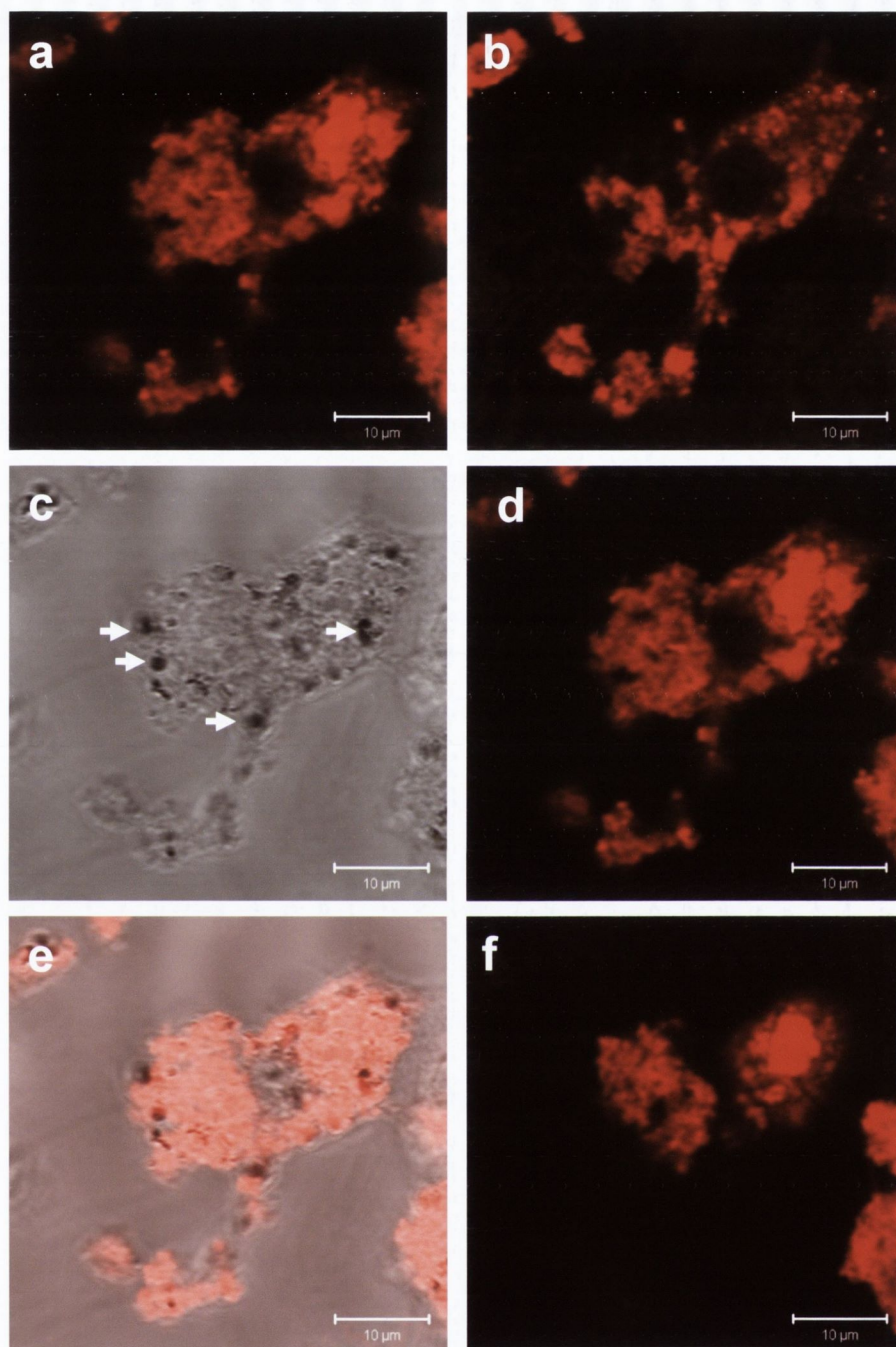


Figure 3.10. Individual confocal microscopy z-sections enables investigation of delineation of nuclues through absence of nanostructure fluorescence.

A representative confocal micrograph of a single mouse primary mixed glial cell following 2 hour incubation in the presence of nanostructures at 2.31×10^{-4} g/ml Fe_3O_4 (a) and corresponding phase contrast (c) and overlay micrograph (e) are presented. Z-section images at 9 μm (b), 12 μm (d) and 15 μm (f) from the distal end of the cell are also shown. Arrows identify membrane localization of the nanostructure suggesting on-going cellular internalization. All micrographs were taken at x40 magnification.

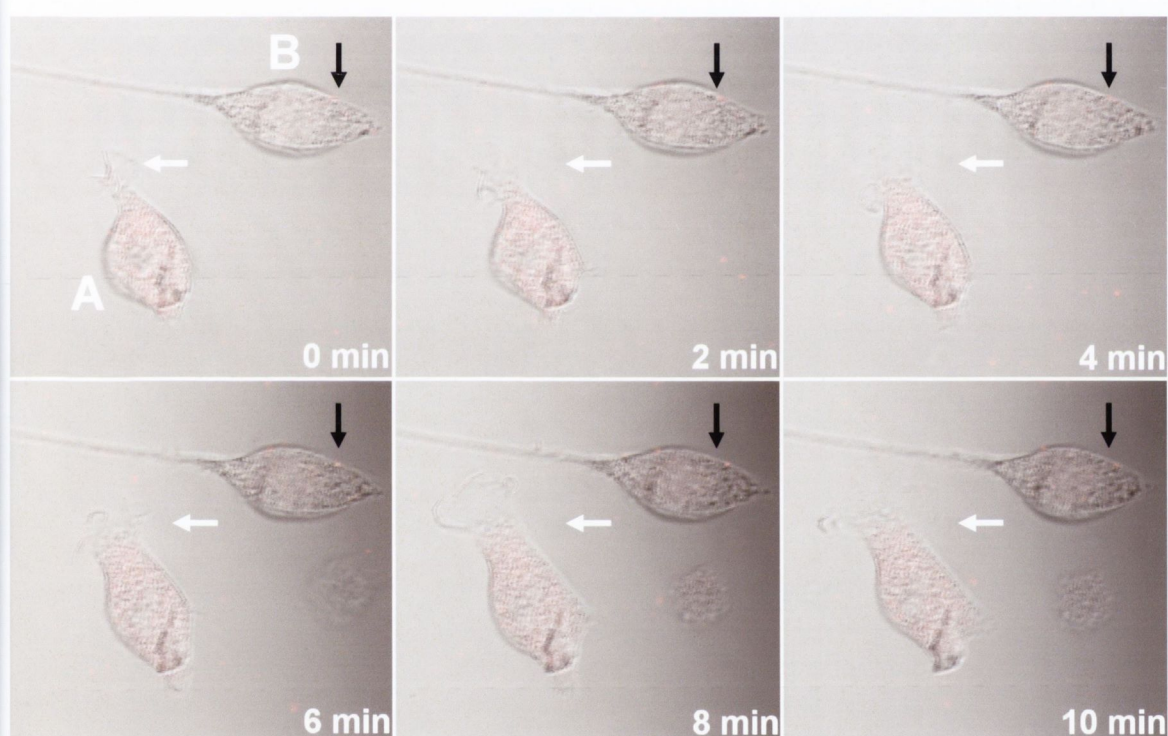
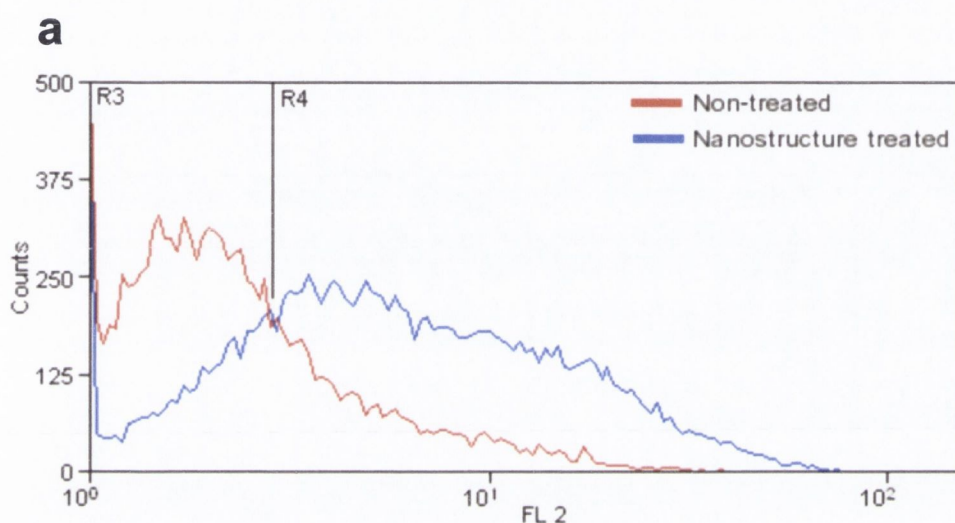


Figure 3.11. Live cell imaging of mouse mixed glial cells incubated with nanostructures.

Serially acquired overlay micrographs, of confocal and phase contrast micrographs, of mouse mixed glial cells incubated with nanostructures are shown. White arrows highlight phagocytic processes in cell A. Black arrows highlight movement of fluorescence across cell membrane in cell B, this may be an endocytic process. All micrographs were taken at x40 magnification.



b

Region	Count	% Hist	% All	Bounds	Mode Count	Mean
Total	15454	100.00	100.00	(1.00,10...	4120	2.83
R3	11559	74.80	74.80	(1.00,2....	4120	1.51
R4	4143	26.81	26.81	(2.75,10...	248	6.54
Total	18755	100.00	100.00	(1.00,10...	328	10.08
R3	3239	17.27	17.27	(1.00,2....	248	2.03
R4	15764	84.05	84.05	(2.75,10...	328	11.62

Figure 3.12. Flow cytometric analysis of non-treated and nanostructure-treated mouse mixed glial cells.

Mean peak fluorescence measurements (a) of control and nanostructure-treated mouse mixed glial cells (2 hour incubation; 2.31×10^{-4} g/ml of Fe_3O_4) are presented. Gates were set to remove non-internalized nanostructures and dead cells. Data for control- and nanostructure-treated cells are given (b).

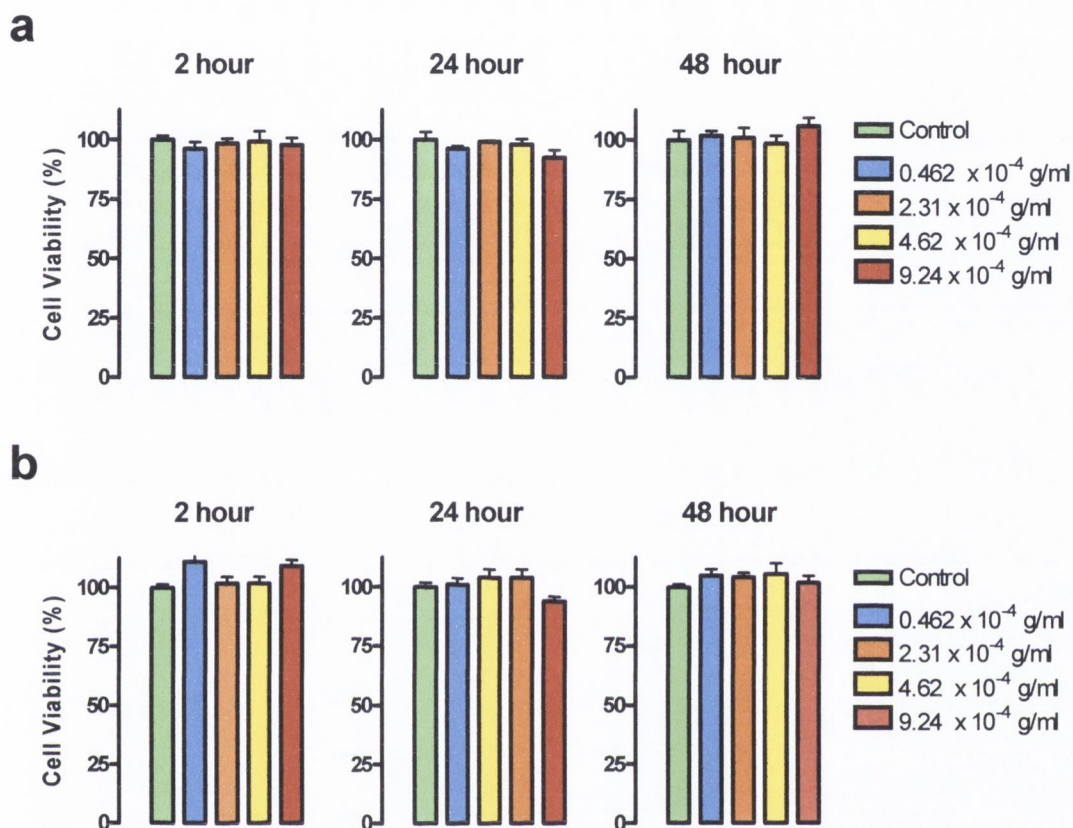


Figure 3.13. Cell viability was not affected by nanostructures.

The cytotoxicity of intact nanostructures (a) and degraded nanostructures (b) was assessed (0.462×10^{-4} , 2.31×10^{-4} , 4.62×10^{-4} , 9.34×10^{-4} g/ml Fe_3O_4) at 3 time points (2, 24 and 48 hours). No change in cell viability was observed.

Data are presented as means $\pm$ SEM, $n = 3$.

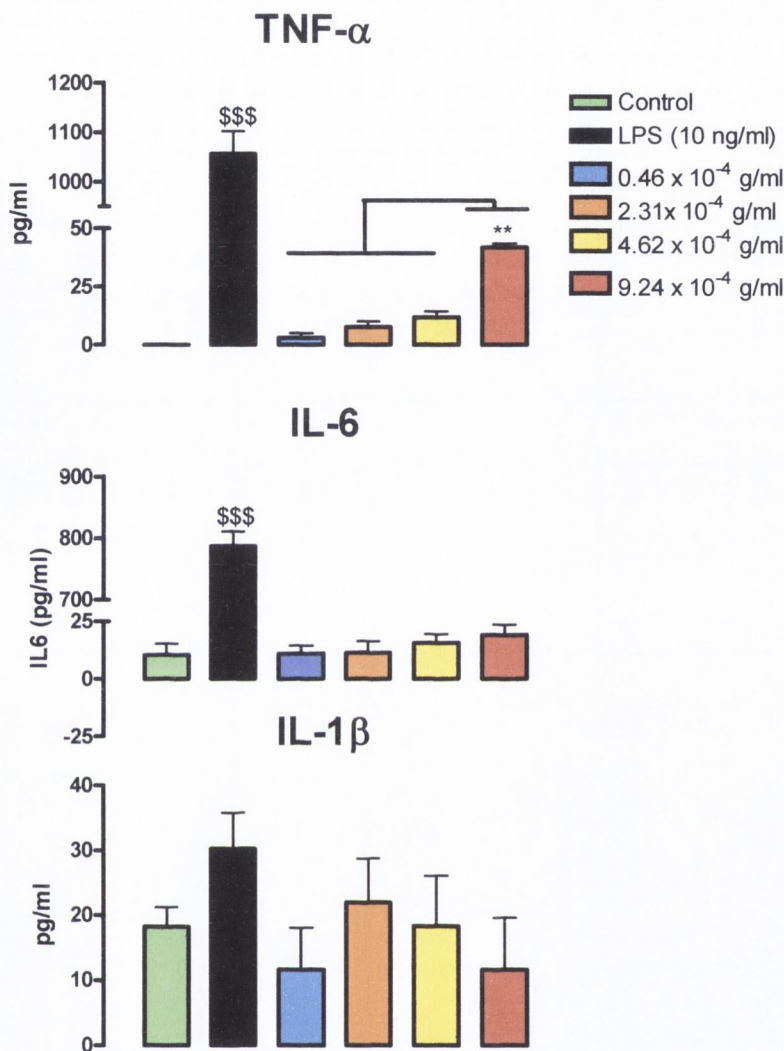


Figure 3.14. Effect of nanostructures on cytokine release.

Concentrations of TNF- α , IL-6 and IL-1 β were assessed in samples of supernatant of mouse mixed glial cells incubated, for 2 hours, in the presence of nanostructures (0.462×10^{-4} , 2.31×10^{-4} , 4.62×10^{-4} , 9.34×10^{-4} g/ml Fe₃O₄). A positive control sample of LPS (10 ng/ml) was also assessed. LPS (10 ng/ml) significantly increased the release of TNF- α and IL-6 (\$\$\$p < 0.001; ANOVA) compared with control samples and nanostructure-treated samples, but had no effect on IL-1 β concentration. Nanostructures did not alter the release of IL-6 or IL-1 β but treatment at 9.34×10^{-4} g/ml Fe₃O₄ significantly increased the release of TNF- α (**p < 0.01; ANOVA).

Data are presented as means $\pm$ SEM, n = 4.

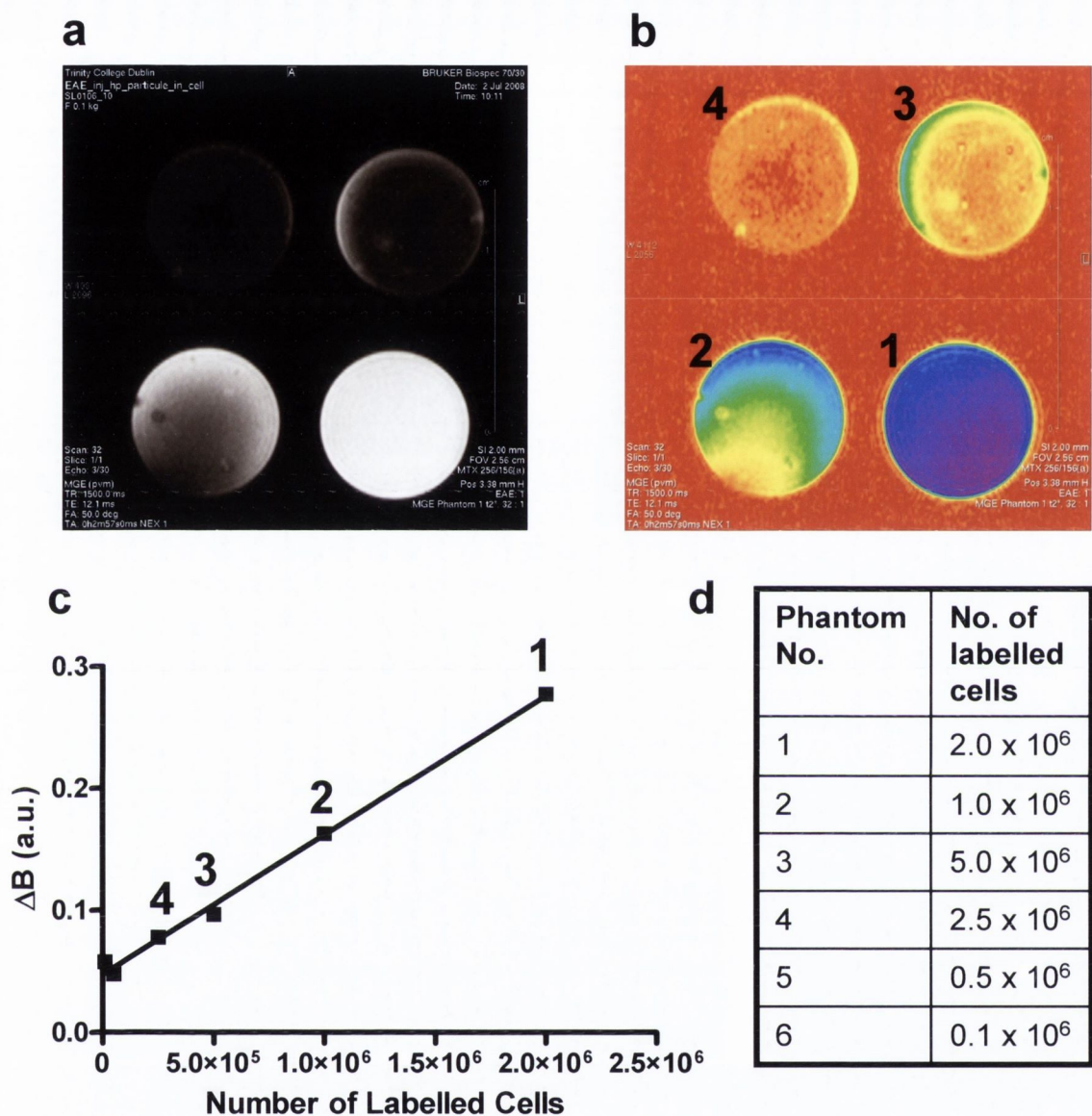


Figure 3.15. MR contrast (ΔB) in agarose phantoms increases with increasing numbers of labelled cells.

A representative T2-weighted image of phantoms (a) is shown. Phantoms displaying the lowest grey level are loaded with nanostructure-labelled cells to the greatest degree. A colour T2 relaxation time map corresponding to (a) is shown; red denotes a T2 time of 0 ms while purple denotes a T2 time of 25 ms (b). The contrast (ΔB) generated by the nanostructures versus number of labeled cells (c) is linear ($p < 0.001$; $R^2 = 0.9967$; Pearson correlation). The number of labelled cells in each phantom is presented (d) and phantoms 1 -4 are identified in the colour T2 relaxation map (b) and the contrast (ΔB) plot.

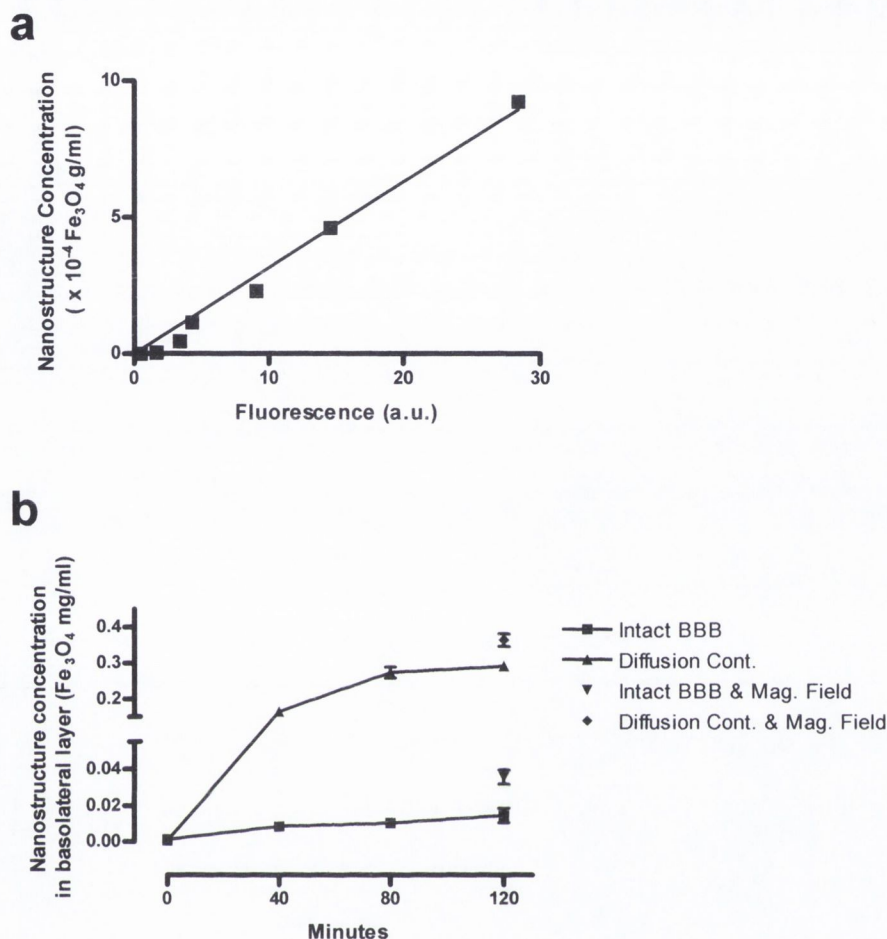


Figure 3.16. Application of a magnetic field allowed nanostructures to cross an endothelial cell layer.

The calibration curve (a) of fluorescence from known concentrations of nanostructures in DMEM at room temperature. ($p < 0.00$; $R^2 = 0.9959$; Pearson correlation) is shown. Nanostructures ($4.62 \times 10^{-4} \text{ Fe}_3\text{O}_4 \text{ g/ml}$) were added to the apical compartment of a transwell system with or without (diffusion control) a confluent endothelial layer (b). The presence of an intact endothelial cell layer decreased significantly the passage of nanostructures ($p < 0.0001$; 2-way ANOVA). Application of a magnetic field ($\sim 0.1 \text{ T}$) stimulated significant movement of nanostructures into the basolateral compartment of the transwell assay, in the presence of an intact endothelial cell layer ($p < 0.05$, Students t-test for independent means) and in the absence of an intact endothelial cell layer ($p < 0.05$, Students t-test for independent means).

All experimental timepoints were run in triplicate.

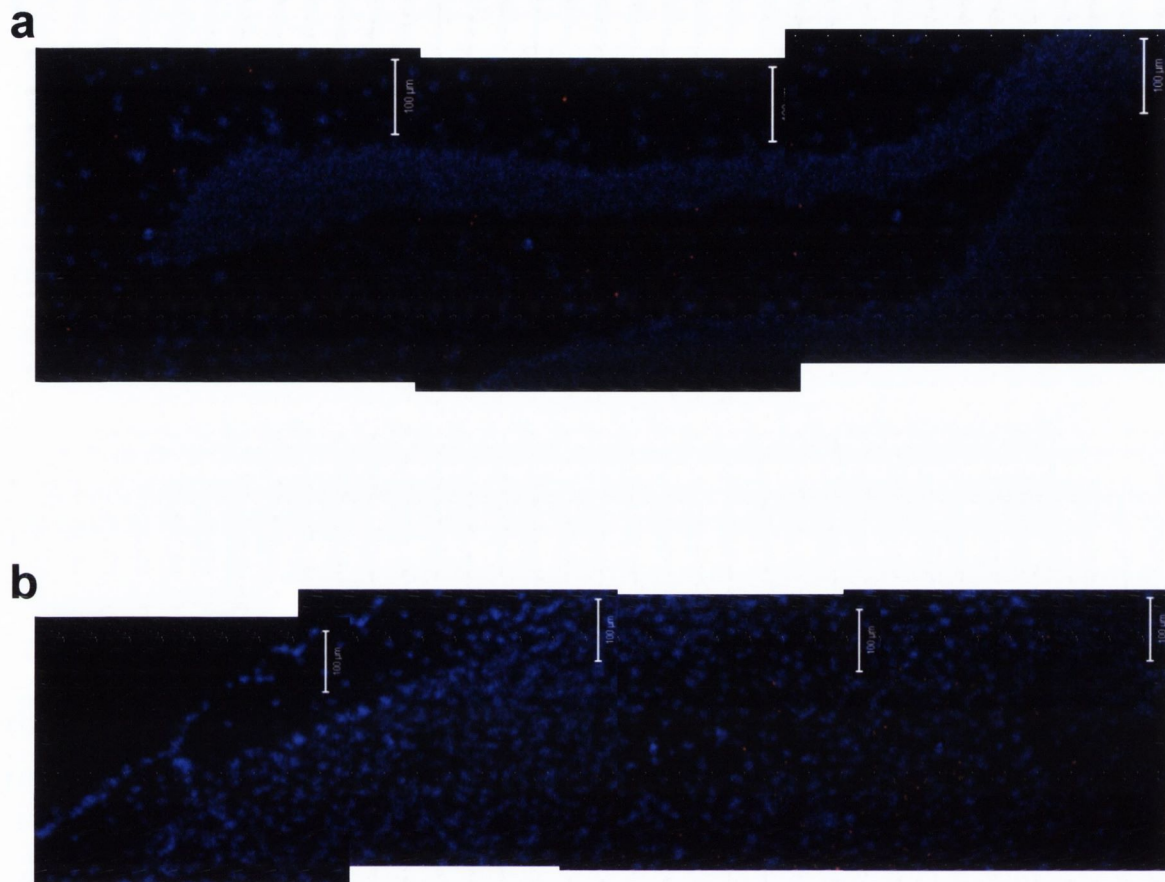


Figure 3.17. Rhodamine fluorescence is detected in the hippocampus and cortex of mice with EAE.

Representative confocal micrographs from brain tissue of a MOG-injected mouse intravenously injected with the nanostructures (90 μM Fe/kg mouse body weight) 24 hours previously are presented. The hippocampus (a) and the cortex (b) display fluorescence associated with rhodamine (red). Nuclei are stained blue using DAPI. Scale bar present on micrographs represents 100 μm . All micrographs were taken at x40 magnification.

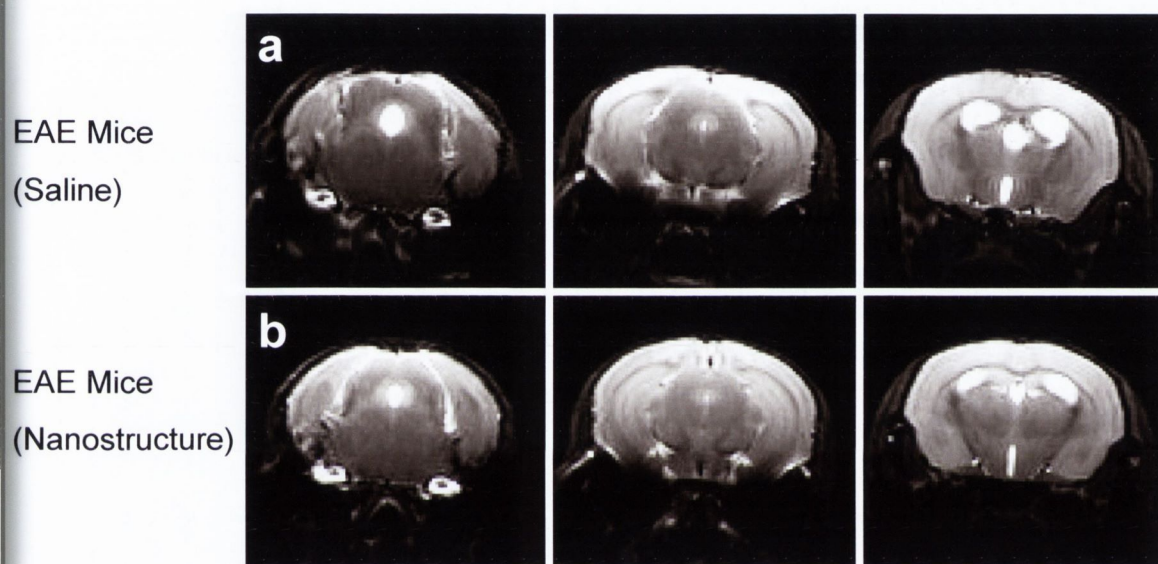


Figure 3.18. T2-weighted images are similar in control mice and mice with EAE following intravenous injection of nanostructures 24 hours previously.

Representative T2-weighted MR images from control mice (a) and mice with EAE (b) 24 hours after intravenous injection of nanostructures ((b); 90 μ M Fe/kg mouse body weight) or a comparable volume of saline (a) are presented. Neither experimental group display T2 attenuations in any area of the brain; notably the hippocampus and cortex.

MRI scan parameters are detailed in section 2.4.2.2

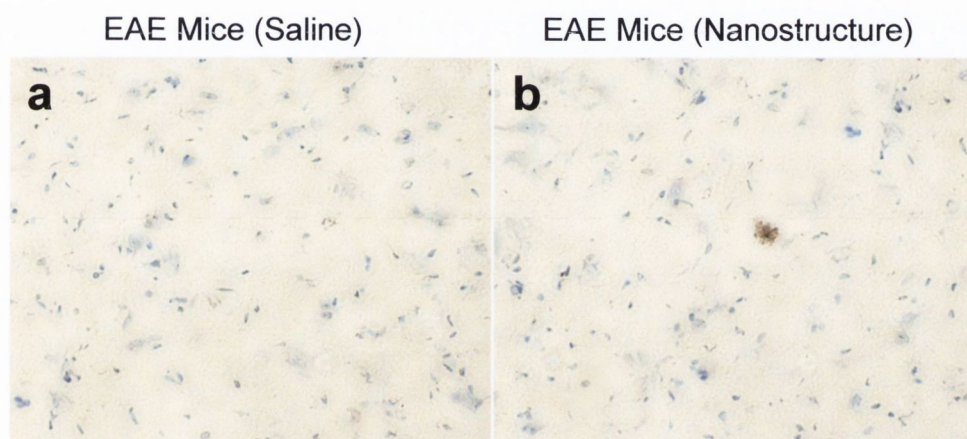


Figure 3.19. DAB-enhanced prussian blue staining identified small deposits of iron in brain sections of mice with EAE intravenously administered nanostructures.

Representative light microscopy images of brain sections prepared from control mice (a) and mice with EAE (b) 24 hours after intravenous injection of nanostructures ((b); 90 μM Fe/kg mouse body weight) or saline (a) are presented. DAB-enhanced prussian blue appears brown and indicates the presence of iron. Gross anatomical staining was carried out with Toluidine blue which appears blue. Both micrographs are taken from S1 cortical region. All micrographs were taken at x40 magnification.

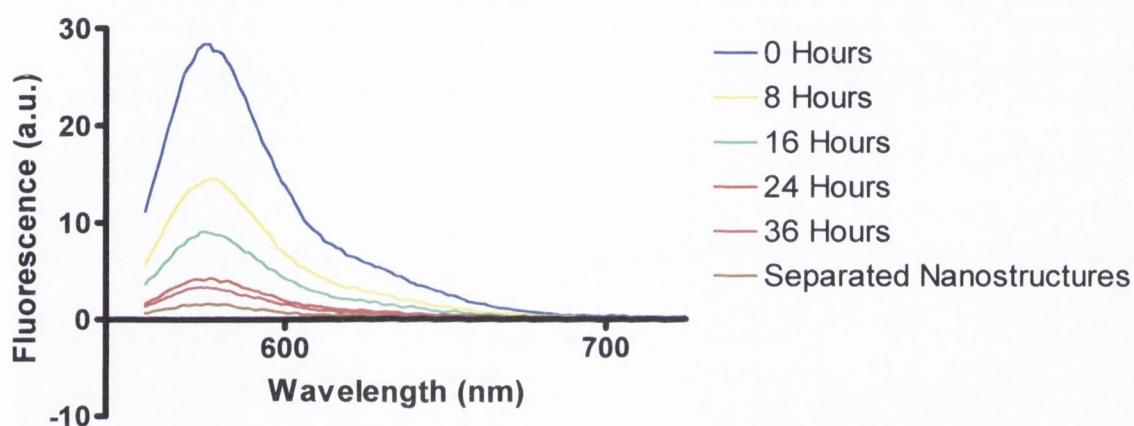


Figure 3.20. Time-related detachment of rhodamine from nanostructure.

Rhodamine B detached from the nanostructure surface incrementally over time. Magnetically-separated and re-dispersed particles, following 48 hours of incubation, show no fluorescence, suggesting the majority of Rhodamine B is detached after 48 hours

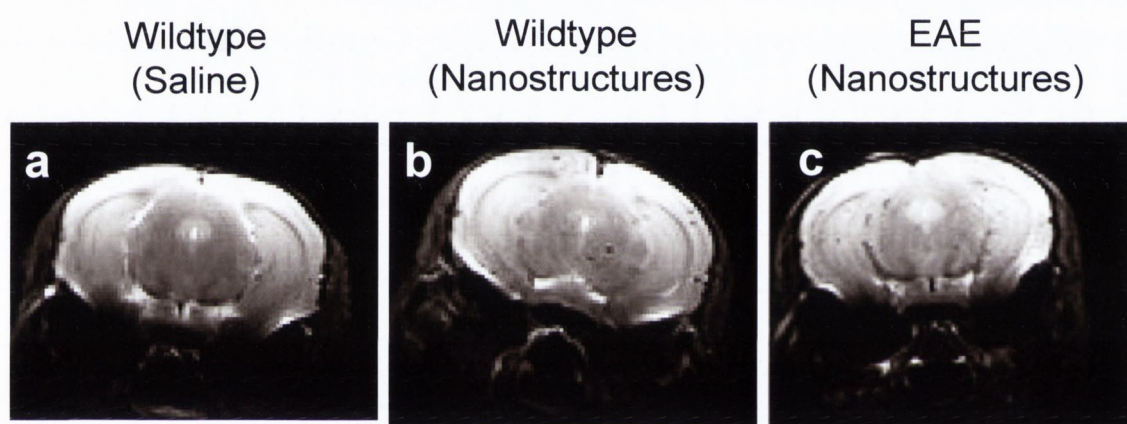


Figure 3.21. Control mice and mice with EAE display T2-signal voids 24 hours after intravenous administration of non-fluorescent nanostructures.

Representative T2-weighted MRI images for sham-injected wild-type mice (a; n = 3), control mice (b; n = 3) and mice with EAE (c; n = 3) 24 hours after intravenous injection of non-fluorescent nanostructures (300 μ M Fe/kg mouse body weight) are presented. Sham group (a) was administered saline. All animals from groups (b) and (c) displayed T2-signal voids, however the exact position of these voids was not consistent throughout or between groups.

MRI scan parameters are detailed in section 2.4.2.2

Wildtype
(Saline)

Wildtype
(Nanostructures)

EAE
(Nanostructures)

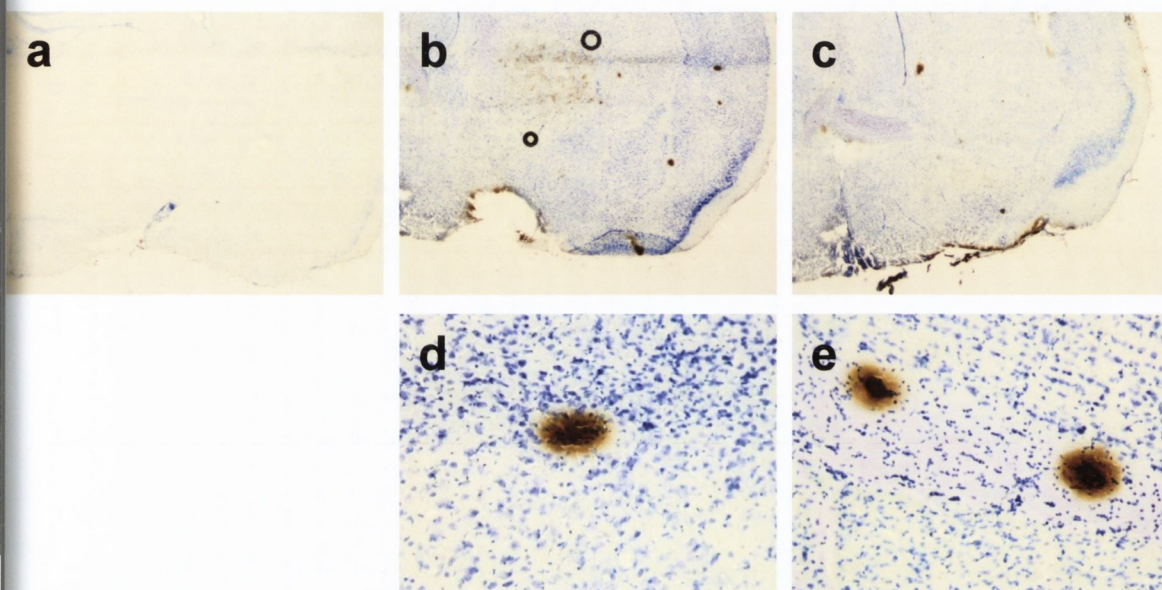


Figure 3.22. Iron is present in brain sections prepared from control mice and mice with EAE.

Representative light microscopy images of brain sections prepared from sham-injected control mice (a; n = 3), control mice (b; n = 3) and mice with EAE (c; n = 3) 24 hours after intravenous injection of non-fluorescent nanostructures (300 μ M Fe/kg mouse body weight) are presented. DAB-enhanced prussian blue appears brown and indicates the presence of iron. Gross anatomical staining was carried out with Toluidine blue which appears blue. Higher magnification of DAB-enhanced prussian blue positive deposits in brain sections prepared from control mice (d) and mice with EAE (e) are presented. (a, b, c) Micrographs were taken at x2.5 magnification. (d, e) Micrographs were taken at x20 magnification.

Chapter 4

Results

4.1 Introduction

Iron, copper and zinc have been identified in A β plaques (Lovell et al., 1998) and are known to accelerate *in vitro* aggregation of A β (Garzon-Rodriguez et al., 1999). These factors, in addition to the abnormal iron, copper and zinc concentration levels observed in AD brain tissue (Zecca et al., 2004) suggests a key role for these metals in AD, that warrants significant research. The majority of studies to date are hampered by a paucity of techniques capable of accurately quantifying metal deposition in biological tissue. Histochemical staining techniques although well characterized, are prone to non specificity, difficult to translate to absolute concentrations and cannot provide information on the valence state of the metal under investigation. Recently techniques, developed primarily for the investigation of the elemental and atomic composition of materials, have been adapted for use with biological tissue. These techniques have enabled the quantification of A β plaque composition, with respect to surrounding tissue, at extremely high spatial resolution (Leskovjan et al., 2009, Miller et al., 2006). However, many of these studies have focused on end-stage A β plaques and it is reasonable to suggest, considering the complex and poorly understood nature of *in vivo* plaque formation and maturation, that A β plaque composition may change with disease progression.

It has been suggested that A β plaques can act as sources of ROS themselves and mediate the activation of microglia leading to microglial production of ROS (Smith et al., 1998, Khan et al., 2006). Recent work has identified iron as having a key role to play in the A β plaque production of ROS, specifically an iron-oxide called magnetite, which has been found in higher concentrations in AD brain tissue compared with age- and sex-matched control brain tissue (Collingwood et al., 2005, Kirschvink et al., 1992, Khan et al., 2006). Transgenic APP/PS1 mouse models of AD, although exhibiting many characteristics of AD pathology, do not display robust

neuronal loss (Lau et al., 2008). It is currently unknown as to whether A β plaques, and brain tissue in general, in these models exhibit similar metal and iron oxide composition when compared with human AD plaques and brain tissue.

The central aim of this study was to assess the composition of early-stage A β plaques in a mouse model of AD, using novel techniques capable of accurate quantification of metals in biological tissue. Secondly, the presence of magnetite, an iron oxide implicated in ROS generation, was assessed in brain tissue prepared from a mouse model of AD exhibiting early-stage plaque deposition.

4.2 Results

Metal content and magnetism were not altered in APP/PS1 transgenic brain tissue.

Brain hemispheres obtained from APP/PS1 transgenic and wildtype mice were freeze-dried and assessed for elemental composition using ICP-MS. The data show that iron, copper and zinc concentration levels were similar in brain tissue prepared from APP/PS1 transgenic, and wildtype, mice (Figure 4.1).

Ferritin behaves as a paramagnet at 150K but behaves as a ferromagnet at 5K and will only contribute a magnetic signal to IRM measurements carried out at 5K. Remanent magnetism measured at 150K is indicative of the presence of a ferromagnetic material or magnetically-blocked particles and can be considered to be a measure of biogenic magnetite. A 5 K IRM curve, obtained from a freeze-dried brain tissue sample prepared from a female APP/PS1 transgenic mouse is presented in Figure 4.2. The IRM curve did not reach saturation at 10,000 Oe indicating the response was due to ferromagnetic material. This response indicated that 10,000 Oe was a suitable magnetic field strength to assess IRM response between samples.

IRM subsequent to exposure to 10,000 Oe at 150 K and 5 K in freeze-dried brain tissue prepared from APP/PS1 transgenic and wildtype mice was assessed (Figure 4.3). The data indicate that the IRM response was similar in tissue prepared

from APP/PS1 transgenic and wildtype mice. These data collected at 150 K suggest no differences existed in the concentrations of biogenic magnetite or magnetically-blocked particles between the groups. However, tissue prepared from APP/PS1 transgenic mice exhibited greater variance in IRM data measured at 150 K, than tissue prepared from wildtype mice. The 5 K IRM measurements indicated no difference existed in the concentration of ferromagnetic material between the groups.

XRF map normalization, brain section creases and contaminant identification.

Previous data indicated no changes in brain tissue concentrations of metals and magnetometry existed between APP/PS1 transgenic and wildtype mice. XRF spectra can be used to map the elemental composition of tissue and preserve spatial characteristics, as opposed to the macroscopic evaluation provided by ICP-MS and SQUID magnetometry. XRF maps were collected from brain sections prepared from a female and male APP/PS1 transgenic mouse and a wildtype mouse.

Prior to analysis, all XRF maps require normalization to the incoming beamline energy (Figure 4.4). Beamline energy fluctuations vary the amount of scatter measured by the detector and thus the spectra peaks of each element. To normalize all pixels in the XRF map, and enable comparison and genuine contrast to be detected, the XRF spectra peaks are normalized to the incident beamline energy. This measurement is obtained from an ion chamber, I_0 , placed in the beamline downstream of all focusing and beamline alignment (Figure 4.4a). A graphical representation of the fluctuations in beamline energy for each pixel is presented (Figure 4.4b). The normalized image (Figure 4.4d) is calculated by dividing the XRF map (Figure 4.4c) by the I_0 measurement during acquisition (Figure 4.4b). Subtraction of the normalized image from the original XRF map enables visualization of the changes in image intensity due to normalization (Figure 4.4e).

The high level of electrostatic attraction between the quartz slides and fresh brain sections (presumably due to the intensive polishing the quartz slide receives during the manufacturing process; H Baumbach Ltd., UK), resulted in poor control over the placement of cryostat sections onto slides and, in some cases, resulted in

small creases in brain sections. Creases in brain sections which are visible in light micrographs of the investigated section, resulted in non-linear increases in the elements measured by XRF mapping. However, during XRF assessment areas of creased brain section require identification to omit data from further analysis. Calcium, which is identifiable on a 60 µm pixel scale, is abundant throughout brain tissue and was used to identify areas of overlapping or creased brain section. The brain section, prepared from a male APP/PS1 transgenic mouse, was removed from further analysis due to creased areas in the brain section (Figure 4.5

Exogenous non-biological contaminant material present on brain sections can be identified by investigating the colocalization of iron, chromium and nickel (Figure 4.6). The presence of any 2 of these 3 metals in high levels is associated with air-borne dust particles, and these are identified with an asterisk. Areas of iron, chromium and nickel colocalization occur on the brain section and also on the quartz slide suggesting contamination could have occurred during the cryosectioning, preparation or mounting process. The electrostatic charge held by the slides would attract air-borne dust particles. Larger contaminant materials, identified with arrows on Figure 4.6, are easily identified and can be seen in the composite light micrograph. Contaminant material not visible on the light micrograph can be visualized at higher magnifications, however in some cases there is no visible contaminant material possibly due to it being washed away during the staining process or being present underneath the brain section.

The large level of nickel deposition in the dorsal 3rd ventricle and the absence of iron and chromium colocalization in the dorsal 3rd ventricle (marked with †) suggests nickel contamination of the perfusant. Nickel deposition in the lateral ventricle (marked with ‡) in the absence of chromium, but colocalized with minimal iron deposition provides further evidence of perfusant contamination.

Amyloid deposition is present in the XRF-assessed brain section prepared from a female APP/PS1 transgenic mouse.

XRF-assessed brain sections were stained with a modified congo red and toluidine blue protocol. Washes were not performed using slide racks to ensure the tissue was not removed from the slide. Congo red-positive amyloid plaque deposition was observed in the cortex and hippocampus of the XRF-assessed brain section prepared from a female APP/PS1 transgenic mouse; no deposition was observed in the thalamus (Figure 4.7). The XRF-assessed brain section prepared from a wildtype mouse did not display any congo red-positive plaques. XRF-assessed amyloid plaques displayed less congo red staining intensity compared with amyloid plaques from the same brain section, that did not undergo XRF assessment (Figure 4.8). These data suggest the structure of XRF-assessed amyloid plaques were affected by the beamline. Estimation of plaque size based on congo red staining may be an unreliable measure in XRF-assessed plaques. Similarly, XRF-assessed brain tissue displayed less toluidine blue staining and appeared damaged.

In the APP/PS1 transgenic brain section areas of high iron deposition were not associated with nickel deposition.

Composite XRF maps of iron, chromium and nickel, represented by red, green and blue respectively, enabled areas of triple and double colocalization to be visualised. In Figures 4.9a,b, white pixels identify areas where iron, chromium and nickel are colocalized and yellow pixels identify areas where iron and chromium are present. Areas of increased iron deposition are present throughout both brain sections and, in some instances, are colocalized with chromium and nickel. Areas of nickel deposition, in the absence of iron and chromium, are identified sporadically throughout the brain section and are suggestive of the patterns associated with cerebral vasculature. The deposition of nickel in the dorsal 3rd ventricle and lateral ventricle was consistent across all brain sections assessed with XRF. The third ventricle, visible only on XRF-assessed brain section prepared from a wildtype mouse, displayed increased levels of nickel deposition.

Analysis of the areas of high iron deposition was carried out in the brain sections prepared from a female APP/PS1 transgenic and wildtype mouse. This

assessment investigated whether areas of high iron concentrations, visible in both brain sections, were due to contamination. The XRF spectra of pixels in the top 5% of iron XRF intensity for each brain section were collected. XRF spectra were normalized to pixel number and background noise spectra associated with the quartz slide was subtracted for each brain section (Figure 4.9c,d).

A nickel peak was present in the XRF spectra from pixels in the brain section prepared from a wildtype mouse only. High levels of iron concentration in the brain section prepared from a female APP/PS1 transgenic mouse were not associated with nickel deposition. A strong calcium peak also indicates that areas of high iron in the APP/PS1 brain section are more likely to be associated with brain tissue. These data indicate that, in the brain section prepared from a female APP/PS1 transgenic mouse, high levels of iron deposition were not associated with nickel and not likely to be contaminant material.

XRF metal mapping produced contrast comparable to MRI scans.

Iron, copper and zinc XRF maps and corresponding composite light micrographs of brain sections prepared from a female APP/PS1 transgenic and wildtype mouse were compared. The contrast obtained using *in vivo* T1-weighted and T2-weighted MRI scans from the same animal at approximately the same region were assessed in the context of the XRF maps (Figure 4.10 – 11). Scan parameters are detailed in Sections 2.4.2.1.

Both copper and zinc are identified in the dorsal 3rd ventricle and lateral ventricle suggesting both metals, despite taking the precautions described above, were present in the perfusant. It appears that copper is present to a greater extent in the lateral ventricle. All sections displayed increased iron in the thalamus that was not accompanied by zinc or copper deposition. This was paralleled by hypointensity in the thalamus in T2-weighted MRI scans. The midbrain did not exhibit this increase in iron concentration. Zinc and copper displayed a decreased concentration in fibre tracts enabling clear identification of the corpus callosum, hippocampal fimbria and

cerebral penduncle in all brain sections assessed. Iron did not exhibit such a decrease in fibre tracts. However in iron XRF maps, the cortex could be clearly distinguished. The reduction of zinc and copper in the alveus and external capsule enabled clear identification of the isocortex and subsequent layers could be visualised in XRF maps of the brain section prepared from a wildtype mouse. These fibre tracts were clearly visible on T2-weighted images and to a lesser extent on T1-weighted images.

The amygdala displayed no appreciable changes in iron and copper concentrations. However, zinc concentration was increased in the amygdala. The increase in zinc appeared to extend from the cortical subregions proximal to the amygdala. However a delineation of the lateral blade of the dentate gyrus and the amygdala was not possible.

The hippocampal structure displayed excellent contrast on all XRF maps and clear delineation due to decreased zinc and copper concentrations in the corpus callosum and associated fibre tracts. These structures were also visible on T2- and T1-weighted MR images. The pyramidal layer of the CA1 field displayed increased iron, zinc and copper deposition. Iron was present to the greatest degree, followed by zinc and then copper, when considering the layer from the fasciola cinerea to the stratum lucidum in the CA3 field. At this point, the pyramidal cellular layer exhibited an increase in zinc and copper concentration. This increase extended to the granular cell layer of the dentate gyrus and the polymorphic layer, which was visible on iron XRF maps due to a decrease in iron concentration compared with the granular cell layer.

The substructures of the CA1 field were identifiable due to an increased concentration of iron, zinc and copper in the molecular layer of the dentate gyrus. The stratum oriens, which encloses the hippocampal structure, was identifiable on the zinc XRF map. The hippocampal structure can be identified due to hypointensity on T2- and T1-weighted MRI scans and the dentate gyrus can be visualized. However, hippocampal substructures such as the molecular layer of the dentate gyrus can only be identified with reference to a brain atlas.

Iron, but not copper or zinc, is associated with amyloid plaque deposition.

Areas of high iron concentration, in the XRF-assessed brain section prepared from a female APP/PS1 transgenic mouse, were assessed to identify if the colocalization of iron and amyloid plaques would enable identification of plaques on iron XRF maps. Areas showing good agreement between high iron deposition and amyloid plaque presence in congo red and toluidine blue light microscopy were identified. Analysis of high iron deposition in the auditory cortex, CA2 and CA1 field of the hippocampus are presented (Figures 4.12 – 15). These data are presented as there was easily identifiable landmarks, helpful in confirming the spatial agreement of high iron deposition in the XRF maps and amyloid deposition in light micrographs; coincident amyloid plaque deposition and high iron concentration were present in all 3 cases. In the auditory cortex an exogenous contaminate deposition enabled identification of 3 areas of high iron deposition that coincided with amyloid plaque deposition. Analysis of the XRF spectra of these areas indicated that only iron was consistently elevated in the 3 areas associated with amyloid deposition, compared with cortical tissue that did not display significant iron deposition. Area 3 exhibited the greatest increase in iron concentration compared with normal cortical tissue (445%), this was associated with a decrease in calcium (-49%) but an increase in cobalt concentration (308%).

In the CA2 field of the hippocampus, identification the dentate gyrus and pyramidal cell layer were used as anatomical landmarks to enable identification of a single pixel that coincided with amyloid deposition (Figure 4.13). The XRF spectra of this pixel was assessed and compared with hippocampal CA2 tissue that did not display significant iron deposition. Increases in iron (141%), cobalt (199%) and calcium (102%) were observed in the area associated with the amyloid plaque deposition relative to other areas of hippocampal CA2 tissue. An increase in manganese was evident but could not be quantified as other areas of hippocampal CA2 tissue did not exhibit a manganese peak.

In the CA1 field of the hippocampus, the pyramidal cell layer enabled identification of an area of high iron deposition that coincided with amyloid deposition (Figure 4.14). The XRF spectra of this area was assessed and compared with hippocampal CA1 tissue that did not display significant iron deposition. Increases in iron (218%), cobalt (44%) and manganese (82%) were observed in the area associated with amyloid plaque deposition relative to other areas of hippocampal CA2 tissue. In comparison, the pyramidal cell layer of the hippocampus displayed only modest increases in iron (34%) and cobalt (13%).

These data indicated that amyloid plaques can be identified in iron XRF maps of brain sections and that iron was consistently increased in areas associated with amyloid plaque deposition. Increases in cobalt and manganese may not be accurate due to the XRF iron peak extending into the areas associated with cobalt and manganese. Copper and zinc displayed no consistent pattern in areas associated with amyloid plaque deposition and any changes observed were modest in all cases.

Mixed valence iron oxide is present in APP/PS1 transgenic brain tissue.

XRF microfocus maps were collected in the dorsal retrosplenial cortex (area A) and the CA2 field of the hippocampus (area B) in the XRF-assessed brain section prepared from a female APP/PS1 transgenic mouse (Figure 4.15). Areas of high iron deposition were identified within each XRF microfocus map and assessed using XANES to determine if mixed valence iron oxides were present in APP/PS1 transgenic brain tissue.

In the dorsal retrosplenial cortex, 3 areas of high iron content chosen for XANES analysis were assessed using XRF spectra analysis (Figure 4.16). It was not possible to determine if these areas were associated with amyloid plaque deposition as effective staining of the tissue following XRF microfocus mapping and XANES spectra acquisition was not possible (Figure. 4.17b). The XRF spectra of the 3 areas of high iron deposition, labeled A1, A2 and A3, were compared with the XRF spectra of cortical tissue that did not display significantly iron deposition. In all 3 areas, iron

and cobalt displayed marked increases in concentration, with Area A3 exhibiting a 4023% increase in iron concentration compared with other areas of the cortex. However, chromium and nickel were also increased in Area A3, but neither increase could be quantified as the background tissue did not exhibit a chromium or nickel peak. These data suggest that Area A3 was an area of exogenous contaminate material, and was therefore removed from further analysis. Area A2 displayed increases in copper (23%) and zinc (47%) in association with an increase in iron (455%). In Area A1, copper and zinc displayed minimal concentration changes while iron increased by 226%.

XANES spectra were collected from areas A1 and A2 and analysed using a linear combination fits method to determine relative weightings of magnetite, maghemite and ferritin in the XANES spectra (Figure 4.17). The XANES spectra collected from these areas were compared with XANES spectra obtained from magnetite, maghemite and ferritin standards and these data are presented in Appendix II, Figure 1 (these standard XANES spectra were prepared by Dr. Joanna Collingwood and are used with permission). None of the XANES spectra displayed good agreement with the profile of maghemite. The XANES spectra from Area A1 was in good agreement with the magnetite (45%) and ferritin spectra (42%; $\chi^2 = 0.03363$; $R = 0.000732$; Figure 4.17b). Area A2 displayed a similar profile with good agreement with the magnetite spectra (52%) but a reduced agreement with the ferritin spectra (22%; $\chi^2 = 0.03744$; $R = 0.001056$). Plots of the linear combination components for Area A1 and A2 are presented in Appendix II, Figures 2 – 3.

In the CA2 field of the hippocampus, a single large area of iron deposition was identified following microfocus XRF mapping (Figure 4.18). XANES analysis of the feature was carried out on two areas, identified as area B1, and a second area identified as area B2. The XRF spectra of these areas were compared with CA2 tissue that did not display significant iron deposition. Area B1 (132%) and B2 (399%) displayed an increase in iron concentration and associated increases in manganese and cobalt. Modest decreases were observed in copper and zinc concentrations. A decrease in calcium was observed in both areas, however no increases in chromium

and nickel were observed suggesting that the increases in iron were biogenic. XANES spectra were collected from areas A1 and A2 and analysed using a linear combination fits method to determine relative weightings of magnetite, maghemite and ferritin in the XANES spectra. The XANES spectra from Area B1 was in excellent agreement with the magnetite spectra (77%) and minimal agreement with the maghemite spectra (20%; $\chi^2 = 0.18669$; $R = 0.002785$; Figure 4.17b). Area B2 showed excellent agreement with the magnetite spectra (71%) and good agreement with the ferritin spectra (38%; $\chi^2 = 0.15782$; $R = 0.002342$). Plots of the linear combination components for Area B1 and B2 are presented in Appendix II, Figures 4 – 5. The weighting values obtained from the linear combination fits analysis do not directly relate to the concentration, of iron oxide under investigation, present at the beam incident site. They provide a means to confirm the presence of a certain oxide and enable comparison between areas. Empirical data indicates a weighting above 50 % relates to a concentration of approximately 10 % (Unpublished, Dr. Joanna Collingwood). These linear combination fit data, obtained in tissue prepared from a APP/PS1 transgenic mouse, indicate the presence of mixed valence iron oxides identified as magnetite, and it's colocalization with ferritin, in areas of high iron deposition.

4.3 Discussion

This study set out to examine the elemental composition of A β plaques in brain tissue prepared from 8 – 9 month-old APP/PS1 transgenic mice and to investigate if the iron present was of a mixed valence. The data indicate a consistent increase in iron in areas associated with plaque deposition, when measured at a 60 μm and a 5 μm spatial resolution. These data are consistent with the findings of studies which used quantitative techniques (Lovell et al., 1998, Leskovjan et al., 2009, Miller et al., 2006, Collingwood et al., 2008, Dhenain et al., 2009) and studies using immunohistochemistry (Meadowcroft et al., 2009, Dhenain et al., 2002). The evidence from the present study also confirms the presence of magnetite, accompanied by ferritin and, in some instances by its oxidative partner maghemite, in areas associated with increased iron and presumed plaque deposition. Studies using

similar techniques have reported the presence of magnetite in human AD tissue (Collingwood et al., 2005).

It is unlikely that the data collected at 60 μm reflect only the increase in iron associated with plaques, as it is conceivable that only a partial volume of plaque would be present in such an area. The cells proximal to plaques have been identified as activated microglia and astrocytes as described in chapter 5 and by others (Itagaki et al., 1989). The data presented here suggests that these cells, in addition the plaque itself, exhibit an increase in iron concentration. This conclusion is supported by a study which reported increased ferritin concentrations in cells proximal to plaques (Quintana et al., 2006).

The identification of magnetite in areas displaying high iron concentration described here is similar to studies which identified magnetite presence in human AD tissue and in A β plaque cores (Collingwood et al., 2008, Collingwood et al., 2005, Pankhurst et al., 2008). However, this is the first demonstration of the presence of magnetite in a mouse model of AD. The evidence indicates that magnetite is present in the core of an elliptical area of high iron deposition that is approximately 50 μm in size, while it also colocalizes with smaller deposits (10 – 15 μm) of increased iron concentration. Previous reports of magnetite presence in amyloid plaques cores suggested that their presence may be due to A β redox chemistry involving, ferritin and ferrihydrite, occurring with the plaque itself (Collingwood et al., 2008), but it is clear from the data presented here that deposits of magnetite are capable of forming in smaller deposits of iron. A possible explanation for this may be a dysfunction in the formation of the ferritin protein, leading to abnormal protein accumulation outside of cells which may subsequently lead to the formation of non-physiological iron such as magnetite. These deposits may then precipitate plaque formation due to the increased levels of non-physiological iron present in the extracellular space. It should be emphasized that a limitation in this technique is the inability to confirm whether the areas analysed using XANES are extracellular or intracellular. Evidence from the present study also identifies maghemite, the oxidation partner of magnetite, in areas of increased iron concentration in brain tissue obtained from female APP/PS1 mice. It

is possible that oxidation of magnetite to maghemite may have occurred post-mortem or during sample preparation, but it must also be considered that *in vivo* conversion of magnetite to maghemite could potentially lead to the generation of free radicals by Fenton chemistry. In any case, it is interesting that previous studies using similar techniques have also identified the presence of maghemite in AD brain tissue (Collingwood et al., 2005). Although the data from the present study indicating the presence of magnetite and maghemite is conclusive, it cannot be confirmed whether A β plaques in this mouse model of AD contain these iron oxides since the areas of brain tissue investigated for iron oxide presence using XANES, were too damaged by the procedure to subsequently stain for amyloid plaque presence.

In contrast to the consistent increase in iron seen in areas associated with amyloid plaques in the present study, no consistent colocalization of copper and zinc with amyloid plaques were observed. Average increases in iron (196 %) and calcium (26 %) were observed in areas associated with amyloid plaques, with copper and zinc increased in some instances and decreased in others. These data do not corroborate previous work using XRF in human plaques, which reported significant increases in iron (573 %), copper (1171 %), zinc (908%) and calcium (9838 %) in amyloid plaque cores compared with surrounding tissue (Leskovjan et al., 2009). A study using proton-induced X-Ray emission (PIXE) analysis of human AD tissue from patients with an average age of 78.8 years, reported significant increases in iron, copper and zinc in A β plaques, with zinc increased to the greatest extent, followed by iron and then copper (Lovell et al., 1998). In both of these studies, it is likely that the tissue was taken from end-stage AD patients and the majority of plaques investigated could therefore be considered mature plaques.

In a recent study which carried out an XRF investigation of plaque composition in 13 – 14 month old APP/PS1 transgenic mice, significant increases in iron (33 %), copper (25 %), zinc (107%) and calcium (50%) were reported in amyloid plaques compared with surrounding tissue (Leskovjan et al., 2009). The authors of this study stained the brain tissue prior to XRF investigation to identify A β plaques. These findings contrast with the absence of copper and zinc increases in areas

associated with amyloid plaques, investigated in the present study; it is possible that the lack of consistency in the data is due to age differences reflecting the maturity of the plaques. Although increasing the accuracy of plaque identification in these specimens, it should be recognized that staining does have the potential to alter tissue metal content through leeching (Chua-anusorn et al., 1997). This may explain the lower iron concentrations observed by Leskovjan et al, 2009, when compared to the concentration levels observed in this study.

The approximately 5-fold increase in concentrations of iron, copper and zinc in human plaques compared with plaques in APP/PS1 transgenic mice may be due to continued aggregation of metals to plaques over time. It has been reported that plaques in the brain parenchyma of mouse models of AD contain both transgene-derived human, and murine, A β (van Groen et al., 2006, Pype et al., 2003), with data indicating plaque cores of predominantly transgene-derived human A β are surrounded by murine A β . Human A β has three histidine residues at position 6, 13 and 14, all of which act as iron binding sites (Atwood et al., 2000, Atwood et al., 1998), and histidine residue-bound iron has been shown to generate H₂O₂ by Fenton chemistry (Huang et al., 1999b, Huang et al., 1999a). However, murine A β lacks a histidine at position 13 and its presence on the perimeter of plaques in APP/PS1 transgenic brain tissue may reduce metal colocalization to the plaque (Radde et al., 2008). This may also explain, at least to some extent, the relative decrease in iron associated with plaques prepared from brains of AD mouse models, though it is unlikely that it could account for the approximately five-fold difference that exists between values in human A β plaques compared with transgenic mouse plaques. Murine A β restriction of metal concentrations in plaques may also diminish the potential neurotoxicity of the plaques by reducing the concentration of metals available for participation in Fenton chemistry. The restriction of metal concentration may result in cells proximal to the plaque being less prone to physiological impairments associated with metals being sequestered to the plaque. Zinc is inert to redox chemistry but known to potentiate A β aggregation. It has also been reported that zinc transporters, key to normal brain physiology, are colocalized with A β plaques (Zhang et al., 2008). If continual plaque colocalization of metals is occurring,

the loss of zinc transporters and zinc in its ionic form (Zn^{2+}) from cells proximal to A β plaques could possibly result in impaired physiological function of these cells (Choi and Koh, 1998).

Evidence from the present study indicates an increase in calcium (26%) in areas associated with plaques and this concurs with the data obtained from similar analysis of A β plaques in human tissue (Lovell et al., 1998), and in tissue prepared from APP/PS1 transgenic mice (Leskovjan et al., 2009). The calcium increase observed in this study may reflect the increase in cellular density proximal to the plaque and, consistent with this, it has been shown that in APP/PS1 transgenic mice, neurites proximal to plaques displayed an increase in calcium concentrations (Kuchibhotla et al., 2008). Since A β has no known binding site for calcium its presence in plaque cores is more likely to be mediated by one of the metals present. This proposal is supported by a study which reported high levels of ferro-calcification in plaques deposited in the thalamus of a transgenic mouse model of AD (Dhenain et al., 2009).

The absence of an alteration in overall brain iron, zinc and copper concentrations, as measured using ICP-MS, suggests that abnormal concentrations of these metals, although reported to be associated with AD (Zecca et al., 2004), may only occur towards the end-stage of the pathology. However, due to the incomplete nature of the AD pathology seen in AD mouse models it is likely that the alterations in overall brain tissue metal content may not show a similar profile as that seen in human AD. This hypothesis is supported by a study which examined brain tissue metal concentrations over time in a mouse model of AD. Overexpression of APP in the Tg2576 mouse model of AD resulted in a significant reduction in copper, and increase in iron, concentration at 18 months of age in the female transgenic mice but not males; no change in zinc concentration was observed (Maynard et al., 2002).

SQUID magnetometry is a technique capable of providing information about the status of iron present in a brain tissue sample and has previously been used to measure the magnetism of non-physiological iron present in AD brain tissue,

specifically the ferromagnetic response of magnetite (Pankhurst et al., 2008). Application of this technique in this study uncovered no evidence of non-physiological iron in the brain tissue obtained from the APP/PS1 transgenic mouse. However, subsequent XANES analysis provided conclusive evidence of non-physiological iron suggesting that the relatively reduced sensitivity of SQUID magnetometry, in comparison with X-ray-based techniques, may only be useful in examining end-stage AD tissue.

Although ICP-MS and SQUID magnetometry are useful tools in evaluating the macroscopic presence of metals, the data presented in this study show that XRF maps of brain sections provide data which preserve spatial characteristics and are highly sensitive. These maps are capable of accurate quantification of iron, copper, zinc and calcium concentration in brain tissue at 60 or 5 μm resolutions. Future work using this technique, now firmly established, could investigate age-related changes in plaque composition and tissue proximal to plaques. It could also prove extremely useful in studying the efficacy of metal chelation therapies developed for AD. It has been reported that zinc-transporter knock-out mice exhibit synaptic and behavioural deficits similar to those observed in AD (Adlard et al, 2010). Zinc-transporters have also been identified in A β plaques which suggesting a potential avenue of toxicity for A β plaques (Zhang et al, 2008). Mouse models of AD in which APP production is under control of the Tet-off promoter offer the potential to examine if A β plaques continue to aggregate metals, and various organo-metal compounds, in the absence of amyloid production (Jankowsky et al, 2008).

The central aim of this study was to assess the composition of early-stage A β plaques in a mouse model of AD. The evidence indicates that iron and calcium, but not copper or zinc, are colocalized with areas associated with early-stage A β plaque deposition in 8 – 9 month old APP/PS1 transgenic mice. Furthermore, the data indicate the presence of magnetite, a mixed valence iron oxide implicated in the generation of ROS species, in this tissue.

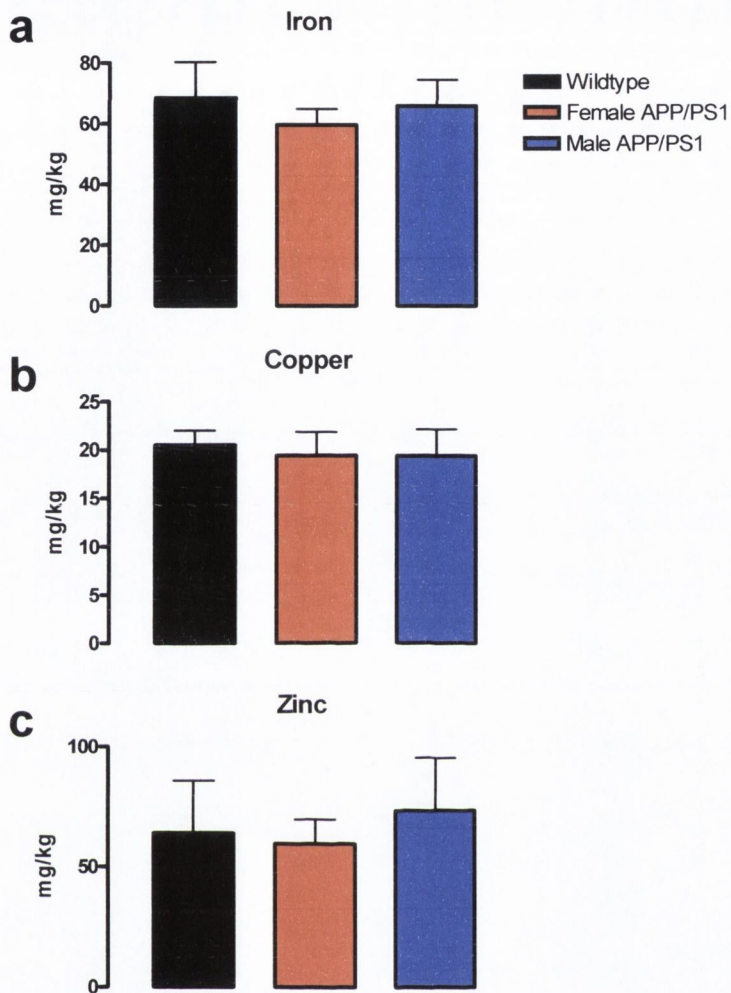


Figure 4.1. Metal content was similar in brain tissue prepared from APP/PS1 transgenic and wildtype mice.

Iron (a), copper (b) and zinc (c) concentrations were similar in brain tissue prepared from APP/PS1 transgenic and wildtype mice.

Data are expressed as mg of metal per kg tissue (dry weight) and means $\pm$ SEM (n = 7 (wildtype) and n = 4 (female and male APP/PS1)).

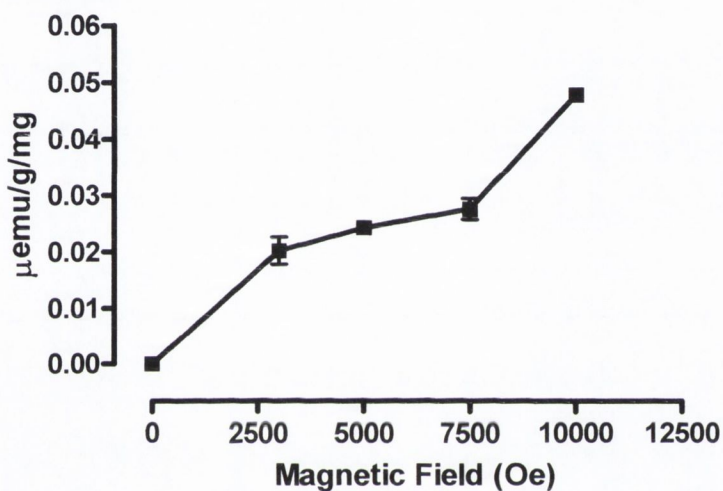


Figure 4.2. Isothermal remanent magnetization, measured at 5 K, did not saturate following exposure to a 10,000 Oe field.

A representative IRM curve, measured at 5 K, obtained from brain tissue prepared from a female APP/PS1 transgenic mouse revealed that IRM increased with field strength but did not saturate following exposure to a 10,000 Oe field.

Data are expressed μemu per grams of iron per mg of brain tissue (dry weight) and are means $\pm$ SEM.

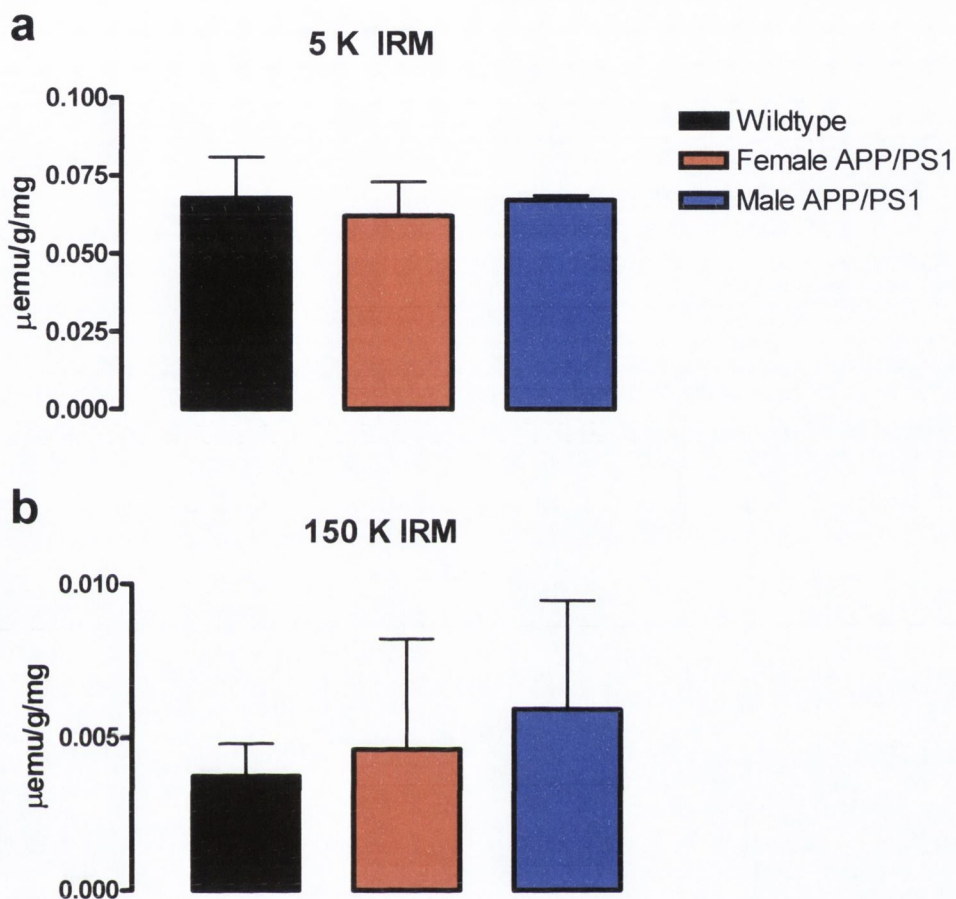


Figure 4.3. IRM was not altered in APP/PS1 transgenic mice.

IRM measured at 150 K (a) and 5 K (b) in brain tissue prepared from APP/PS1 transgenic and wildtype mice, following exposure to a magnetic field of 10,000 Oe, was similar.

Data are expressed as μemu per grams of iron per mg of brain tissue (dry weight) and means $\pm$ SEM ($n = 6$ (wildtype); $n = 4$ (female APP/PS1); $n = 3$ (male APP/PS1)).

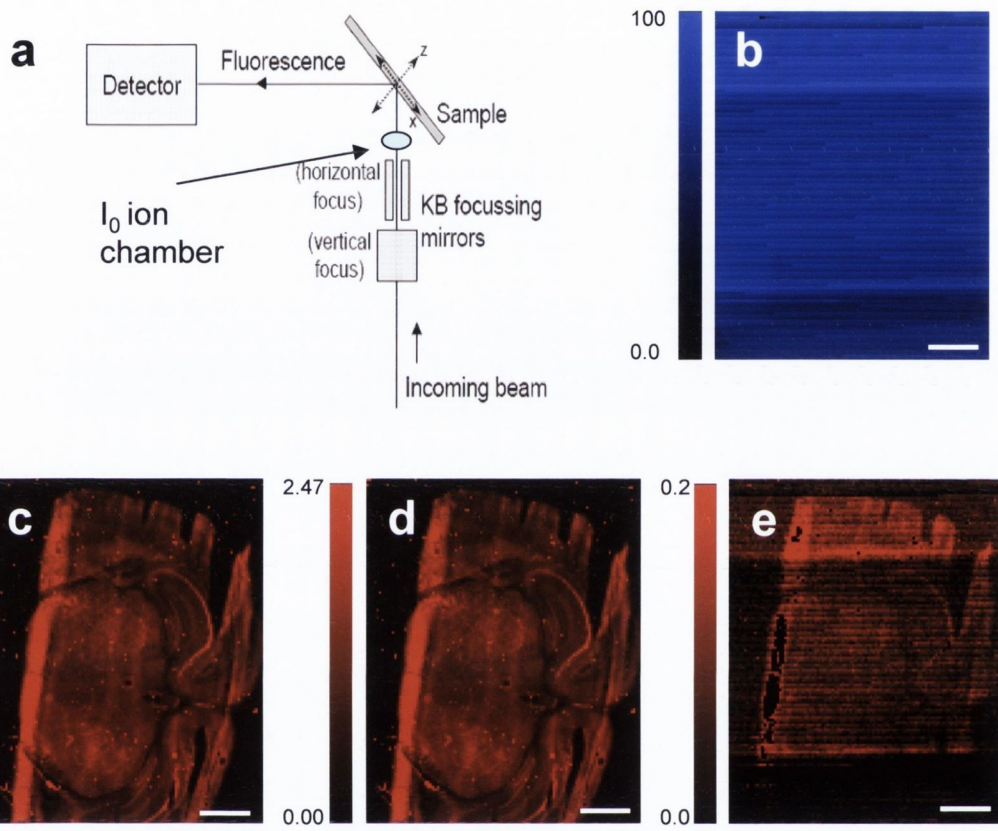


Figure 4.4. XRF map normalization.

The placement of I_0 ion chamber in the beamline (a) and the I_0 measurement (b) obtained during XRF analysis of a brain section from a male APP/PS1 transgenic mouse is shown. The iron XRF map of the brain section, from the male APP/PS1 transgenic mouse, prior to normalization is presented (c). The normalized image (d) is calculated by dividing (c) by (b). Subtraction of the normalized image from the original XRF map (e) enables visualization of the changes in XRF intensity due to normalization. XRF scale bar = 1200 μm and pixel dimensions = 60 x 60 μm . Intensity bars represent I_0 energy (a), total iron fluorescence counts (b, d) and normalized iron fluorescence (c); fluorescence is proportional to total iron present.

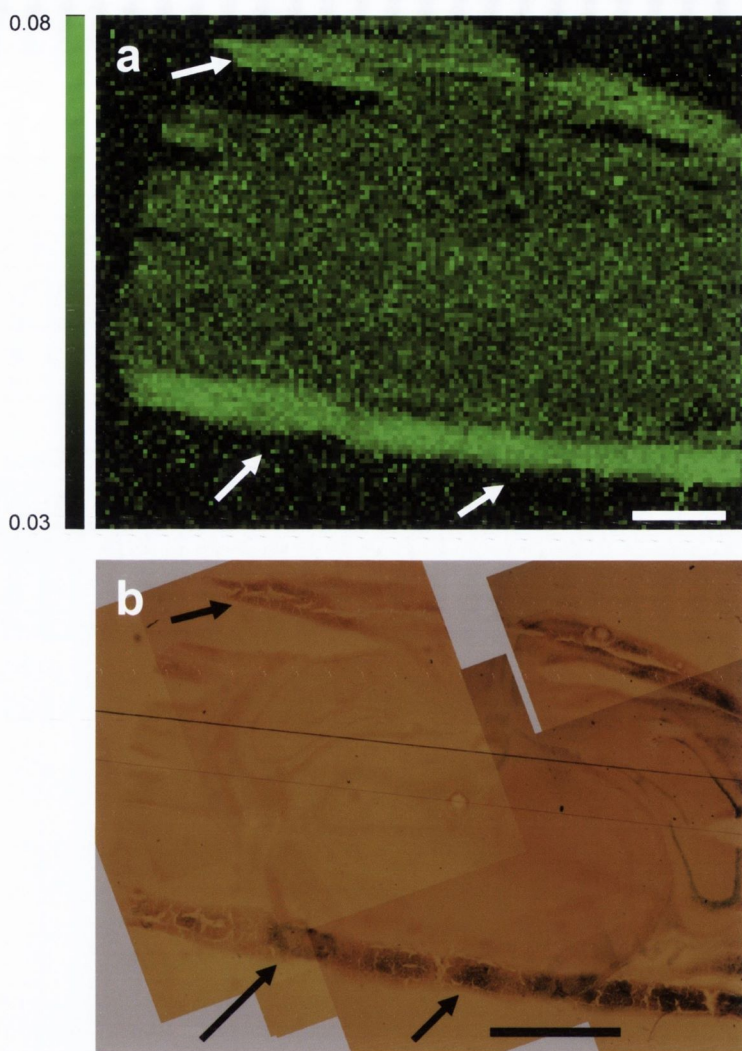


Figure 4.5. Calcium XRF maps enabled identification of creased brain sections.

A calcium XRF map (a) and corresponding composite congo red and toluidine blue stained light micrograph (b) of a brain section from a male APP/PS1 transgenic mouse is presented. Areas of creased brain sections were identified in the calcium XRF map and were confirmed in the composite light micrograph (arrows). XRF scale bar = 1200 μm and pixel dimensions = 60 x 60 μm . Intensity bar represents normalized calcium fluorescence and is proportional to total calcium present. All light micrographs used in the composite image were taken at x2.5 magnification, scale bar = 1200 μm .

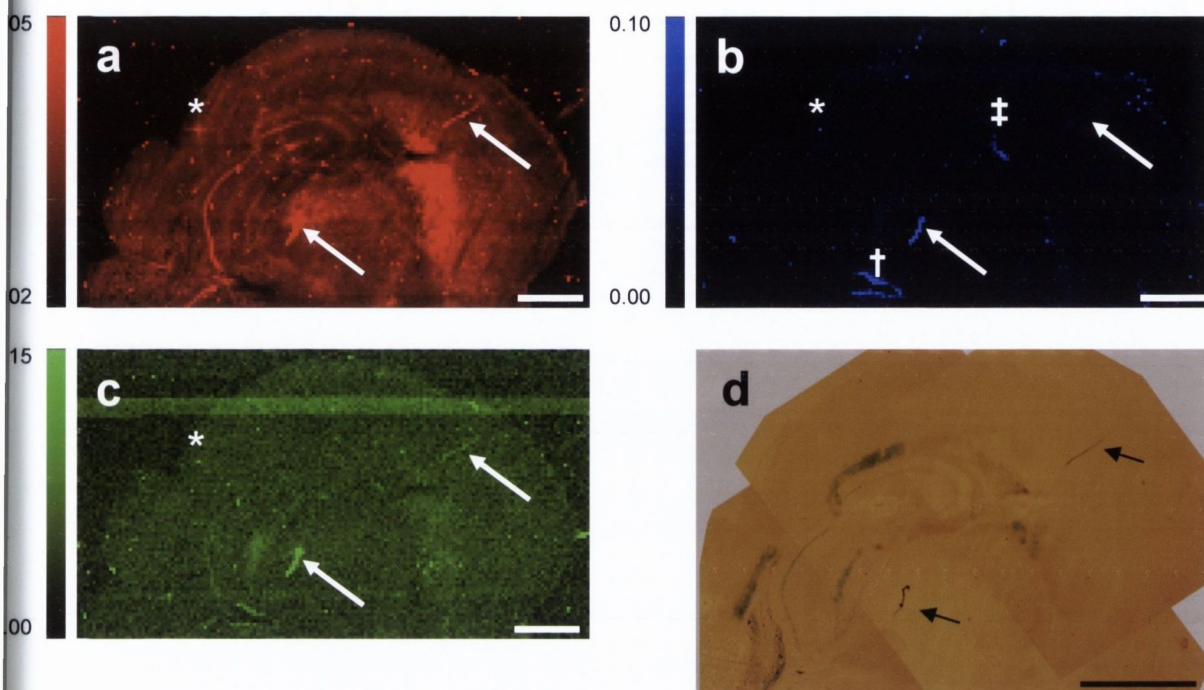


Figure 4.6. Identification of exogenous non-biological contaminant material.

Iron (a), chromium (b) and nickel (c) XRF maps of a brain section prepared from a female APP/PS1 transgenic mouse and corresponding composite congo red and toluidine blue stained composite light micrograph (d) are presented. The arrows identify examples of large exogenous non-biological material. Smaller deposits of contaminant particles are identified with an asterisk (these are not visible in the composite light micrograph). Nickel deposition in the dorsal 3rd ventricle (†) and the lateral ventricle (‡) is also identified. In order to enable visualisation of the gross anatomy of the brain section, chromium and nickel XRF maps did not undergo background subtraction. XRF scale bar = 1200 μm and pixel dimensions = 60 x 60 μm . Intensity bars represent normalized metal fluorescence and are proportional to total metal present. All light micrographs used in the composite image were taken at x2.5 magnification, scale bar = 1200 μm .

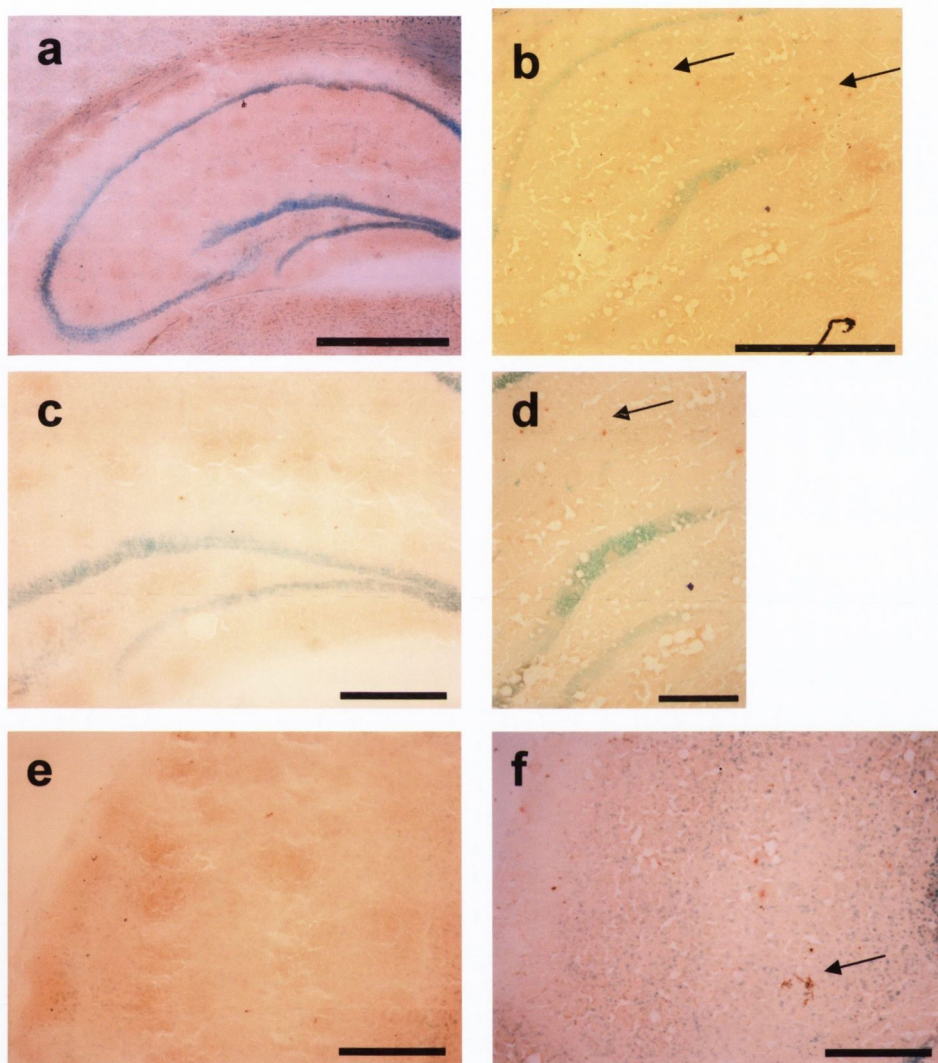


Figure 4.7. Amyloid deposition was identified in an XRF-assessed brain section prepared from a female APP/PS1 transgenic mouse.

Congo red and toluidine blue staining of XRF-assessed brain sections prepared from a wildtype mouse (a, c, and e) and female APP/PS1 transgenic mouse (b, d and f) are shown. Micrographs from the hippocampus (a – d) and the cortex (e – f) are presented. Amyloid deposition (arrows) is present in the micrographs prepared from APP/PS1 transgenic tissue only. (a, b) were taken at x5 magnification, scale bar = 500 μm . (c – f) were taken at x10 magnification, scale bar = 200 μm .

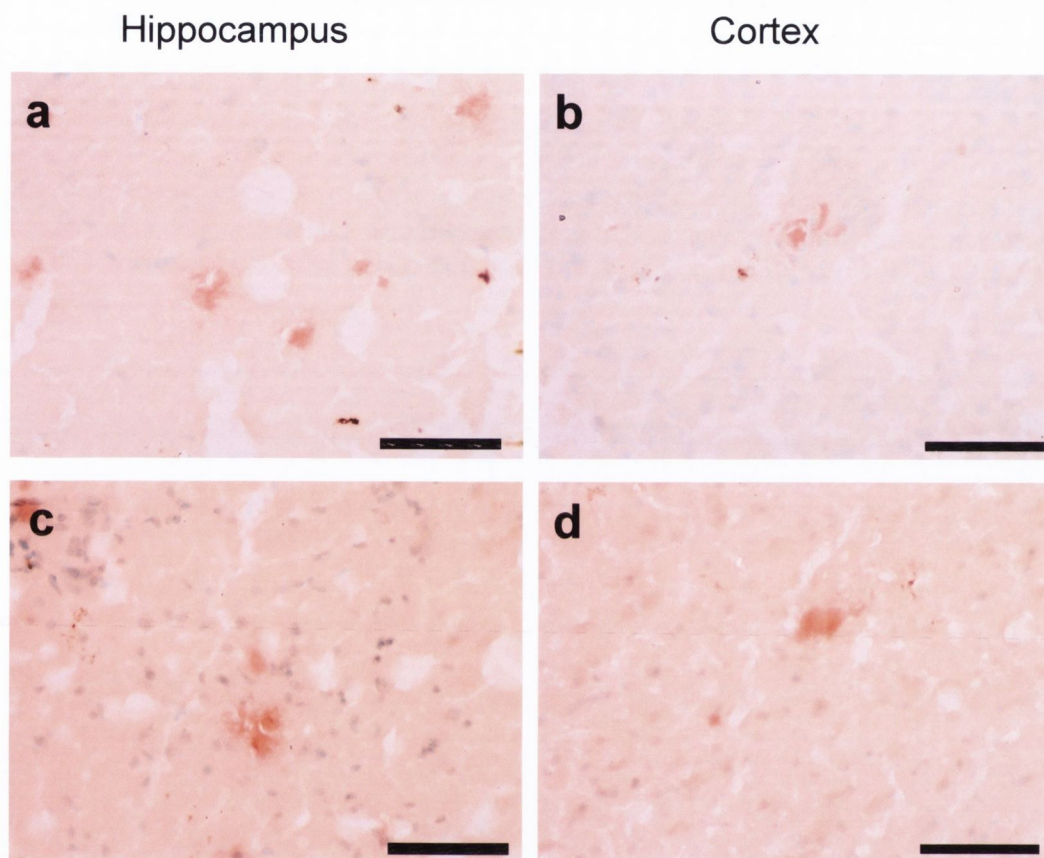


Figure 4.8. XRF-assessed amyloid plaques exhibited decreased congo red intensity.

Congo red and toluidine blue staining of a brain section prepared from a female APP/PS1 transgenic mouse is presented. Areas assessed using XRF (a, b) and corresponding contralateral areas that were not assessed (c, d) are presented. Micrographs of the hippocampus (a, c) and the cortex (b, d) are presented. XRF-assessed amyloid plaques exhibited decreased congo red staining intensity compared with amyloid plaques that did not undergo XRF assessment. All micrographs were taken at x40 magnification, scale bar = 60 μ m.

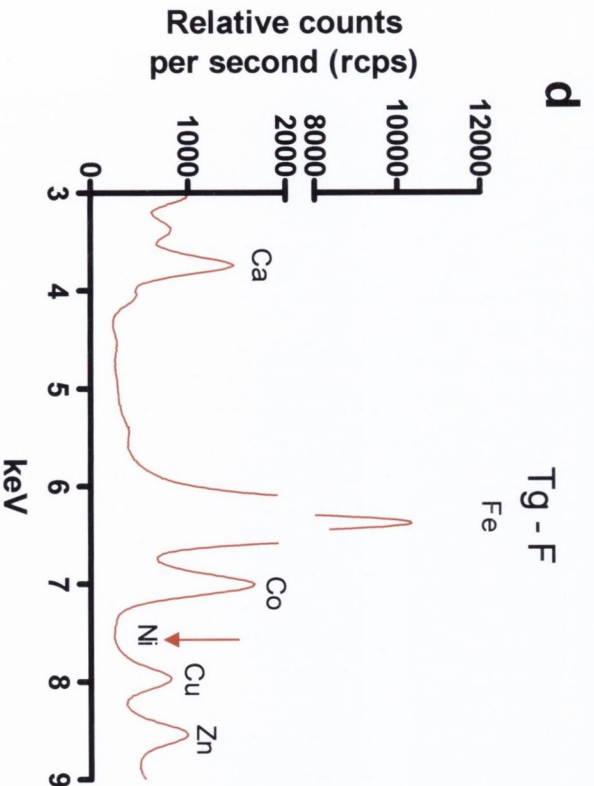
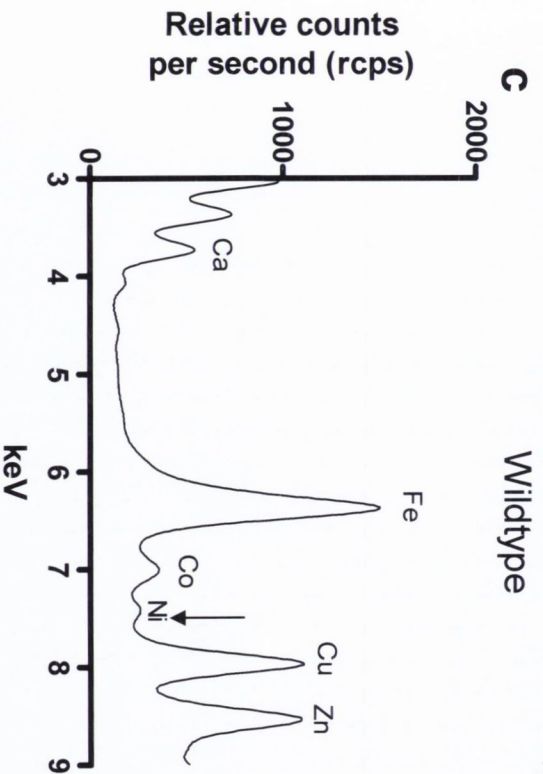
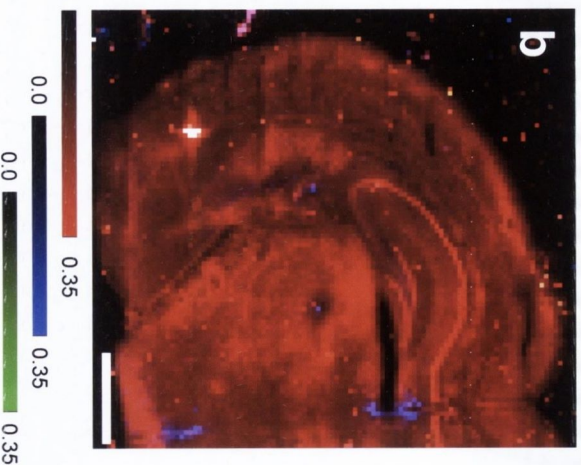
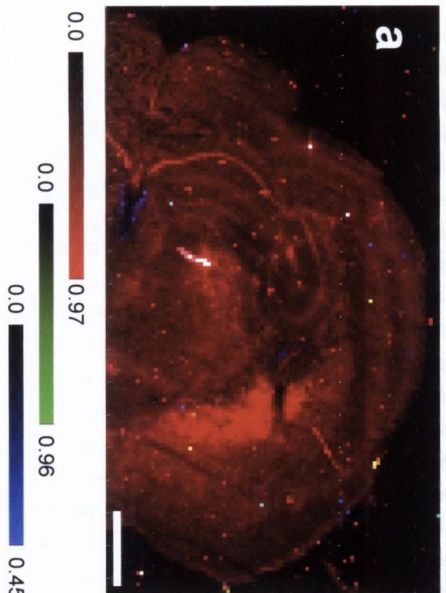


Figure 4.9. Increased iron deposition in brain sections obtained from APP/PS1 transgenic mice does not colocalize with nickel deposition.

Composite XRF maps of brain sections prepared from a female APP/PS1 transgenic (a) and a wildtype (b) mouse are shown, with red representing iron, green representing chromium and blue representing nickel. The XRF spectra of pixels in the top 5% of iron XRF intensity for the brain section prepared from a wildtype (c) and female APP/PS1 transgenic (d) mouse are presented. A nickel peak, identified by arrows, is present in the XRF spectra from pixels in the brain section prepared from a wildtype mouse only. XRF spectra were normalized to pixel number, and for each brain section, background noise spectra associated with the quartz slide was subtracted. XRF scale bar = 1200 μm and pixel dimensions = 60 x 60 μm . Intensity bars represent normalized metal fluorescence and are proportional to total metal present.

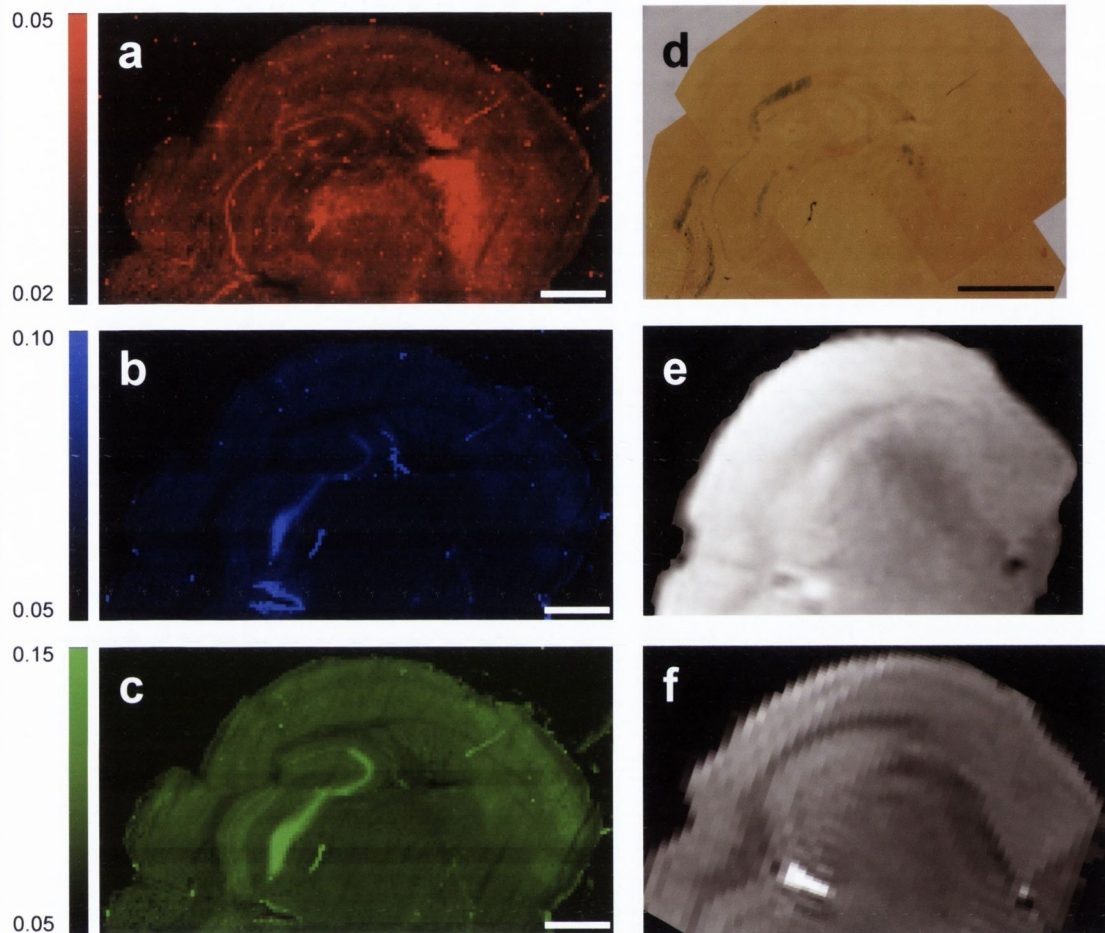


Figure 4.10. Comparison of metal XRF contrast with MRI contrast in a brain section prepared from a female APP/PS1 transgenic mouse.

Iron (a), zinc (b) and copper (c) XRF maps of a brain section prepared from a female APP/PS1 transgenic mouse and the corresponding composite congo red and toluidine blue stained composite light micrograph (d) are shown; corresponding T1-weighted (e) and T2-weighted (f) MRI scans are also presented. XRF scale bar = 1200 μm and pixel dimensions = 60 x 60 μm . All light micrographs used in the composite image were taken at x2.5 magnification, scale bar = 1200 μm . Intensity bars represent normalized metal fluorescence and are proportional to total metal present.

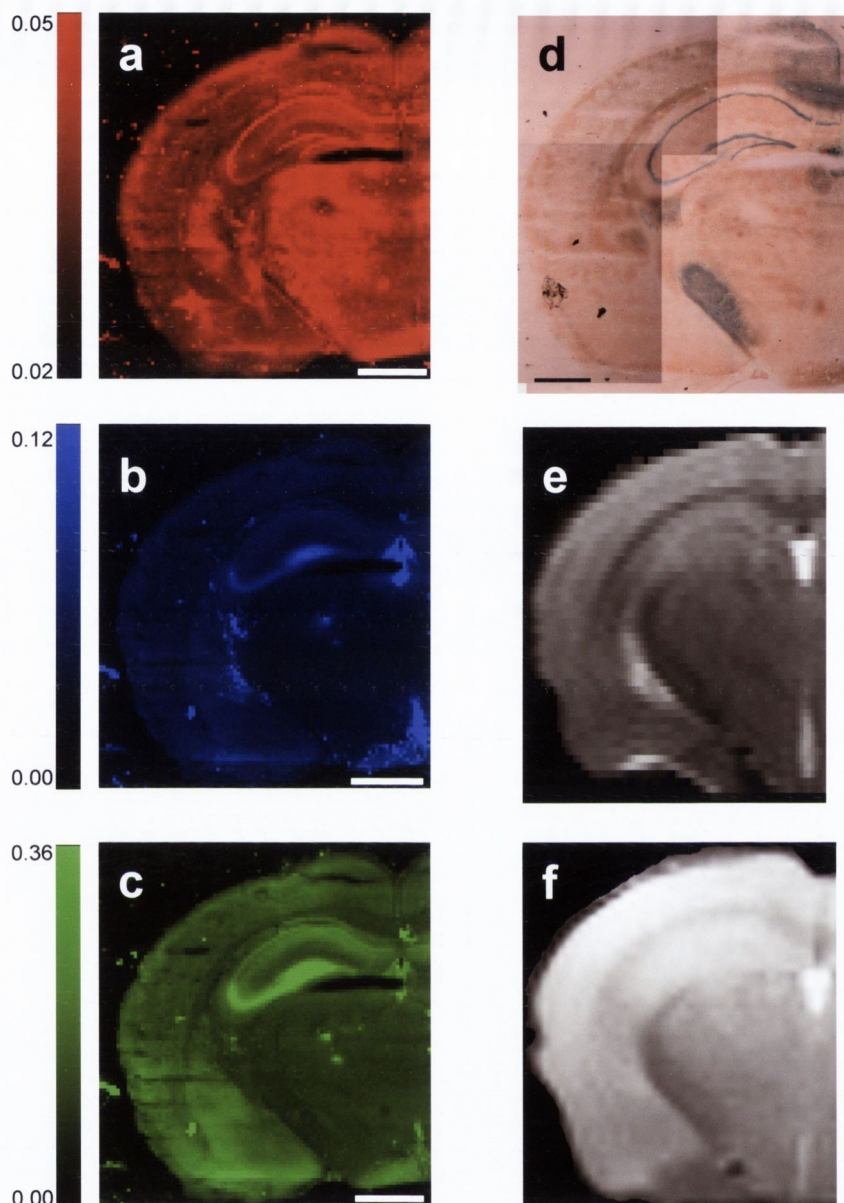
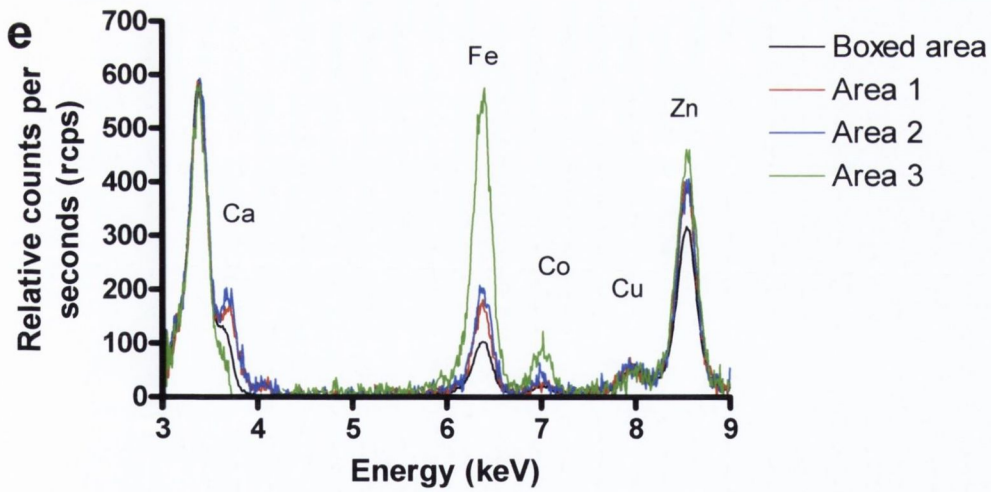
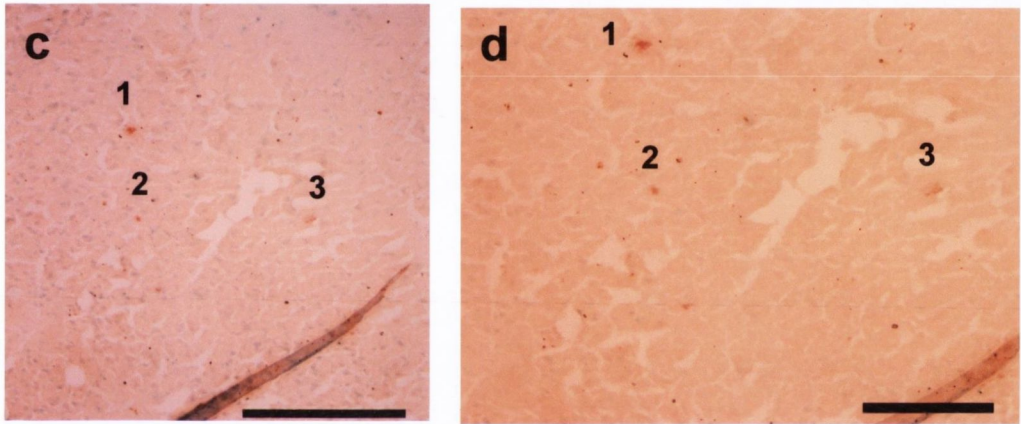
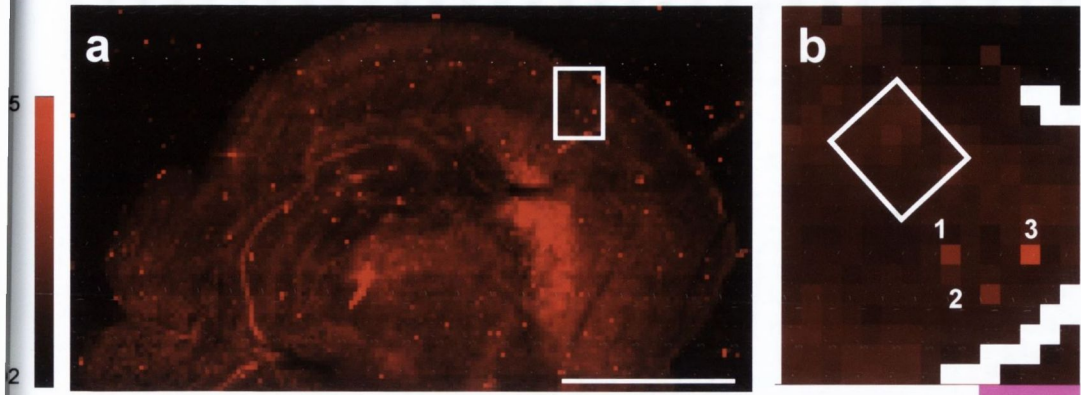


Figure 4.11. Comparison of metal XRF contrast with MRI contrast in a brain section prepared from a wildtype mouse.

Iron (a), zinc (b) and copper (c) XRF maps of a brain section prepared from a wildtype mouse and the corresponding composite congo red and toluidine blue stained composite light micrograph (d); corresponding T1-weighted (e) and T2-weighted (f) MRI scans. XRF scale bar = 1200 μm and pixel dimensions = 60 x 60 μm . All light micrographs used in the composite image were taken at x2.5 magnification, scale bar = 1200 μm . Intensity bars represent normalized metal fluorescence and are proportional to total metal present.



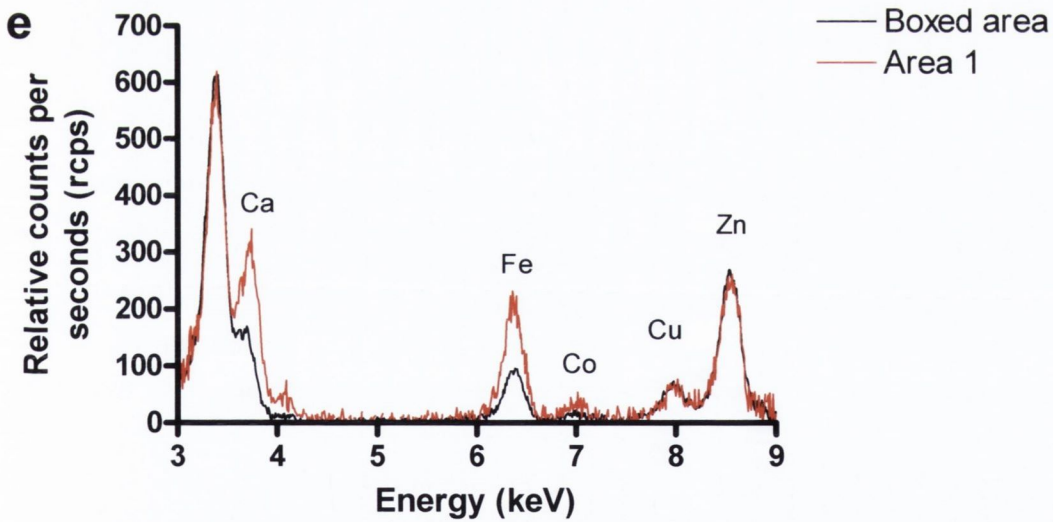
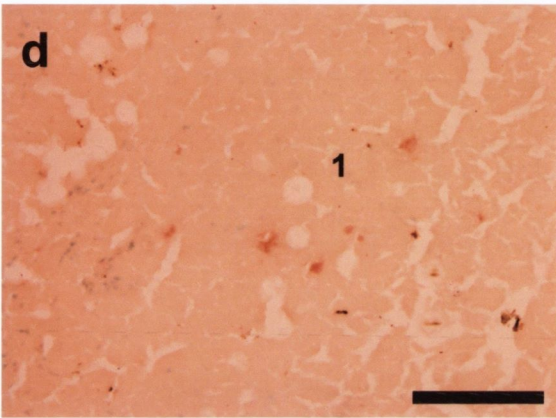
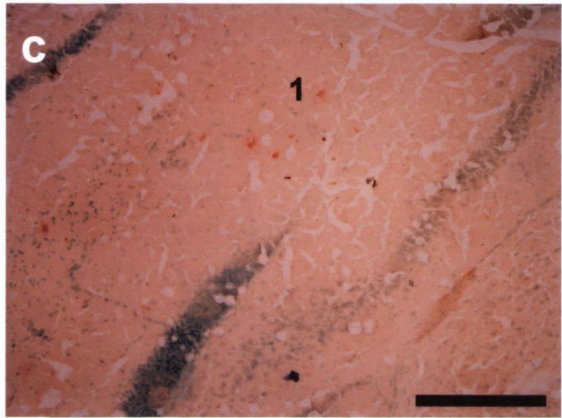
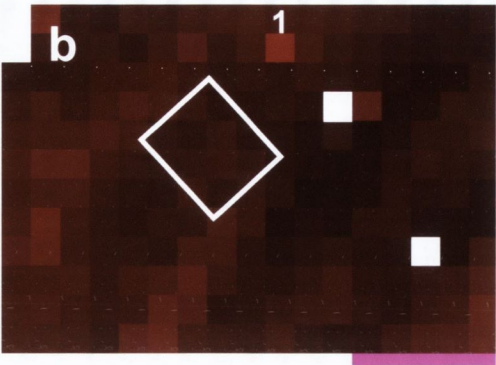
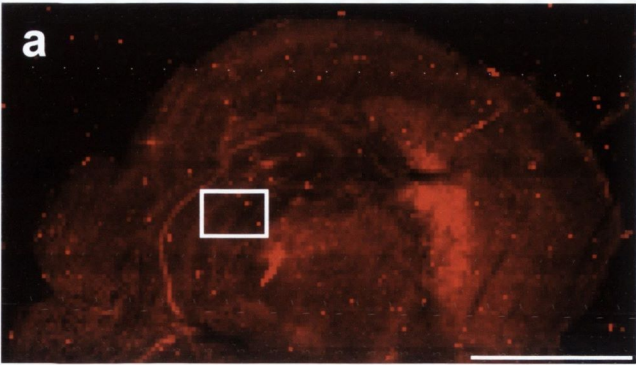
f % difference compared to cortical tissue (boxed area)

	Ca	Cr	Mg	Fe	Co	Ni	Cu	Zn
Area 1	35%	-	-	75%	-	-	19%	26%
Area 2	53%	-	-	95%	323%	-	-2%	13%
Area 3	-49%	-	-	455%	308%	-	3%	45%

Figure 4.12. Analysis of amyloid deposition in the auditory cortex of a female APP/PS1 transgenic mouse.

Areas of high iron deposition (labelled 1, 2, and 3) and an area without increased iron deposition (boxed area) in the dorsal auditory cortex of a brain section prepared from a female APP/PS1 transgenic mouse are indentified. Iron XRF maps (a, b) and associated congo red and toluidine blue light microscopy (c, d), XRF spectra (e) and associated XRF peak analysis (f) are presented.

The boxed area in (a) is magnified and displayed in (b). Light microscopy revealed that congo red-positive amyloid plaques were spatially correlated with areas of high iron XRF intensity. XRF spectra and peak analysis indicated only iron was consistently increased in areas associated with amyloid plaque deposition. (a) XRF scale bar = 1200 μm and pixel dimensions = 60 x 60 μm . (b) XRF scale bar = 300 μm and pixel dimensions = 60 x 60 μm , the boxed area was used as the cortical tissue background. White pixels identify contamination due to high nickel concentration. Intensity bars represent normalized metal fluorescence and are proportional to total metal present. (c) Image taken at x10 magnification, scale bar = 200 μm . (d) Image taken at x10 magnification, scale bar = 100 μm . (e) XRF spectra were normalized to pixel number, and for each brain section, background noise spectra associated with the quartz slide was subtracted.



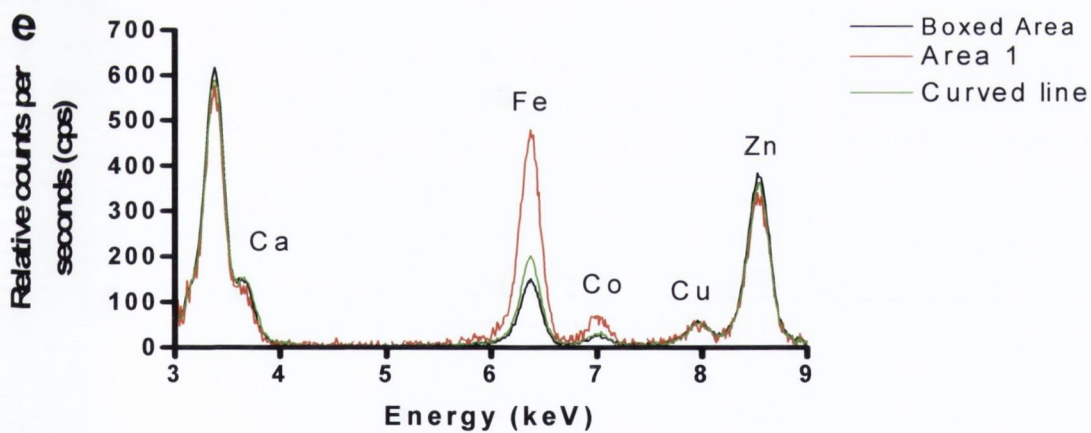
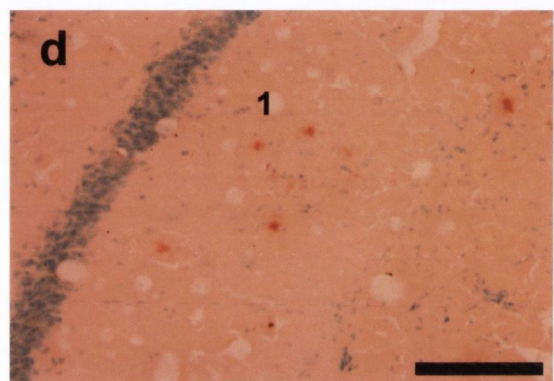
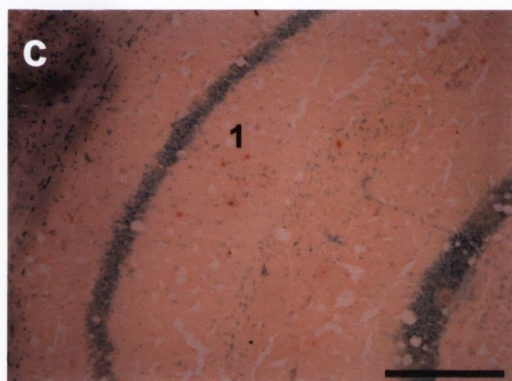
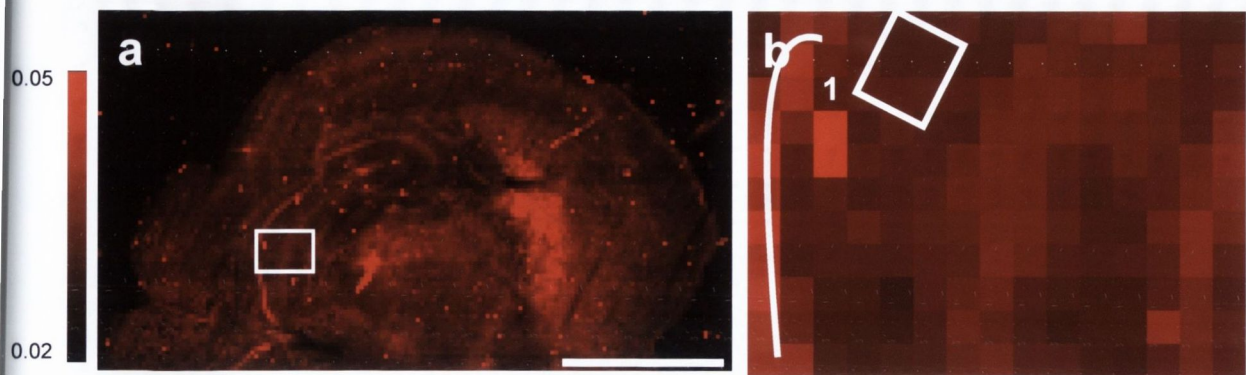
f

	% difference compared to hippocampal CA2 tissue (boxed area)							
	Ca	Cr	Mg	Fe	Co	Ni	Cu	Zn
Area 1	102%	-	↑	141%	199%	-	5%	-5%

Figure 4.13. Analysis of amyloid deposition in the CA2 field of a female APP/PS1 transgenic mouse.

Areas of high iron deposition (labelled 1) and an area without increased iron deposition (boxed area) in the CA2 field of the hippocampus of a brain section prepared from a female APP/PS1 transgenic mouse are identified. Iron XRF maps (a, b) and associated congo red and toluidine blue light microscopy (c, d), XRF spectra (e) and associated XRF peak analysis (f) are presented.

The boxed area in (a) is magnified and displayed in (b). Light microscopy revealed that congo red-positive amyloid plaques were spatially correlated with an area of high iron XRF intensity. XRF spectra and peak analysis indicated only iron was increased in the area associated with amyloid plaque deposition. (a) XRF scale bar = 1200 μm and pixel dimensions = 60 x 60 μm . (b) XRF scale bar = 300 μm and pixel dimensions = 60 x 60 μm , the boxed area was used as background for hippocampal CA2 field tissue. White pixels identify contamination due to high nickel concentration. Intensity bars represent normalized metal fluorescence and are proportional to total metal present. (c) Image taken at x10 magnification, scale bar = 200 μm . (d) Image taken at x20 magnification, scale bar = 100 μm . (e) XRF spectra were normalized to pixel number, and for each brain section, background noise spectra associated with the quartz slide was subtracted.



f % difference compared to hippocampal CA1 tissue (boxed area)

	Ca	Cr	Mg	Fe	Co	Ni	Cu	Zn
Area 1	-8%	-	82%	218%	44%	-	14%	-11%
Pyramidal layer (curved line)	1%	-	-6%	34%	13%	-	-12%	-6%

Figure 4.14. Analysis of amyloid deposition in the CA1 field of a female APP/PS1 transgenic mice.

An area of high iron deposition (labelled 1), an area without increased iron deposition (boxed area) and the pyramidal cell layer (curved line) in the hippocampus of a brain section prepared from a female APP/PS1 transgenic mouse are identified. Iron XRF maps (a, b) and associated congo red and toluidine blue light microscopy (c, d), XRF spectra (e) and associated XRF peak analysis (f) are presented.

The boxed area in (a) is magnified and displayed in (b). Light microscopy revealed that congo red-positive amyloid plaques were spatially correlated with areas of high iron XRF intensity. XRF spectra and peak analysis indicated only iron was increased in the area associated with amyloid plaque deposition. (a) XRF scale bar = 1200 μm and pixel dimensions = 60 x 60 μm . (b) XRF scale bar = 300 μm and pixel dimensions = 60 x 60 μm , the boxed area was used as the background in hippocampal CA1; the pyramidal cell layer is represented by the curved white line. White pixels identify contamination due to high nickel concentration. Intensity bars represent normalized metal fluorescence and are proportional to total metal present. (c) Image taken at x10 magnification, scale bar = 200 μm . (d) Image taken at x20 magnification, scale bar = 100 μm . (e) XRF spectra were normalized to pixel number, and for each brain section, background noise spectra associated with the quartz slide was subtracted.

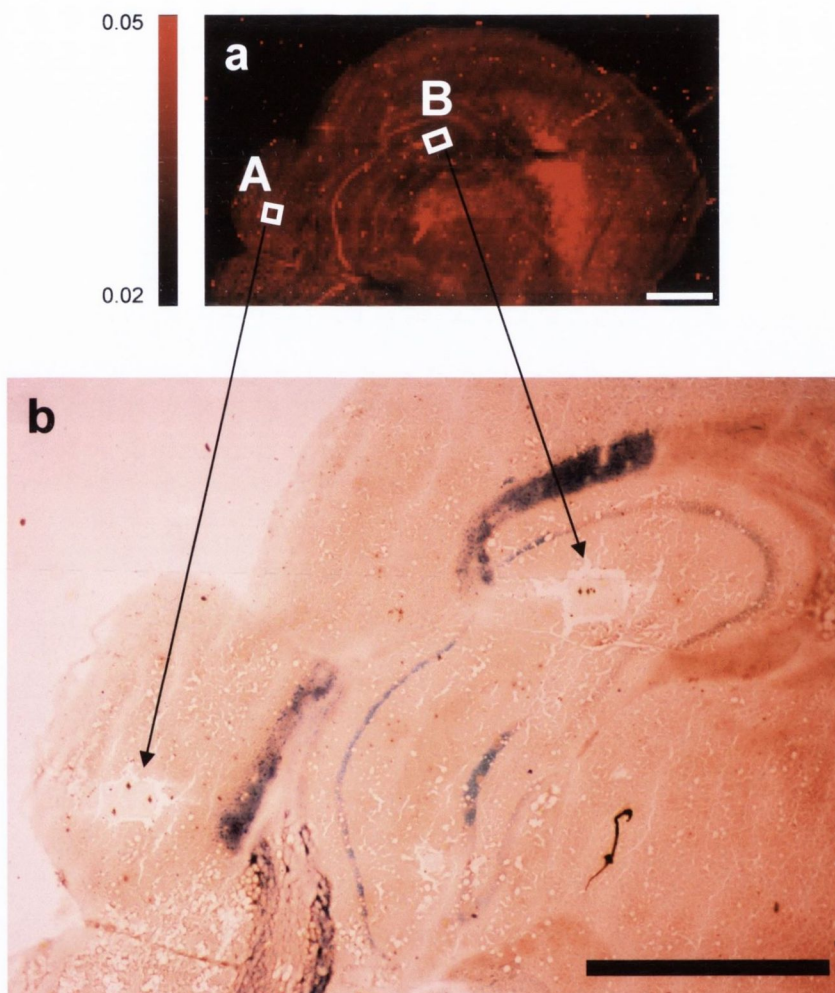


Figure 4.15. Microfocus mapping locations in an XRF-assessed brain section prepared from a female APP/PS1 transgenic mouse.

Two areas in an XRF-assessed brain section prepared from a female APP/PS1 transgenic mouse were chosen for microfocus mapping, the dorsal retrosplenial cortex (Area A) and the CA2 field (Area B) of the hippocampus. These are identified on the XRF iron map (a) and on the corresponding congo red and toluidine blue-stained light micrograph (arrows; b). XRF scale bar = 1200 μm and pixel dimensions = 60 x 60 μm . Intensity bar represents normalized iron fluorescence and is proportional to total iron present. All light micrographs used in the composite image were taken at x2.5 magnification, scale bar = 1200 μm .

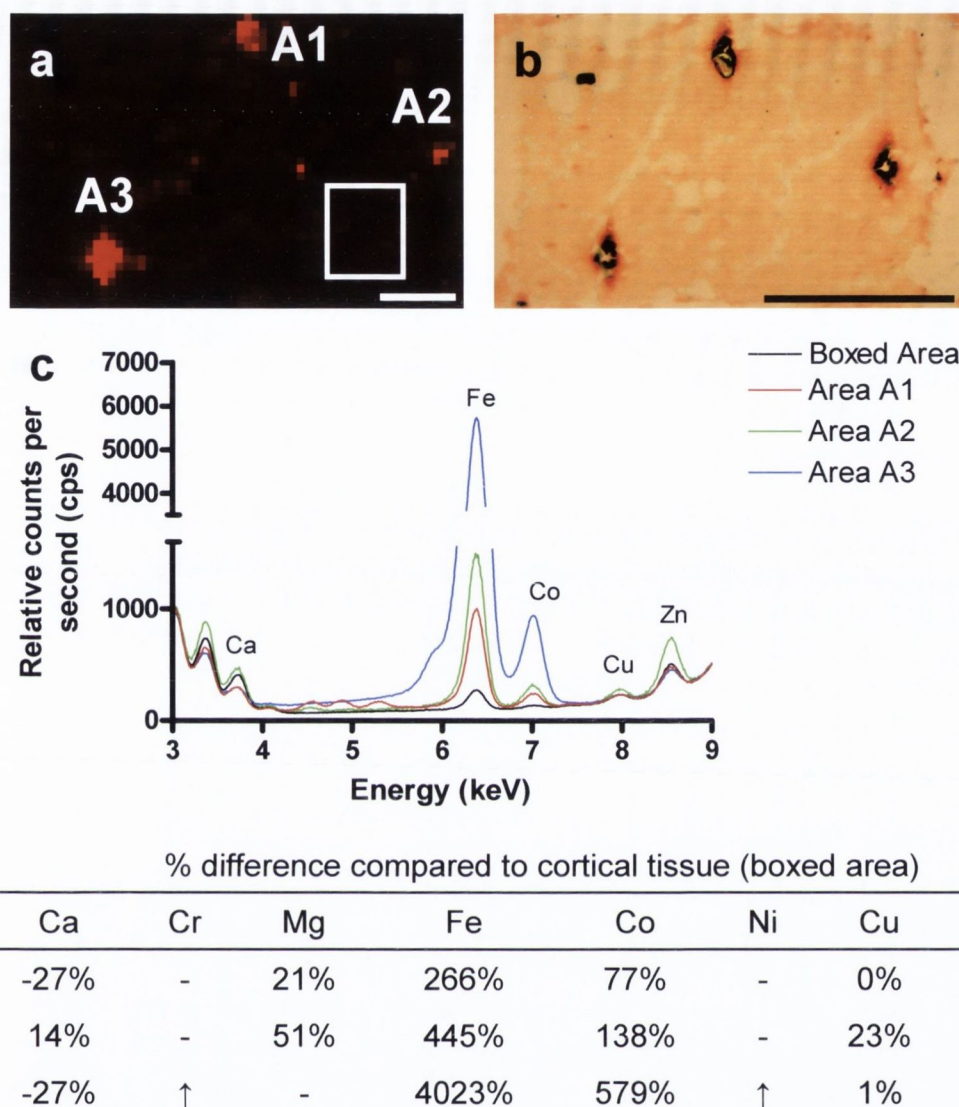


Figure 4.16. Microfocus XRF analysis in the dorsal retrosplenial cortex of a female APP/PS1 transgenic mouse.

Microfocus XRF analysis was undertaken in areas of high iron deposition in the dorsal retrosplenial cortex of a brain section prepared from a female APP/PS1 transgenic mouse. Microfocus iron XRF maps (a) and associated congo red and toluidine blue light microscopy (b), XRF spectra (c) and associated XRF peak analysis (d) are presented. Areas of high iron deposition labelled in (a) were chosen for XANES analysis. The boxed area was used as the background and termed cortical tissue in XRF spectra peak analysis. (a) XRF pixel dimensions = 5 x 5 μm and scale bar = 50 μm . Intensity bar represents normalized iron fluorescence and is proportional to total iron present. (b) Light microscopy image was taken at x20 magnification, scale bar = 100 μm . (c) XRF spectra of areas labelled in (b), spectra were normalized to pixel number and background noise spectra associated with the quartz slide was subtracted.

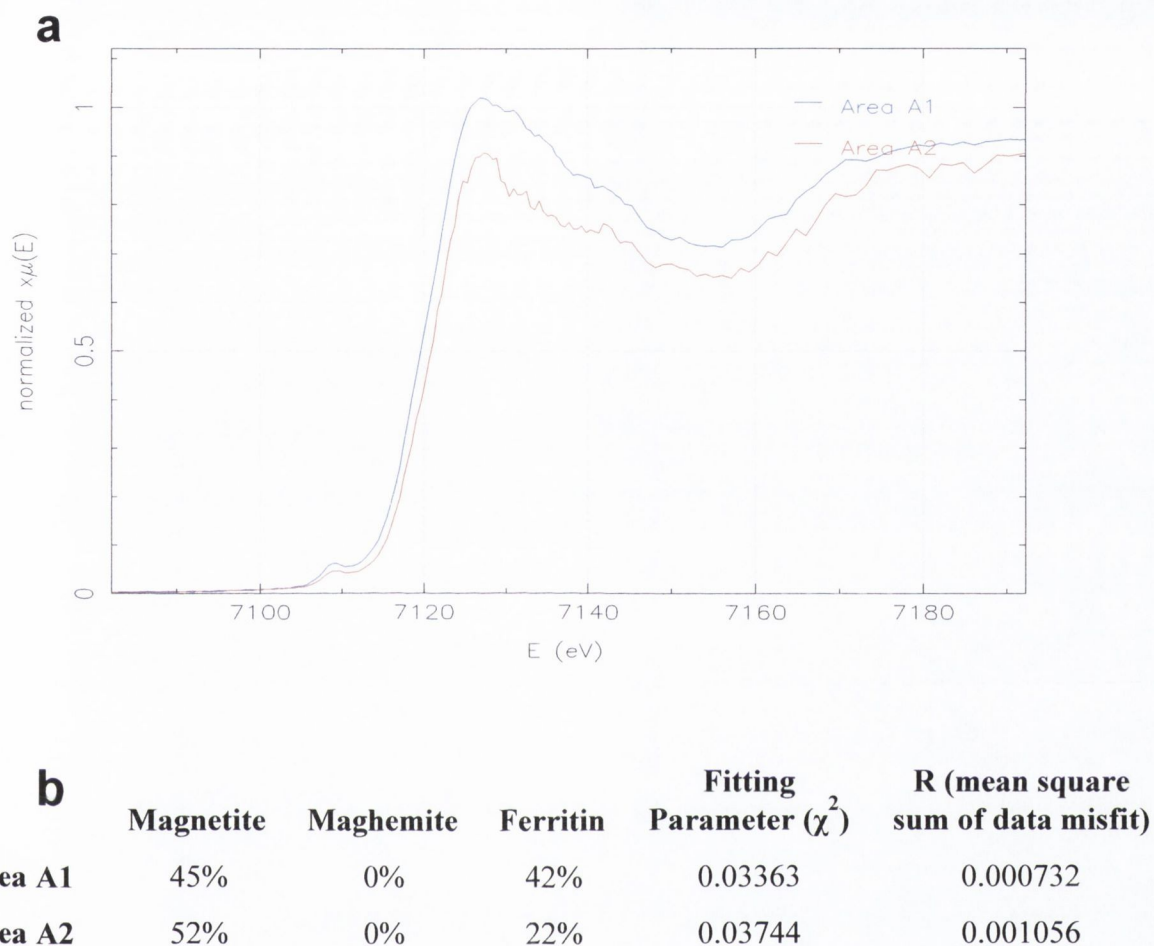


Figure 4.17. XANES spectra and linear-combination fits in Area A.

XANES spectra (a) and linear-combination fits (b) of areas of high iron deposition in Area A. XANES spectra were normalized, background subtracted and linear combination fits to XANES spectra of magnetite, maghemite and ferritin standards were assessed.

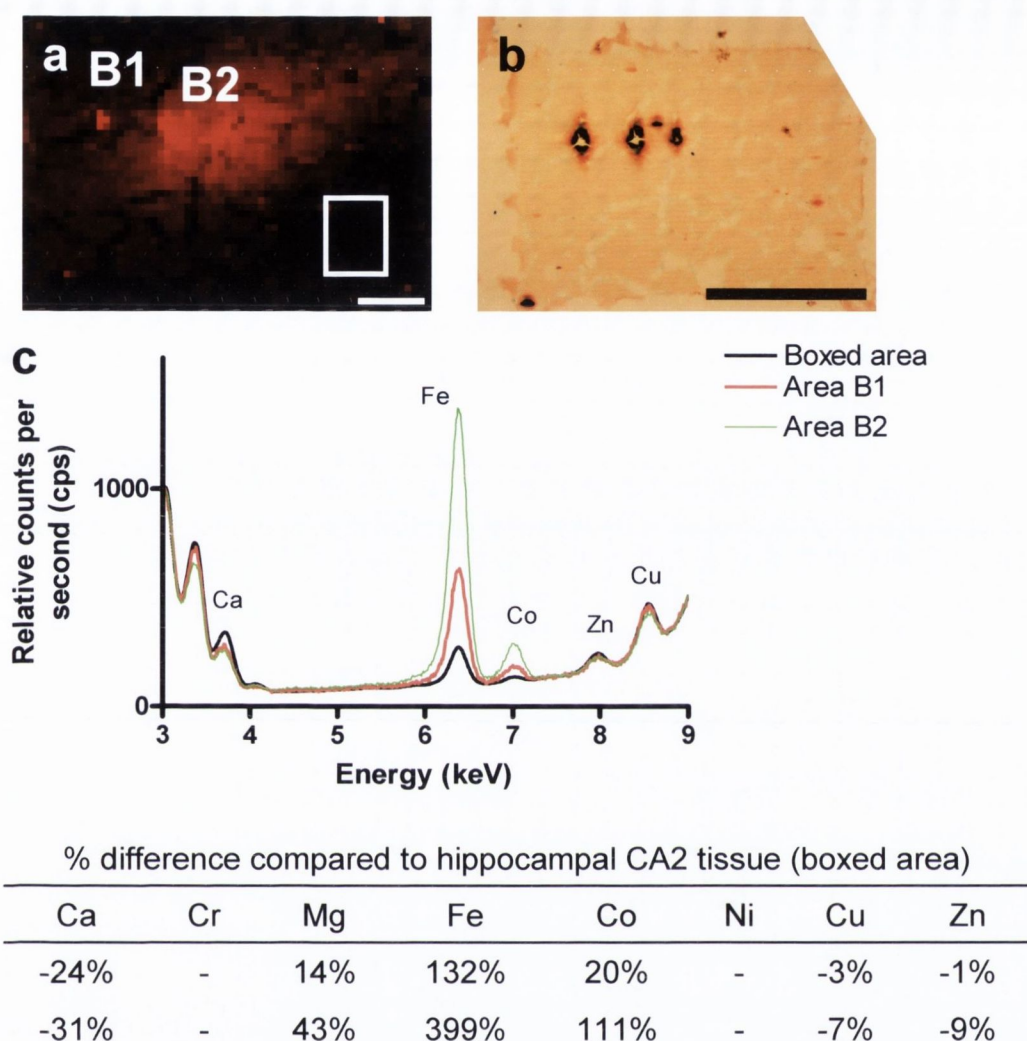
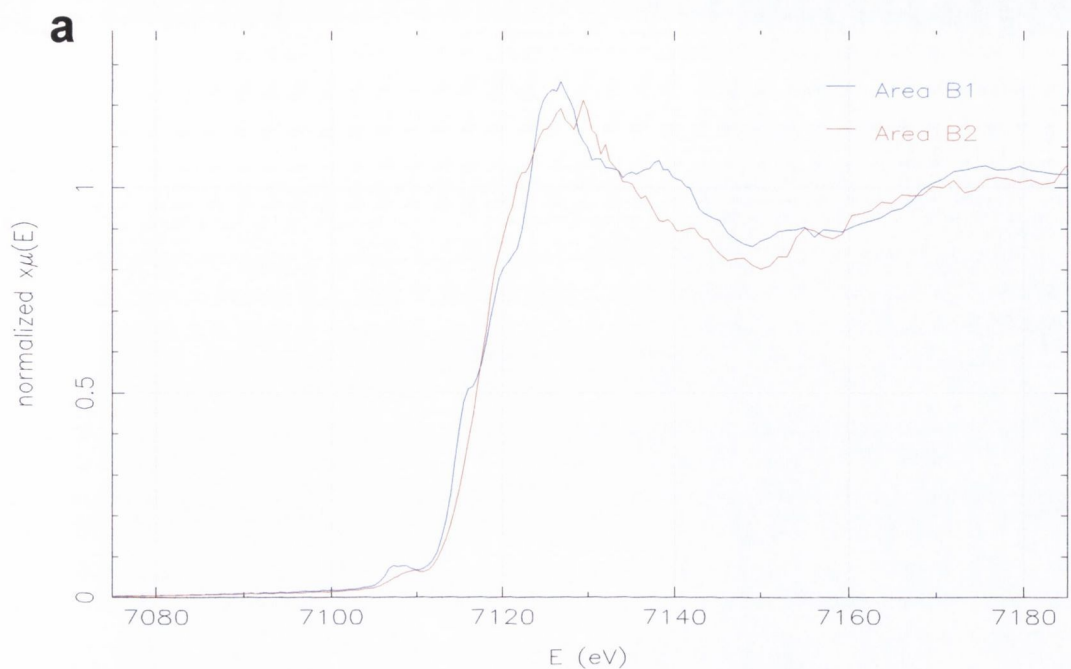


Figure 4.18. Microfocus XRF analysis in the CA2 field of the hippocampus of a female APP/PS1 transgenic mouse.

Microfocus XRF analysis was undertaken in areas of high iron deposition in the CA2 field of the hippocampus of a brain section prepared from a female APP/PS1 transgenic mouse. Microfocus iron XRF maps (a) and associated congo red and toluidine blue light microscopy (b), XRF spectra (c) and associated XRF peak analysis (d) are presented. Areas of high iron deposition labelled in (a) were chosen for XANES analysis. The boxed area was used as the background and termed hippocampal CA2 tissue in XRF peak analysis. (a) XRF pixel dimensions = 5 x 5 μm and scale bar = 50 μm . (b) Light microscopy image was taken at x20 magnification, scale bar = 100 μm . Intensity bar represents normalized iron fluorescence and is proportional to total iron present. (c) XRF spectra of areas labelled in (b), spectra were normalized to pixel number and background noise spectra associated with the quartz slide was subtracted.



b

	Magnetite	Maghemite	Ferritin	Fitting Parameter (χ^2)	R (mean square sum of data misfit)
Area B1	77%	20%	0%	0.18669	0.002785
Area B2	38%	9%	51%	0.15782	0.002342

Figure 4.19. XANES spectra and linear-combination fits in Area B.

XANES spectra (a) and linear-combination fits (b) of areas of high iron deposition in Area B. XANES spectra were normalized, background subtracted and linear combination fits to XANES spectra of magnetite, maghemite and ferritin standards were assessed.

Chapter 5

Results

5.1 Introduction

There is considerable evidence implicating neuroinflammation in AD, with microglial activation and associated increases in ROS and proinflammatory cytokine production (Griffin et al., 1989, Wood et al., 1993, Cacabelos et al., 1994). Senile plaques, the presence of which is a diagnostic hallmark of AD, are primarily composed of fibrillar A β ₁₋₄₂, and have been shown to be colocalized with iron, copper and zinc (Lovell et al., 1998). Activated microglia and astrocytes have been shown to surround senile plaques in human AD tissue (Itagaki et al., 1989). Aggregated A β has been shown to exert neurotoxic effects *in vitro* and *in vivo*; this lends support to the amyloid hypothesis which suggests that A β is the primary causative agent in AD (Walsh and Selkoe, 2007). Recent advances have led to the development of transgenic APP/PS1 mouse models of AD, in which transgene derived human A β concentration levels are increased through the expression of human APP and A β ₁₋₄₂ production is favoured through the expression of the PS1 gene. Although A β plaque deposition increases with age, these models do not exhibit neuronal loss (Lau et al., 2008, Maheswaran et al., 2009), thus providing the opportunity to assess changes due to A β presence only. This may more closely model early-stage A β deposition in human AD.

The central aim of this study was to assess the neuroinflammation, and particularly microglial activation, associated with early-stage A β plaque deposition in an APP/PS1 transgenic mouse model of AD. The data indicate that microglial activation was increased, particularly around A β -containing plaques in brain sections prepared from APP/PS1 transgenic mice. These animals exhibited impaired performance in spatial memory tasks which was more profound in female, compared with male, mice and which correlated with changes in A β deposition in brain tissue and in the cerebromicrovasculature.

5.2 Results

The performance of 8 – 9 month old APP/PS1 mice was impaired in the reversal phase of the MWM task.

Tail snips were obtained from mice, cDNA was prepared and APP/PS1 transgenic mice were identified by genotyping (Figure 5.1). The average ages of mice in wildtype and APP/PS1 transgenic groups were similar at the start of the experiment (3 – 4 months of age) and at this time, mean weights were similar in all groups. Age was a significant factor in animal weights (female, $p < 0.0001$; (F(1, 57.61); male, $p < 0.0001$; (F(1, 67.80); 2-way ANOVA); when animals were 8 – 9 months of age, male APP/PS1 transgenic mice were significantly heavier than the other experimental groups ($p < 0.05$; ANOVA Bonferroni post-tests; Figure 5.2).

Animals were assessed for muscle strength and coordination using the hangwire and inverted screen tests and the data indicate that the performance of mice in each group was similar at 8 – 9 months of age (Figure 5.3). Spatial learning was assessed in the MWM and the data indicate that all mice learned the task; the mean pathlength, and mean latency to find the hidden platform, on the final day of training, was similar in wildtype and APP/PS1 transgenic mice. Training was a significant factor in the decrease in mean pathlength taken by both male and female animal groups (male, $p < 0.05$; F(4, 3.664); female, pathlength, $p < 0.001$; F(4, 6.170); 2-way ANOVA; Figure 5.4). Training also lead to significant decreases in latency in both groups (male, $p < 0.0001$; (F(4, 5.940); female, $p < 0.001$; F(4, 7.563); 2-way ANOVA). Analysis also indicated that genotype was a significant factor in the latency of male mice ($p < 0.01$; F(1, 7.481); 2-way ANOVA), but this effect did not translate to inter-group differences on any training day.

During the reversal phase of the MWM task animals, are tested on the ability to learn a modified version of the original task. All groups learnt the modified task

(Figure 5.5). Analysis indicated that time was significant factor in pathlength in both male ($p < 0.001$; $F(3, 9.509)$; 2-way ANOVA) and female mice ($p < 0.001$; $F(3, 9.347)$; 2-way ANOVA). Repeated trials also lead to a significant decrease in latency in both male ($p < 0.001$; $F(3, 10.930)$; 2-way ANOVA) and female mice ($p < 0.001$; $F(1, 21.06)$; 2-way ANOVA). However, genotype was a significant factor in the performance of female mice only (latency, $p < 0.001$; $F(1, 21.06)$; 2-way ANOVA) leading to female APP/PS1 mice exhibiting impaired MWM performance on day 2 (latency, $**p < 0.001$; Bonferroni post-tests). Female APP/PS1 transgenic mice continued to exhibit a decrease in latency on day 4 ($p < 0.01$; ANOVA) when compared with female wildtype mice; similar effects were not observed in male experimental groups.

The impaired performance of the female APP/PS1 transgenic mice in the reversal phase of the MWM was not due to a difference in average swim speeds (Figure 5.6), although during the training phase average swim speed was significantly lower in female APP/PS1 transgenic mice compared with that of female wildtype mice ($**p < 0.05$; ANOVA).

Plaque deposition is greater in the brain tissue of female APP/PS1 mice.

Deposition of amyloid plaques in the cortex and hippocampus of APP/PS1 transgenic mice was confirmed using congo red staining and pan-amyloid immunofluorescence. Amyloid plaques displayed morphology consistent with that described in the literature. Female APP/PS1 transgenic mice display an increased level of cerebral amyloid plaque deposition compared with male APP/PS1 transgenic mice (Figure 5.7). Plaque deposition was observed throughout the cortex and the majority of the hippocampus, however the dentate gyrus consistently displayed reduced deposition compared with other hippocampal structures. No plaque deposition was observed in the thalamus. Plaques ranged in size but no plaques greater than 50-60 μm in diameter were observed using histochemistry or immunofluorescence. Immunofluorescent labeling of pan-amyloid species enabled the identification of smaller deposits of amyloid, possibly amyloid fibrils in the early stages of plaque

formation (Figure 5.8). Deposits of this size, although present, were not easily identifiable using congo red staining; this may indicate low levels of β -pleated sheet structures in smaller deposits. The presence of small deposits of pan-amyloid immunofluorescence proximal to plaques was observed throughout all brain sections.

Further investigation of amyloid plaque composition confirmed the presence of iron in plaque cores (Figure 5.9). Plaques displayed two distinct morphologies; compact, dense plaques were characterized by higher levels of congo red staining in the core of the plaque, whereas diffuse plaques displayed notably less congo-red staining in the plaque core (Figure 5.10). This consistent with the literature and suggests a reduction in β -pleated structures in the core of diffuse plaques when compared to compact plaques.

No vascular amyloid plaques were observed in congo-red stained brain sections from APP/PS1 transgenic mice. However, minimal amyloid immunofluorescence was observed close to blood vessels in APP/PS1 brain sections (Figure 5.11); this image was prepared by Belinda Grehan and is shown with her permission.

Soluble and insoluble amyloid- β peptides were present in the brain tissue of APP/PS1 transgenic mice.

Soluble and insoluble amyloid- β peptide concentrations were assessed in brain tissue prepared from female APP/PS1 transgenic, male APP/PS1 transgenic and wildtype mice. These data are presented in Figures 5.12 and 5.13.

Soluble $A\beta_{1-42}$, $A\beta_{1-40}$ and $A\beta_{1-38}$ were significantly greater in brain tissue prepared from APP/PS1 transgenic, compared with, wildtype mice (* $p < 0.05$; ** $p < 0.01$; ANOVA; Figure 5.12). A similar increase was observed in the 3 insoluble $A\beta$ species (** $p < 0.01$; *** $p < 0.001$; ANOVA; Figure 5.13). Soluble $A\beta_{1-38}$ was the dominant soluble peptide and its concentration was significantly higher than the other species (* $p < 0.05$, ANOVA; Figure 5.12). The concentrations of soluble $A\beta_{1-42}$ and

A β ₁₋₄₀ were negligible in tissue prepared from wildtype mice but in brain tissue prepared from APP/PS1 transgenic mice soluble A β ₁₋₄₂ was significantly greater compared with soluble A β ₁₋₄₀ concentrations (*p < 0.05; **p < 0.01; Students t-test for independent means).

A comparison of the concentration of A β in the brain tissue of male and female APP/PS1 transgenic mice revealed that, whereas there was no gender difference in soluble A β , concentrations of insoluble A β were higher in female, compared with male APP/PS1 transgenic mice and the difference was significant in the case of A β ₁₋₄₂ (### p<0.001; ANOVA; Figure 5.13). A β ₁₋₄₂ was the dominant peptide in female APP/PS1 transgenic brain tissue and was present in significantly greater concentrations than A β ₁₋₃₈ and A β ₁₋₄₀ (p < 0.001, ANOVA); A β ₁₋₄₂ and A β ₁₋₄₀ concentration levels were similar in male APP/PS1 transgenic brain tissue. This suggests that A β ₁₋₄₂ was the primary peptide involved in amyloid aggregation and plaque formation.

MHCII- and CD68- positive cells surround amyloid plaques.

Double-staining of brain sections for plaques and markers of microglial activation was carried out and example confocal micrographs obtained from the brain of a female APP/PS1 transgenic mouse are presented in Figure 5.14. Nuclei are seen to encircle plaques but there are no nuclei within the plaque cores; this was a consistent finding in every plaque investigated. Cells proximal to plaques express MHCII to a greater extent than cells distal to plaques, suggesting activated microglia may migrate to plaques.

Brain sections from female APP/PS1 transgenic mice were further investigated for amyloid plaques and CD68 by immunohistochemical staining, since CD68 is a marker of activated, perhaps phagocytic, microglia. Marked CD68 expression was observed proximal to amyloid plaques compared with CD68 immunofluorescence in other areas of the brain section and semi-quantitative analysis

revealed that immunofluorescence was significantly increased in sections prepared from female APP/PS1 transgenic, compared with wildtype mice (** $p < 0.01$; Student's t-test for independent means; Figure 5.15) and that the immunofluorescence proximal to the plaques was increased compared with that distal to the plaques in APP/PS1 transgenic mice ($^{§§}p < 0.01$; Student's t-test for independent means).

Microglial and astrocytic activation is upregulated in brain tissue prepared from 8 – 9 month old APP/PS1 transgenic mice.

Previous data, Figures 5.14 - 15, provided evidence for an overall increase in markers of microglial activation. Brain tissue was assessed for markers of microglial activation, CD11b and CD68, and astrocytic activation, GFAP. The data show that CD11b and CD68 mRNA were significantly increased in brain tissue prepared from female APP/PS1, compared with wildtype, mice (* $p < 0.05$; *** $p < 0.001$; ANOVA; Figures 5.16a, b). Similar increases were observed in tissue prepared from male APP/PS1 mice but the increase was statistically significant only in the case of CD68 mRNA (* $p < 0.05$; ANOVA). GFAP mRNA was significantly increased in tissue prepared from APP/PS1 transgenic, compared with, wildtype mice (** $p < 0.01$; *** $p < 0.001$; ANOVA; Figure 5.16c) but the value was significantly greater in tissue prepared from male, compared with female APP/PS1 transgenic mice ($^{§}p < 0.05$; ANOVA).

To investigate if the increase in markers of glial activation were paralleled by changes in cytokines, IL-1 β mRNA and TNF- α concentration were assessed in aliquots of the same tissue. IL-1 β mRNA expression was significantly increased in tissue prepared from female, but not male, APP/PS1 mice compared with wildtype mice (** $p < 0.01$; ANOVA; Figure 5.17). Similarly, TNF- α concentration was significantly increased in tissue prepared from female, but not male, APP/PS1 transgenic mice (* $p < 0.05$; ANOVA).

No changes in gray matter volume due to ageing.

Volumetric analysis, of high resolution T2-weighted MRI brain scans, was performed using VBM. Assessment of gray matter volume changes from 3 – 4 months to 8 – 9 months of age for each experimental group is presented in Figure. 5.18. No differences in gray matter volume were observed, the statistical overlay was thresholded at $p < 0.001$.

MRI relaxometry was differentially affected by age and genotype.

T1 and T2 relaxation times were assessed when mice were aged 3 – 4 months and again at 8 – 9 months. Hippocampal and cortical relaxometry data are plotted in Figures 5.19 and 5.20.

Age was not a significant factor in hippocampal and cortical T1 relaxation times, Figure 5.19. Further analysis of these data indicated that differences due to genotype were present when mice were 3 – 4 months of age and isolated to male APP/PS1 transgenic mice. T1 relaxation times were significantly higher in male compared with female APP/PS1 transgenic and wildtype mice (male and female) T1 relaxation times at 3 – 4 months of age (hippocampus, $p < 0.05$; cortex, $p < 0.05$; ANOVA Bonferroni post-tests). This difference was not present at 8 – 9 months of age.

Hippocampal and cortical T2 relaxation time was significantly affected by age in male mice only (hippocampus, $p < 0.001$; $F(1, 29.74)$; cortex, $p < 0.001$; $F(1, 28.36)$; 2-way ANOVA, Figure 5.20). Male APP/PS1 transgenic and wildtype mice exhibited a significant age-related decline in T2 relaxation time in the hippocampus (transgenic, $p < 0.001$; wildtype, $p < 0.001$; Students t-test for independent means; values at 8-9 months versus 3-4 months) and cortex (transgenic, $p < 0.001$; wildtype, $p < 0.05$; Students t-test for independent means; values at 8-9 months versus 3-4 months). Female wildtype and female APP/PS1 transgenic mice did not exhibit an

age-related decline in T2 relaxation time as displayed by male APP/PS1 transgenic and male wildtype mice.

Female APP/PS1 transgenic mice had an increased cortical and hippocampal $rCBV_{lw}$ and $rCBF$ and display an age-related decrease in hippocampal $rCBF$.

Due to previous reports of deficits in vasculature autoregulation in APP/PS1 transgenic mice and the complex effect this may have on age-related changes, only age-related differences within each experimental group were examined statistically (Niwa et al, 2002). Cerebral blood flow ($rCBF_{lw}$) was assessed in female APP/PS1 transgenic mice and female wildtype mice at 3 – 4 months of age; this analysis was repeated when the animals were 8 – 9 months of age. $rCBV_{lw}$ was assessed using a novel ASL technique (Kelly and colleagues, 2009). All ASL data are presented in Table 3, Appendix III, no age-related changes in CTT or MTT were observed.

Female wildtype mice and female APP/PS1 transgenic mice did not display a statistically significant age-related decline $rCBV_{lw}$. However, $rCBF$ in the hippocampus of female APP/PS1 transgenic mice was significantly decreased at 8 – 9 months of age compared to 3 – 4 months of age ($p < 0.01$, Students t-test for independent means; values at 8 – 9 months versus 3 – 4 months of age; Figure 5.22). Female wildtype mice did not display a statistically significant age-related decline in $rCBF$.

5.3 Discussion

This study set out to assess the neuroinflammation associated with early-stage A β plaque deposition. The data indicate that plaque deposition, which was more profound in female, compared with male, 8 – 9 month old APP/PS1 transgenic mice, was associated with microglial and astrocytic activation, evidence of increased proinflammatory cytokine expression and deficits in spatial learning.

Significant plaque deposition, accompanied by increased soluble and insoluble A β was observed in tissue prepared from APP/PS1 mice. The data indicate that female APP/PS1 transgenic mice exhibited greater plaque deposition and accordingly higher levels of insoluble A β_{1-42} , although not A β_{1-38} or A β_{1-40} , than male APP/PS1 mice. This finding concurs with a previous report indicating that female transgenic mouse models of AD display higher concentrations of plaques (Wang et al., 2003). Soluble A β_{1-42} was increased in male, but not female, APP/PS1 transgenic mice compared with wildtype mice suggesting that the increased number A β plaques seen in the female APP/PS1 transgenic mice are not due to increased amounts of soluble A β_{1-42} . Plaque deposition exhibited similar spatial profiles in APP/PS1 transgenic mice suggesting the mechanism determining *in vivo* formation of plaques is not brain structure associated. In addition, similar numbers of diffuse plaques were seen in both female and male APP/PS1 transgenic mice suggesting that there is no gender bias in the mechanism responsible for the maturation of diffuse plaques into dense core plaques. These findings provide evidence for A β plaque mediated activation of microglia being similar in male and female APP/PS1 transgenic mice but *in vivo* plaque formation being more likely in female APP/PS1 transgenic mice.

Microglial activation was assessed in this study by evaluating MHC II immunoreactivity and expression of CD11b and CD68 mRNA and the data indicate that all measures were increased in brain tissue prepared from APP/PS1 transgenic mice, compared with wildtype, mice. It is known that CD11b mRNA is expressed at very low levels under resting conditions and is markedly increased when microglia are activated in response to any stress (Lynch, 2009). In the present study, CD11b

mRNA was increased in both male and female APP/PS1 transgenic mice although the increase failed to reach statistical significance in the male animals because of the greater variability. Expression of CD68 was markedly increased in both male and female APP/PS1 transgenic mice. CD68 is thought to be indicative of lysosomal activity (Lynch, 2009), which would suggest on-going phagocytosis in these cells. Importantly, while its expression was observed throughout the brain of APP/PS1 transgenic mice, CD68 expression was more marked proximal to A β plaques. However the current literature suggests that minimal phagocytosis of plaques occurs (Meyer-Luehmann et al., 2008) and therefore it must be suggested that the CD68-positive cells are involved in phagocytosis of cell debris adjacent to the A β plaques or perhaps smaller A β aggregates. In parallel with the increases in expression of CD11b and CD68, the evidence presented here indicates that both MHC II immunofluorescence and mRNA expression are increased in tissue prepared from APP/PS1 transgenic mice. This finding concurs with similar work which also observed an increase in MHC II in APP/PS1 transgenic mice at similar ages (Gordon et al., 2002). There is a great deal of evidence indicating that A β induces microglial activation in vitro and, similarly that injection of A β induces microglial activation in vivo (Walsh et al., 2002b) but there is considerable debate in the literature regarding this issue in AD. While there are obvious concerns in extrapolating from an animal model, the literature suggests that plaque formation precedes microglial activation (Gordon et al., 2002).

Like the markers of microglial activation, the present findings indicate that GFAP mRNA expression was increased in transgenic APP/PS1 mice and this is consistent with a previous report which also revealed that GFAP expression was increased in tissue prepared from APP/PS1 transgenic mice at similar ages (Gordon et al., 2002).

In the present study, changes were observed in both male and female transgenic APP/PS1 mice, although male mice exhibited a significantly higher level of GFAP expression compared with female APP/PS1 transgenic mice. The reason for this gender bias is unclear. One possible explanation is that soluble A β ₁₋₄₂, which is

significantly greater in tissue prepared from male APP/PS1 transgenic mice, may be predominantly responsible for activation of astrocytes.

It is known that microglia are the primary cell source of inflammatory cytokines, which they release when appropriately activated, although activated astrocytes are also involved in cytokine production (Lieberman et al., 1989). The present data reveal that IL-1 β mRNA and TNF- α concentration were increased in female, but not male, APP/PS1 transgenic mice. Thus parallel gender differences in plaque number, insoluble A β_{1-42} concentration, proinflammatory cytokines and, to a lesser extent, markers of microglial activation are identified here and lend some support to the view that deposition of A β plaques may mediate microglial activation and consequently upregulation of inflammatory cytokines. It is significant that proinflammatory cytokines like TNF- α can induce expression of a number of proinflammatory genes, such as those for adhesion molecules (ICAM-1, VCAM-1), and for the inflammatory cytokines, IL-6 (Lee and Benveniste, 1999) and that these signals can contribute to impairment of synaptic function and eventual neurodegeneration (Akassoglou et al., 2003).

Consistent with the negative impact of these signals on synaptic function, the data indicate that spatial learning was impaired in transgenic mice at 8 – 9 months of age. While no deficits were observed in the learning phase of the task, APP/PS1 transgenic mice exhibited an impairment in the reversal phase of the task with more profound and persistent changes in female, compared with male, mice. Although, there are reports of memory impairments in transgenic mouse models of AD, this is the first report of a gender bias in such measures (Puolivali et al., 2002, Cao et al., 2007). Previous studies reported a correlation between hippocampal concentration levels of total A β_{1-42} and performance in spatial memory tasks. Here A β analysis was not undertaken in hippocampal tissue and no significant correlations were observed between behavioural performance and concentration of insoluble or soluble A β_{1-42} , A β_{1-40} and A β_{1-38} . The absence of a correlation may be due to the fact that the task is hippocampal-dependent and A β was not assessed in this area but it may also reflect

the dynamic nature of A β plaque- or microglial- mediated hippocampal functional impairment, since the deposition of A β plaques varied between transgenic animals.

The poorer performance of female APP/PS1 transgenic in the spatial memory task may be associated with the increases in proinflammatory cytokines, which were observed only in tissue prepared from female APP/PS1 transgenic mice, although no positive correlation between these measures was found. Similarly although soluble A β potentially inhibits synaptic plasticity in the hippocampus (Walsh et al., 2002a), the impaired spatial memory observed in female mice did not correlate with changes in soluble A β_{1-42} although soluble A β_{1-40} and A β_{1-38} were increased. Overall, the present data provide support for the proposal that activation of microglia, together with the upregulation of proinflammatory cytokines, mediate the impairment in hippocampal function and highlight the fact that female APP/PS1 transgenic mice are particularly affected by the ongoing neuropathology present in this mouse model of AD.

Female APP/PS1 transgenic mice exhibited an age-related decline in rCBF in the hippocampus but not the cortex. This finding was not observed in a recent study which examined perfusion changes in an APP/PS2 transgenic mouse model of AD using a different theoretical model of CBF (Weidensteiner et al., 2009). These authors examined the change from 10 months to 17 months of age and age-related decreases in CBF and CBV were observed in the occipital and frontal cortices only. Studies on vascular function in APP/PS1 mice are rare. However, whole brain rCBV was reported to be decreased in 15 month old APP/PS1 transgenic mice when assessed using dynamic contrast enhanced MRI (Hooijmans et al., 2007). More recently, reduced perfusion in the cortex of APP/PS1 transgenic mice at 33, 54, and 62 weeks compared with wildtype mice, as measured by ASL, has been reported (Dhenain et al., 2008). Interestingly, several studies have reported a decrease in CBV and CBF in young, APP-mutated transgenic mice prior to amyloid plaque deposition (Niwa et al., 2002, Wu et al., 2004).

The present data indicate that female APP/PS1 transgenic and female wildtype mice do not display an age-related decline in rCBV_{Iw}. The rCBV_{Iw}

parameter calculated in this study contains a contribution from the extravascular space and this may therefore be one possible explanation for the finding being contrary to reports from the literature (Dhenain et al., 2008). The possible masking of putative intravascular deficits due to the use of this technique and conflicting reports from the literature suggest results derived from competing ASL methods are difficult to accurately compare across experiments.

Although plaque deposition was not confirmed in these animals at 3 – 4 months of age, others have reported the presence of plaques at this stage in APP/PS1 mice (Garcia-Alloza et al., 2006). Confocal microscopy carried out in the present study indicates that amyloid is present in the vasculature in 8 – 9 month old APP/PS1 transgenic mice and this is consistent with previous findings which indicated that similar deposition occurred in hippocampus and cortex of 6 month-old APP/PS1 transgenic mice (Gordon et al., 2002) and amyloid deposition in the leptomeningeal vasculature from 6 months of age onward (Garcia-Alloza et al., 2006). It is reasonable to suggest early-stage amyloid deposition would affect smaller blood vessels to a greater degree, due to the reduced area of the blood vessel. Selective interrogation of the intravascular ASL-derived CBV signal from such vessels, via selective crusher gradients, may position investigators to assess the earliest possible deficits in cerebral perfusion.

No observable decline in gray matter volume was observed in these animals with age. This finding is well supported by reports from the literature suggesting that modest volumetric changes occur, but only in much older animals (Lau et al., 2008, Delatour et al., 2006, Maheswaran et al., 2009). Therefore it might be concluded that the age-related decline in rCBF may be associated with CAA-mediated cerebrovasculature changes or A β -mediated neuronal impairment, but not neuronal loss. It is also possible that both of these factors contribute to the rCBF change observed since decreases in rCBF are usually associated with decreased neuronal function. Another possible explanation for the age-related decrease in rCBF may be a consequence of impaired astrocytic modulation of neuronal blood flow demands due

to neuroinflammation although it must be acknowledged that the perfusion demands of inflammatory tissue compared with normal tissue are not completely understood.

The relaxometry data presented here indicate the absence of any age-related in T2 relaxation time in female APP/PS1 transgenic and female wildtype mice, although a significant age-related decrease was observed in male APP/PS1 transgenic and wildtype mice. The underlying physiology of an age-related T2 relaxation time decline in non-pathological aging has been suggested to be due to changes in cell density and decreased water content (Weidensteiner et al, 2009). Previous reports of T2 relaxation time declines in mouse models of AD, although in older mice than those used here, have suggested the change is due to iron colocalization to the A β plaque, which has been confirmed here, as well as the hydrophobic nature of plaques (El Tannir El Tayara et al., 2006). However a decrease in T2 relaxation time in the subiculum of a mouse model of AD prior to amyloid deposition has been reported (El Tayara Nel et al., 2007). It is clear, however, that the level of plaque deposition observed in APP/PS1 transgenic mice in the present study is not sufficient to induce a decrease in T2 relaxation time.

The presence of the age-related decline in male APP/PS1 transgenic mice considering the significantly lower level of plaque deposition when compared to female APP/PS1 transgenic mice suggests plaque presence and any putative plaque-mediated changes in brain tissue are not the causative agent of the T2 relaxation time decline. The parallel of this gender differential in wildtype mice suggests this effect is entirely gender-specific. The reason for this bias is unclear and difficult to explain with reference to current interpretations of the biophysical basis of T2 relaxation time.

Male APP/PS1 transgenic mice displayed an increased cortical and hippocampal T1 relaxation time at 3 – 4 months, but not 8 – 9 months, of age. In contrast with this, two studies have reported a decrease in T1 relaxation time in mouse models of AD [Tayara 2006, Hooijmans, 2009]. The reason for the increase seen in the male APP/PS1 transgenic mice is unclear, one possible explanation may

be delayed maturation of brain tissue in male APP/PS1 transgenic mice. Such a process would explain the absence of the difference at 8 – 9 months, and, if this is the case, it must be suggested that the age-related difference is not associated with amyloid plaque deposition.

This study set out to assess the neuroinflammation associated with early-stage A β plaque deposition and the evidence presented indicates an increased level of neuroinflammation, characterized by microglial activation and proinflammatory cytokine production. These changes are postulated to contribute to changes in spatial memory and cerebral vasculature observed in the APP/PS1 mouse model of AD.

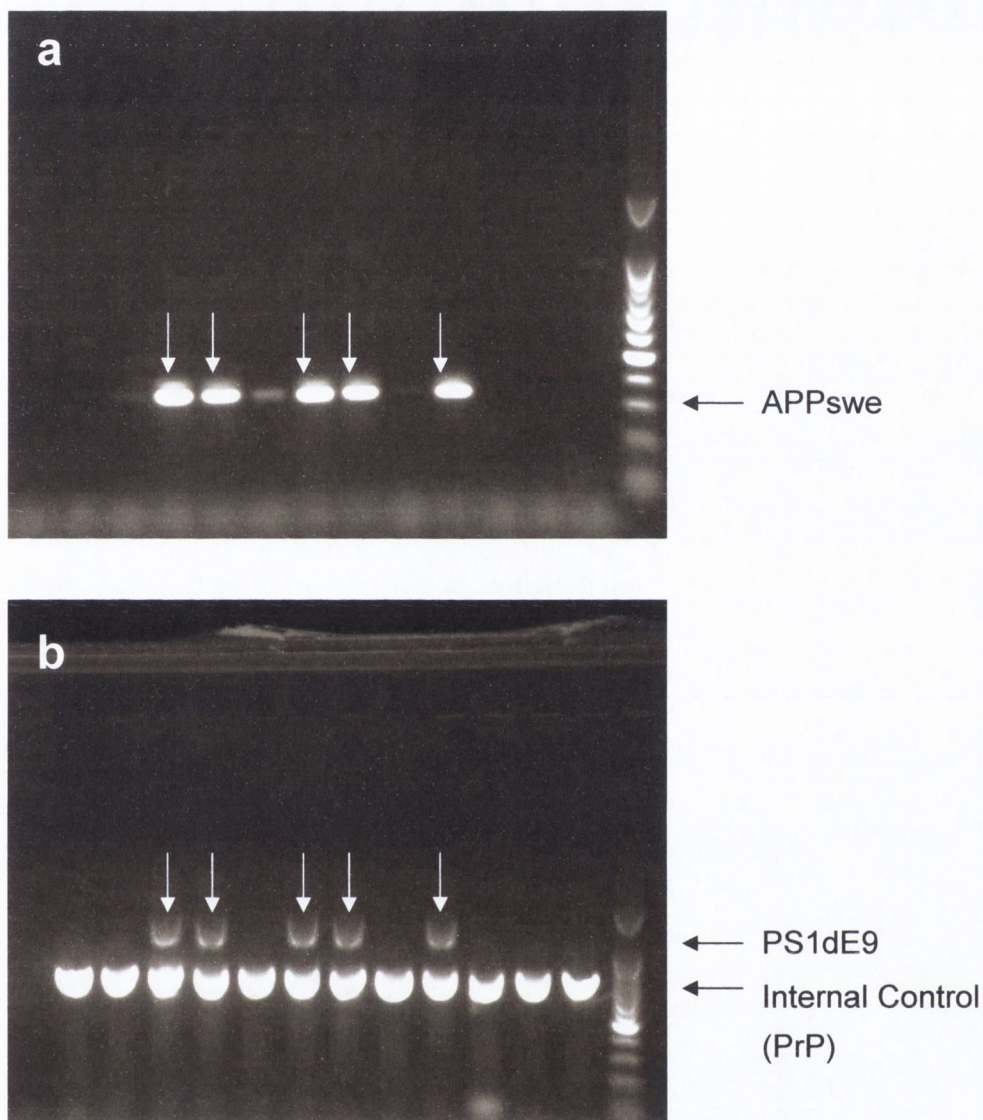


Figure 5.1. Genotype analysis of APPswe and PS1dE9 presence.

Representative gel electrophoresis of isolated cDNA from mouse tail snips. APPswe gene (a) and PS1dE9 and internal control (b) are shown. White arrows identify the transgenic animals. The lower band present in all samples in (b) is the internal control (PrP gene).

a

	Female Wildtype		Female APP/PS1		Male Wildtype		Male APP/PS1	
	Age (days)	n	Age (days)	n	Age (days)	n	Age (days)	n
3-4 months	151 ± 7.79	7	156 ± 13.5	11	143 ± 4.24	14	138 ± 7	7
8-9 months	292 ± 8.19	10	278 ± 12	8	312 ± 4.35	14	294 ± 15.5	8

b

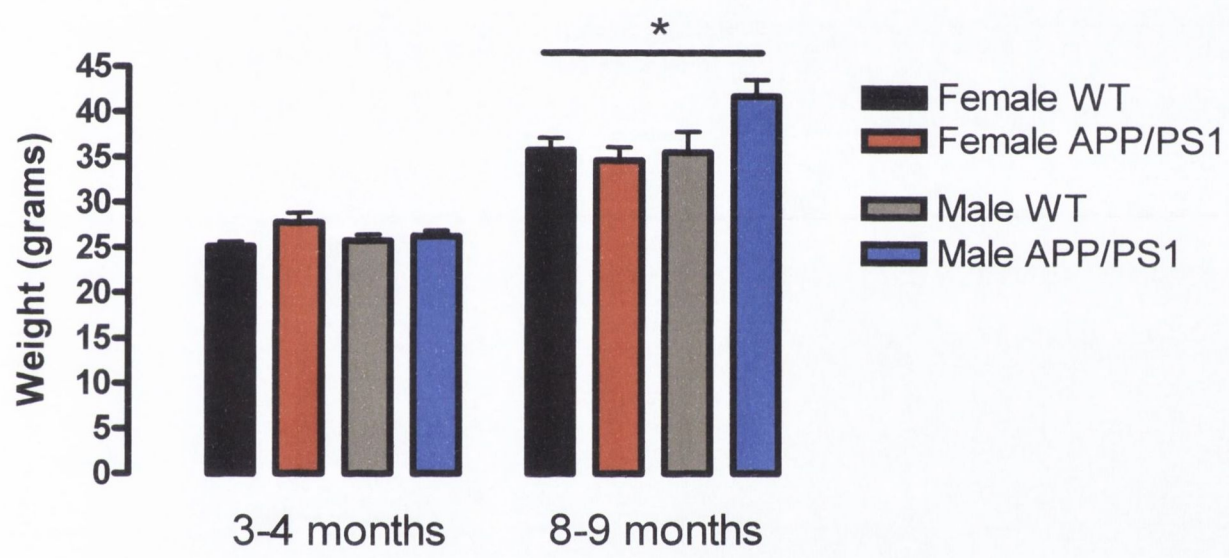


Figure 5.2. Animal ages and weights at experiment timepoints.

Mean age (a) and weights (b) of animals in the 3 groups are presented. Data are expressed as means ± SEM. Age was significant factor in animal weight (female, $p < 0.0001$; (F(1, 57.61); male, $p < 0.0001$; (F(1, 67.80); 2-way ANOVA). Genotype was a significant factor in the weights of male mice (F(1,4.885); $p < 0.05$; 2-way ANOVA), this effect was not observed in female mice. At timepoint 2, male APP/PS1 mice were significantly heavier than all other groups (* $p < 0.05$, ANOVA Bonferroni post-tests). The number of animals in each group is shown in (a).

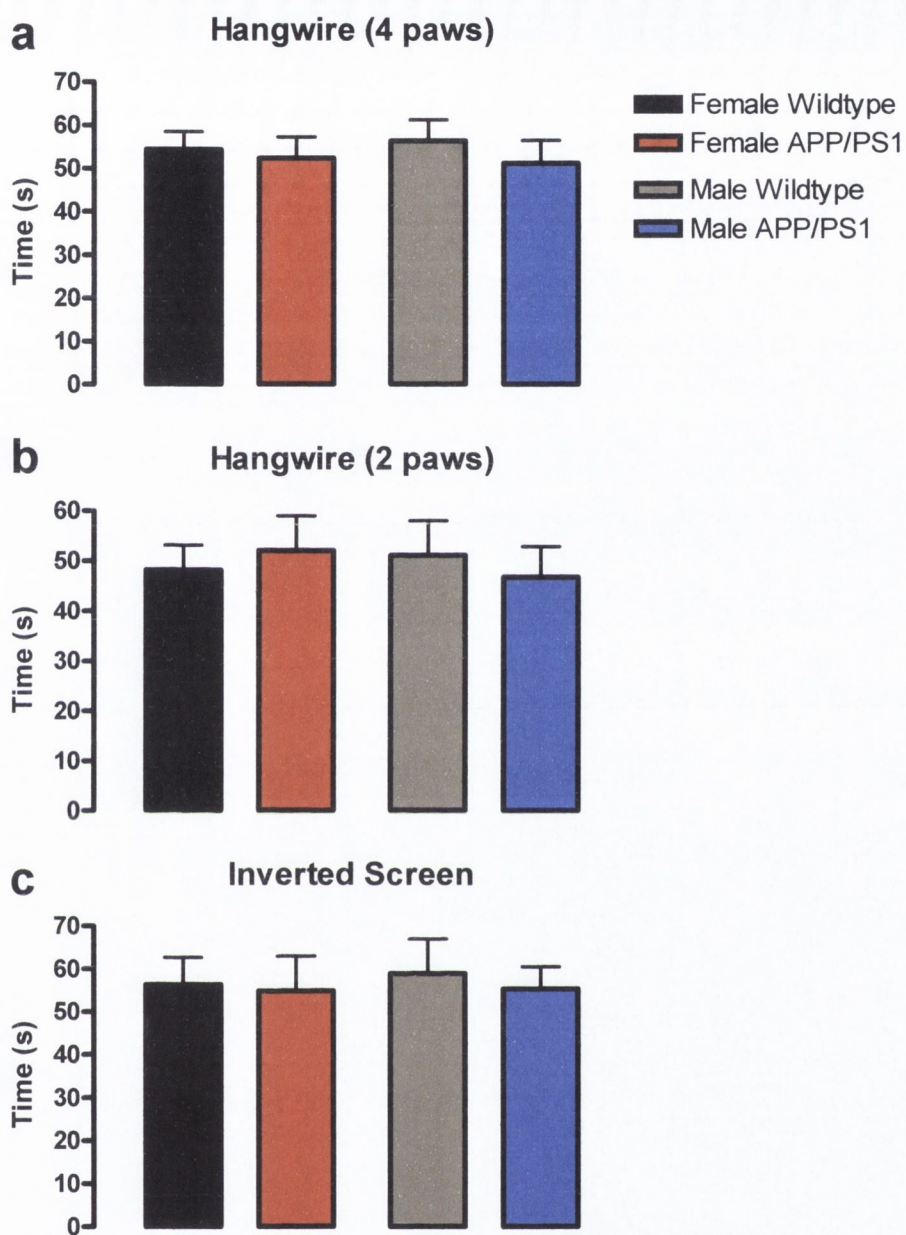


Figure 5.3. Muscle strength and coordination was similar in APP/PS1 transgenic and wildtype mice.

Muscle strength and coordination, assessed using the hangwire 4-paw (a) and 2-paw (b) tests, and the inverted screen test (c), were similar in mice in the 4 groups.

Data are means $\pm$ SEM (n values are given in Figure 5.2a).

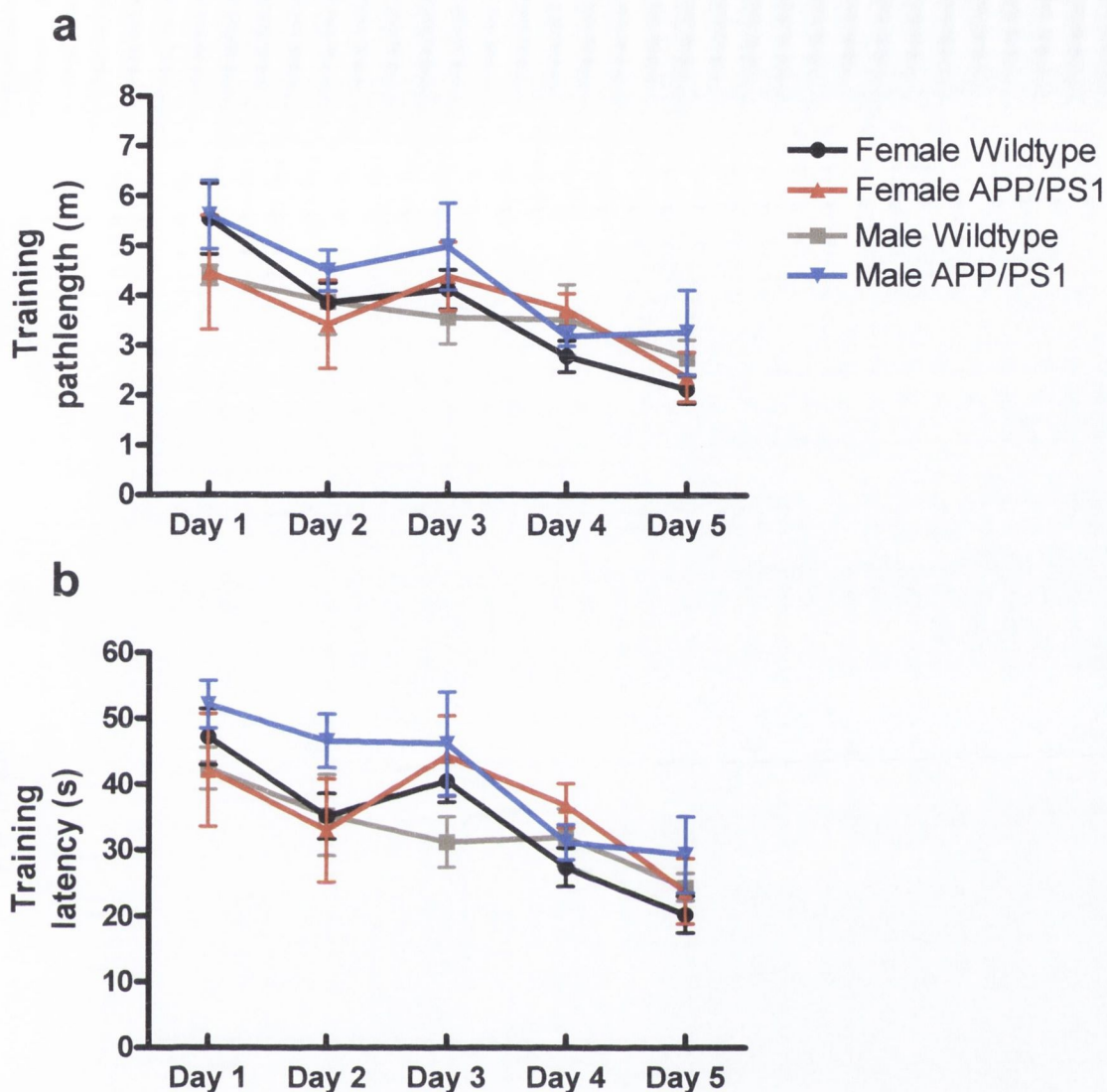


Figure 5.4. APP/PS1 transgenic and wildtype mice learn the Morris water maze.

Pathlength to (a) and latency in (b) locating the platform in the training phase of the MWM task. All groups learned the position of the platform after 5 days. Training was a significant factor in both measures of performance in male (pathlength, $p < 0.05$; ($F(4, 3.664)$); latency, $p < 0.0001$; ($F(4, 5.940)$); 2-way ANOVA) and female mice (pathlength, $p < 0.001$; ($F(4, 6.170)$); latency, $p < 0.001$; ($F(4, 7.563)$)). Genotype was also a significant factor in latency in male mice ($p < 0.01$; ($F(1, 7.481)$); 2-way ANOVA), but this effect did not translate to inter-group differences on any day. Genotype was not a significant factor in the performance of female mice.

Data are means $\pm$ SEM (n values are given in Figure 5.2a).

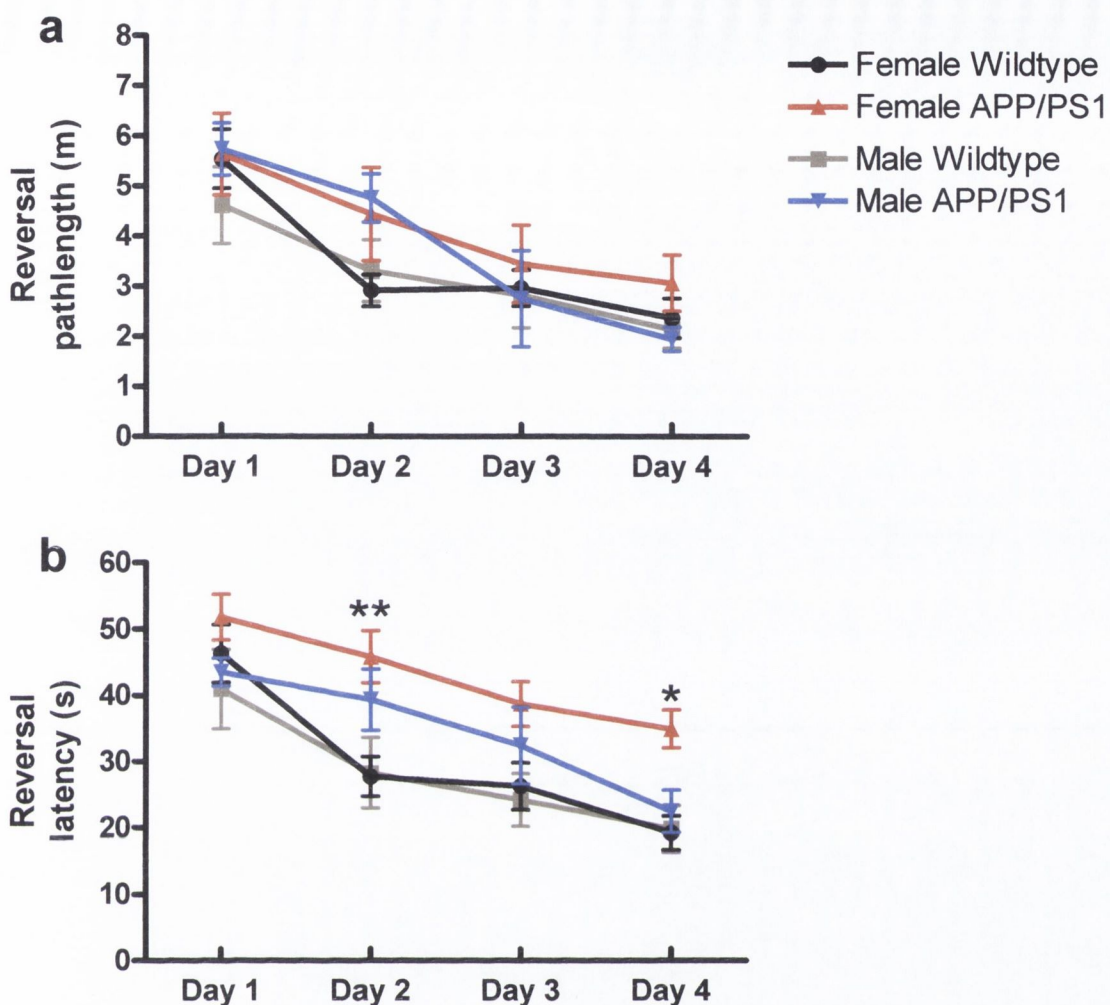


Figure 5.5. Female APP/PS1 transgenic mice display an impaired performance in the reversal phase of the Morris water maze task.

Pathlength to (a) and latency in (b) locating the platform in the reversal phase of the MWM task. Time was a significant factor in both measures for male (pathlength, $p < 0.001$; $F(3, 9.509)$; latency, $p < 0.001$; $F(3, 6.937)$; 2-way ANOVA) and female mice (pathlength, $p < 0.001$; $F(3, 9.347)$; latency, $p < 0.001$; $F(3, 10.930)$; 2-way ANOVA). Genotype was a significant factor in the latency performance of female mice only ($p < 0.001$; $F(1, 21.06)$; 2-way ANOVA). On day 2, female APP/PS1 transgenic mice performed significantly worse than female wildtype mice (latency, $**p < 0.001$; Bonferroni post-tests). The performance of female APP/PS1 transgenic mice continued to be impaired on days 3 and 4, significant differences were observed on day 4 only ($*p < 0.01$; Bonferroni post-tests), while male APP/PS1 transgenic and wildtype mice performed similarly.

Data are means $\pm$ SEM (n values are given in Figure 5.2a).

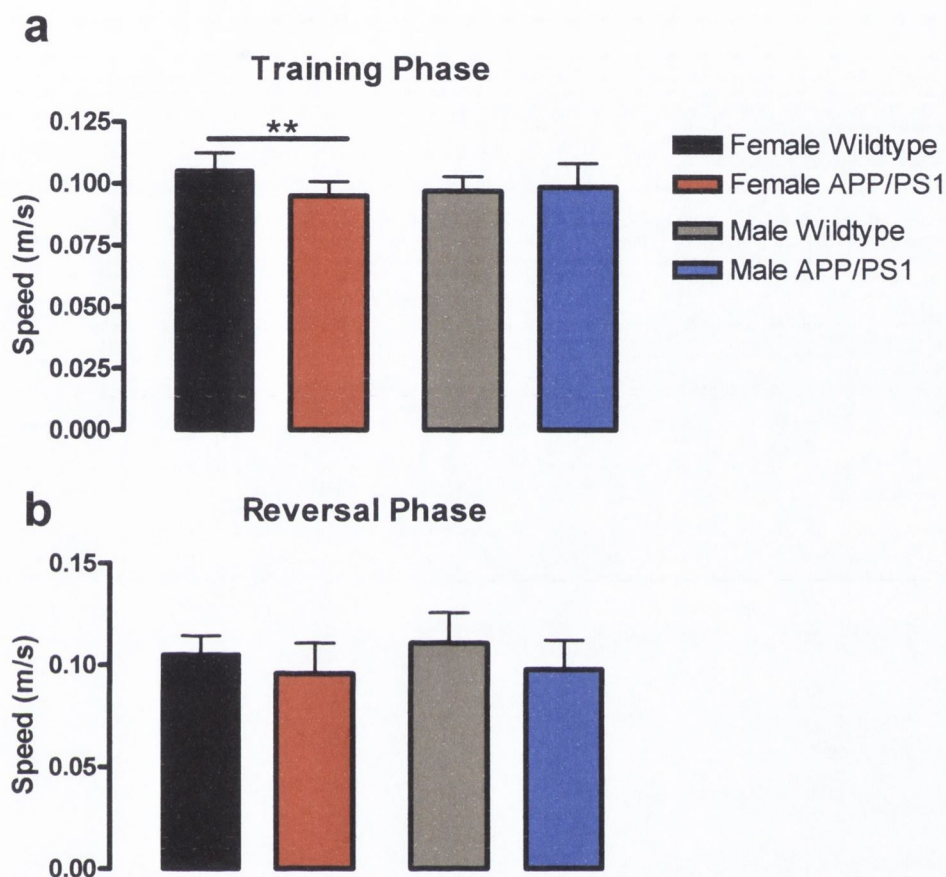


Figure 5.6. Swim speed of female wildtype mice was greater than that of female APP/PS1 transgenic mice in the training, but not the reversal phase, of the MWM task.

The average swim speed of female wildtype mice during the training phase of the MWM task (a), was greater than that of female APP/PS1 transgenic mice ($**p < 0.01$; Students t-test for independent means; female wildtype average swim speed versus female APP/PS1 average swim speed). This effect was not observed in the male mice. Average swim speed during the reversal phase of the MWM navigation task (b) was similar in APP/PS1 transgenic and wildtype mice.

Data are means $\pm$ SEM (n values are given in Figure 5.2a).

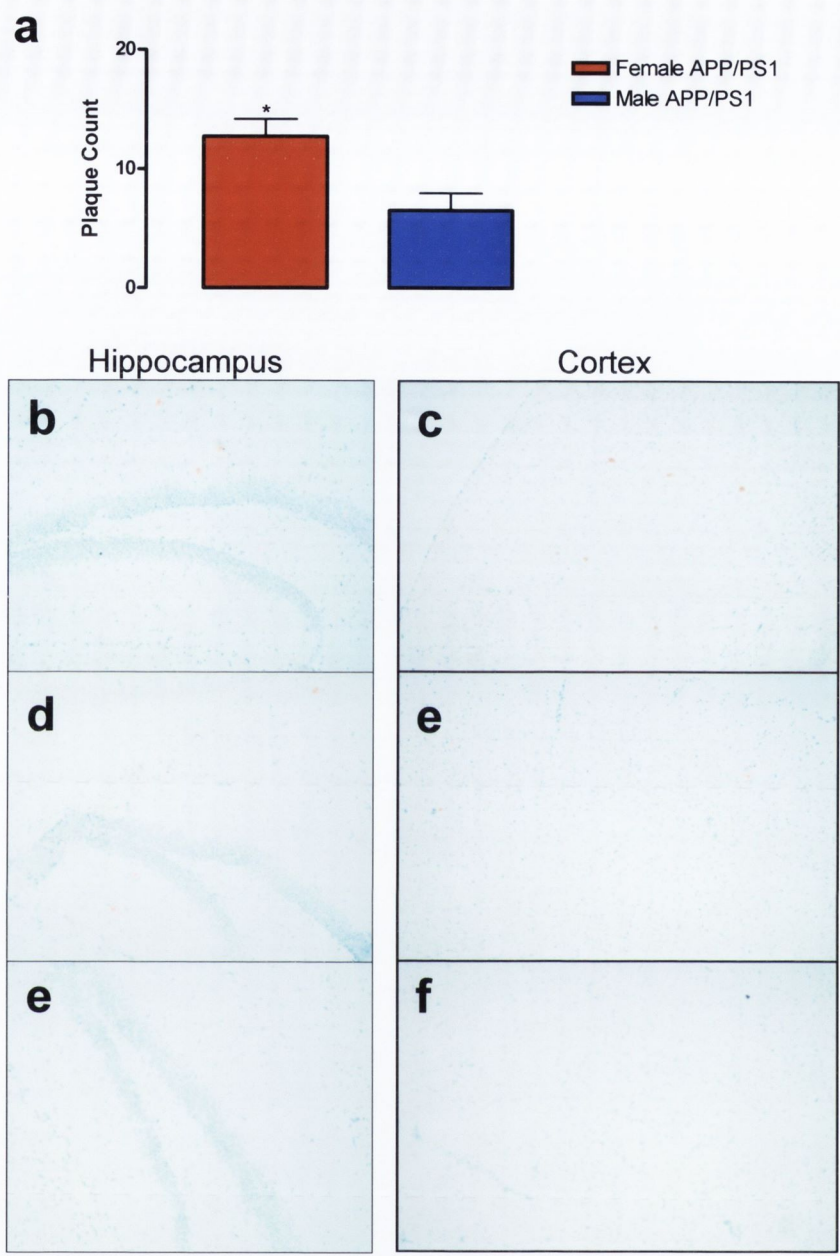


Figure 5.7. Female APP/PS1 transgenic mice exhibit higher levels of plaque deposition than male APP/PS1 transgenic mice.

Brain sections prepared from female APP/PS1 transgenic mice display a significantly greater number of congo-red positive plaques than brain sections prepared from male APP/PS1 transgenic mice (* $p < 0.05$; Student's t-test for independent means). Congo red-stained brain sections from female APP/PS1 transgenic (b, c), male APP/PS1 transgenic (d, e) and wildtype (e, f) mice. All micrographs taken at x10 magnification.

Data in (a) are expressed as average plaque count per brain section per animal and are means $\pm$ SEM ($n = 4$).

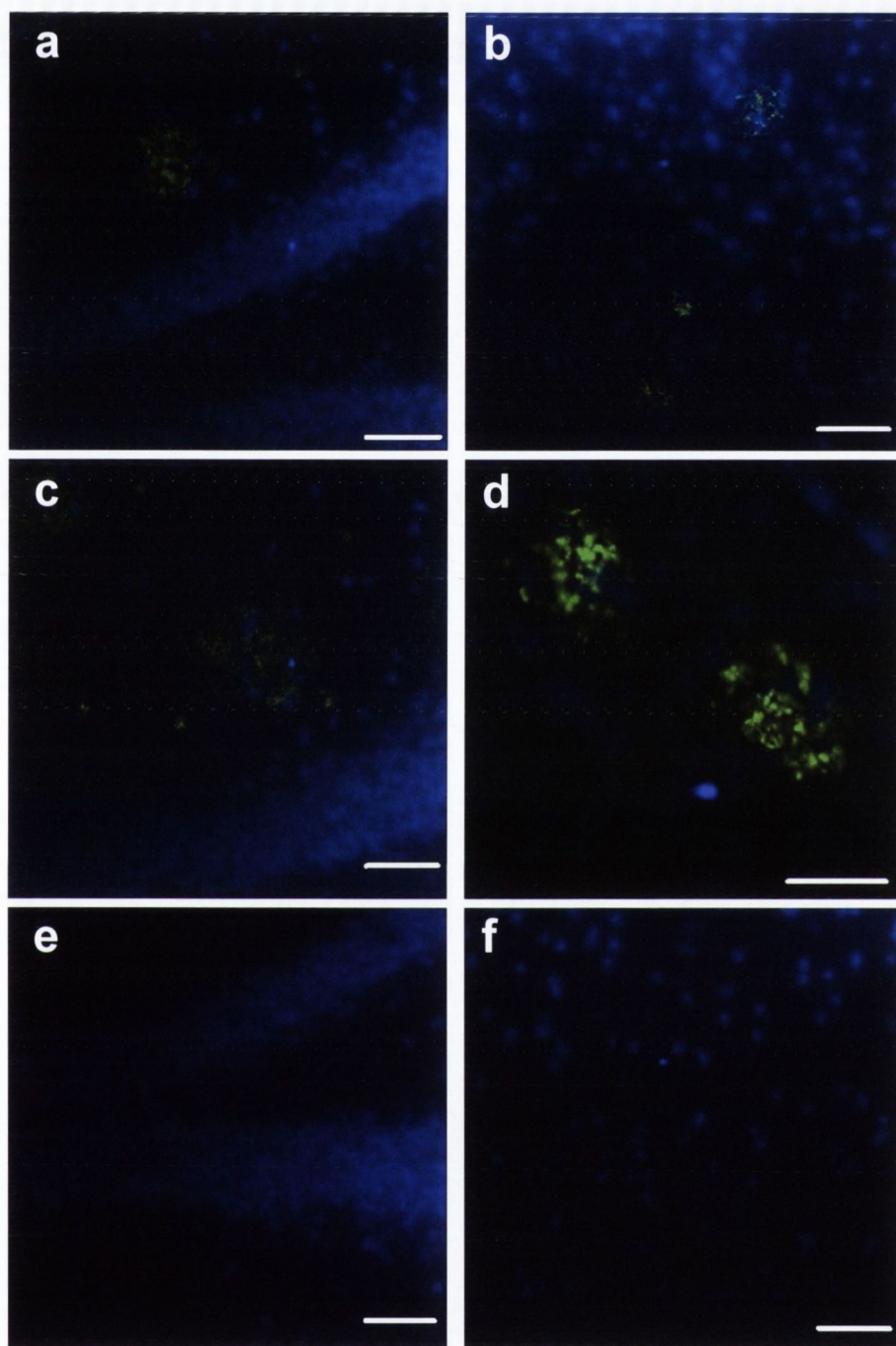


Figure 5.8. Brain sections prepared from female APP/PS1 transgenic mice display amyloid immunofluorescence in the cortex and hippocampus.

Brain sections prepared from female APP/PS1 transgenic mice display amyloid immunofluorescence (green) in the hippocampus (a, c) and the cortex (b, d). Nuclei are stained blue with DAPI. Confocal microscopy of brain sections from wildtype control mice display no amyloid immunofluorescence, in the hippocampus (e) or the cortex (f). All confocal micrographs taken at x40 magnification, scale bar = 50 μ m.

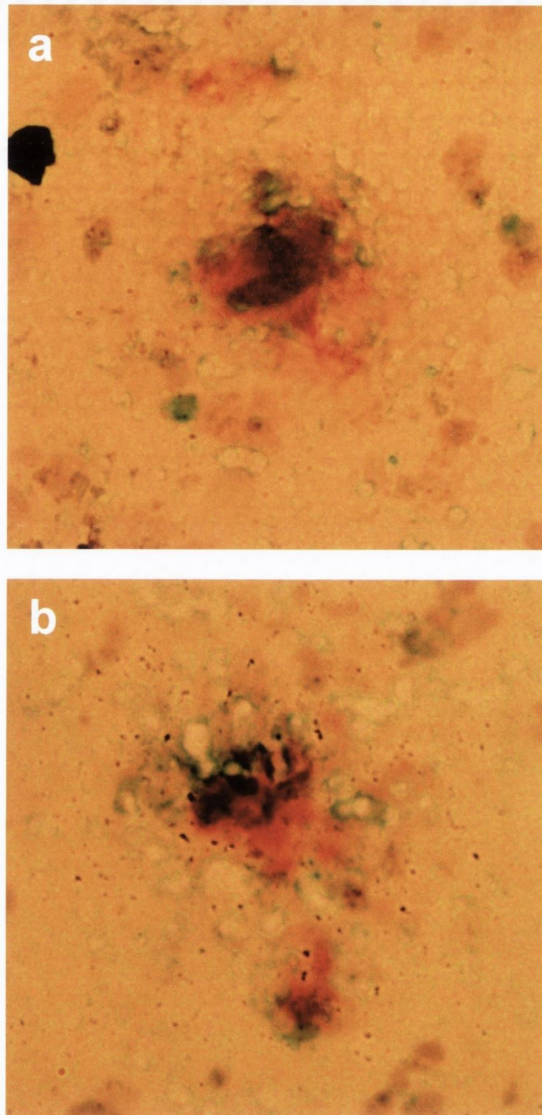


Figure 5.9. DAB-enhanced prussian blue staining confirms the presence of iron in congo red-positive plaques.

The colocalization of amyloid plaques and iron was evaluated in brain sections prepared from APP/PS1 transgenic mice by congo red- and DAB-enhanced prussian blue- staining (dark brown/black in the presence of iron). A methyl green counterstain was used to visualize nuclei. A representative light micrograph of amyloid and iron colocalization in a cortical plaque (a) and a hippocampal plaque (b) is shown. All micrographs taken at x40 magnification.

0.5 % Pixel saturation and
histogram equalization.

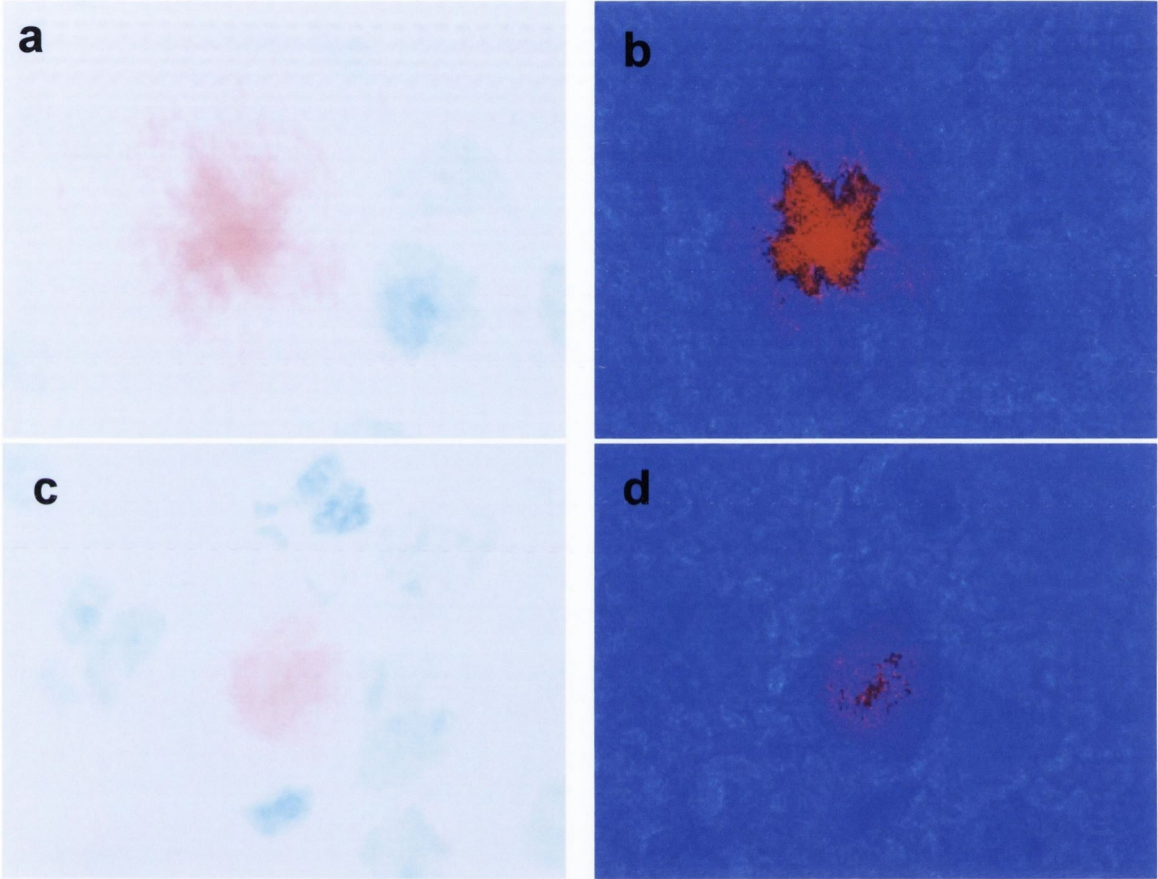


Figure 5.10. Diffuse and compact plaques display distinct morphology.

Representative congo-red staining of cortical dense (a, b) and diffuse (c, d) amyloid deposits in brain sections prepared from female APP/PS1 transgenic mice. A methyl green counterstain was used to visualize nuclei. Pixel saturation (0.5 %) and histogram equalization enables visual inspection of the core densities. All micrographs were taken at x100 magnification.

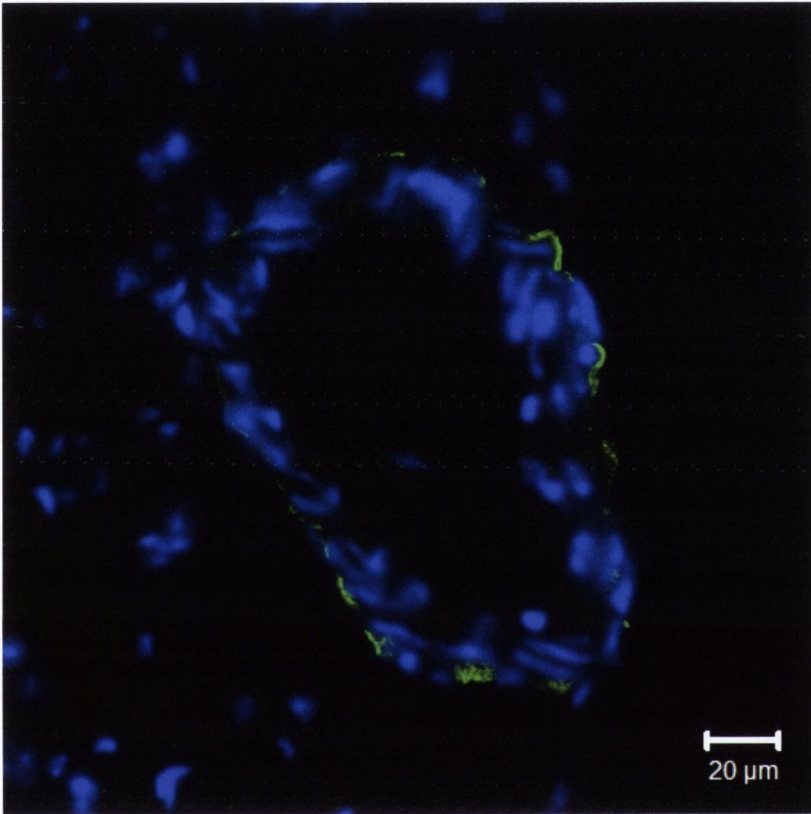


Figure 5.11. Modest vascular amyloid deposition was observed in brain sections prepared from 8-9 month old APP/PS1 transgenic mice

Representative brain section prepared from a female APP/PS1 transgenic mouse showing amyloid immunofluorescence (green) at a blood vessel. Nuclei are stained blue with DAPI. Micrograph was taken at x63 magnification.

Image courtesy of Belinda Grehan.

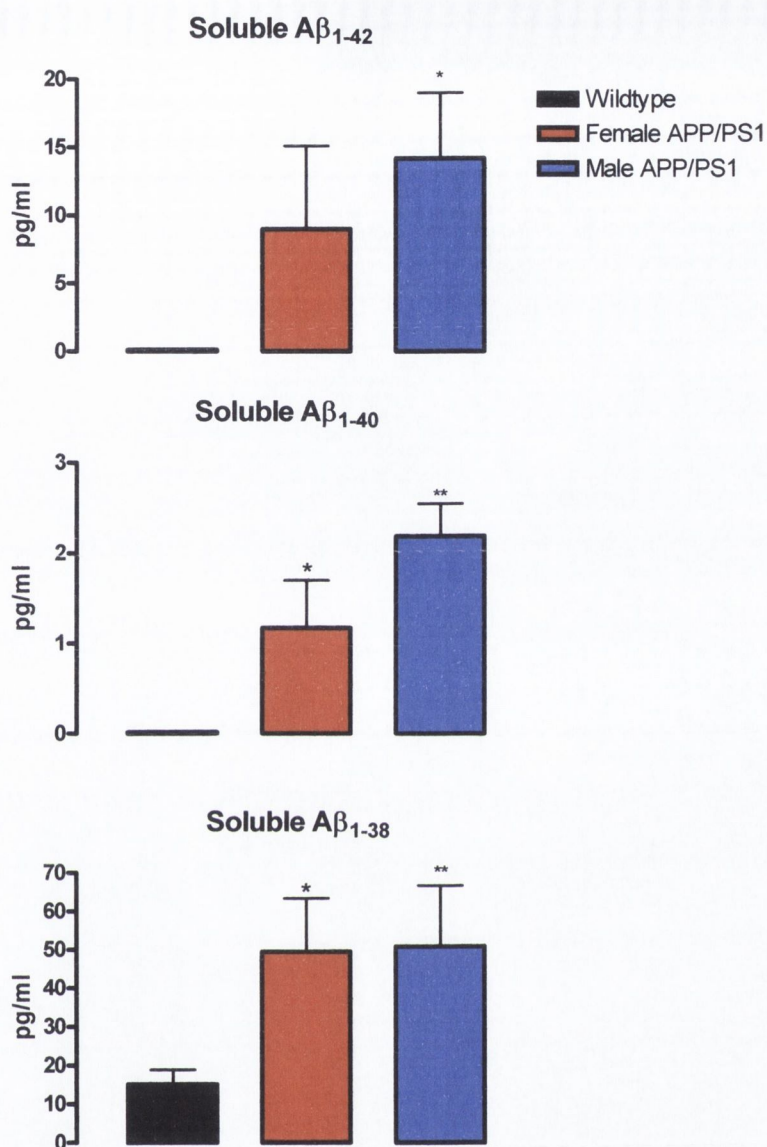


Figure 5.12. Soluble Aβ peptides were increased in brain tissue prepared from APP/PS1 transgenic mice compared with wildtype mice.

Soluble Aβ₁₋₃₈ and Aβ₁₋₄₀ were significantly increased in brain tissue prepared from APP/PS1 transgenic, compared with wildtype, mice (*p < 0.05; **p < 0.01; ANOVA); a significant increase in soluble Aβ₁₋₄₂ concentration was observed only in brain tissue prepared from male APP/PS1 transgenic mice (*p < 0.05; ANOVA). Soluble Aβ₁₋₃₈ was the dominant peptide in all groups with concentrations which were significantly higher than Aβ₁₋₃₈ and Aβ₁₋₄₀ (p < 0.001; ANOVA).

Data are expressed as pg/ml and are means ± SEM (n = 5).

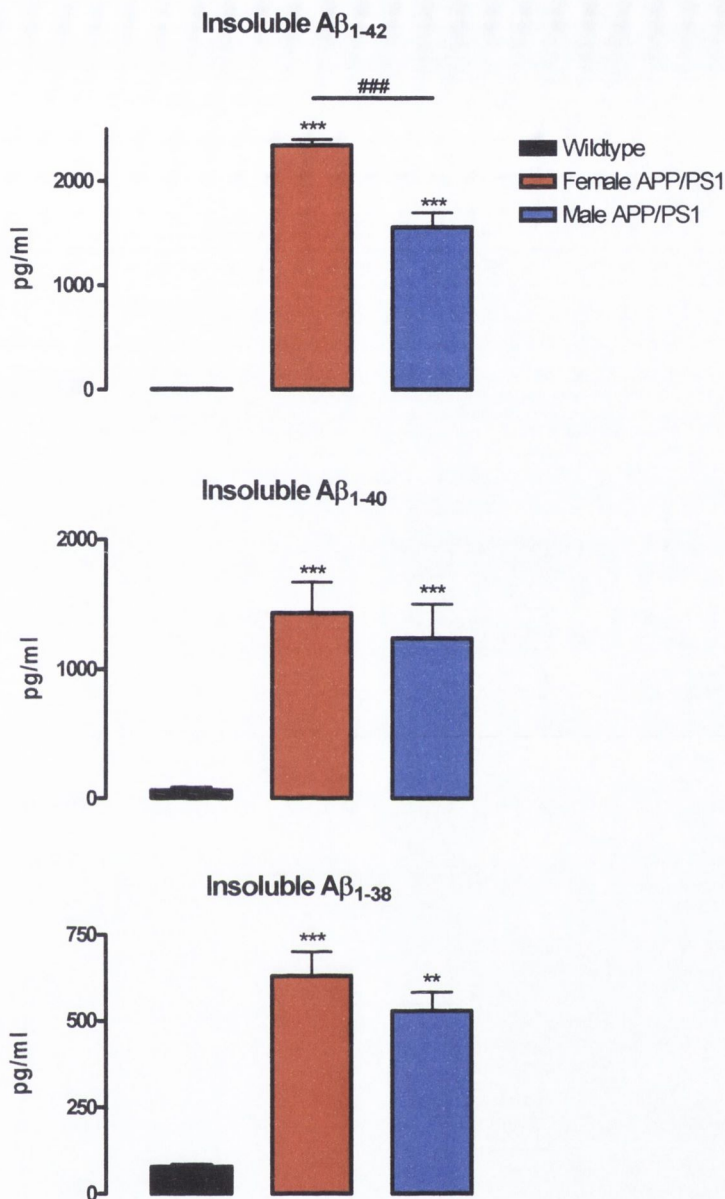


Figure 5.13. Insoluble A β peptides were increased in brain tissue prepared from APP/PS1 transgenic mice compared with wildtype mice.

Insoluble A β ₁₋₃₈, A β ₁₋₄₀ and A β ₁₋₄₂ were significantly increased in brain tissue prepared from APP/PS1 transgenic, compared with wildtype, mice (** $p < 0.01$; *** $p < 0.001$; ANOVA); A β ₁₋₄₂ concentration was significantly higher in brain tissue prepared from female, compared with male, APP/PS1 mice (### $p < 0.001$; Student's t-test for independent means). A β ₁₋₄₂ was the dominant peptide in female APP/PS1 brain tissue, with concentration levels significantly higher than A β ₁₋₃₈ and A β ₁₋₄₀ ($p < 0.001$; ANOVA).

Data are expressed as pg/ml and are means $\pm$ SEM ($n = 5$).

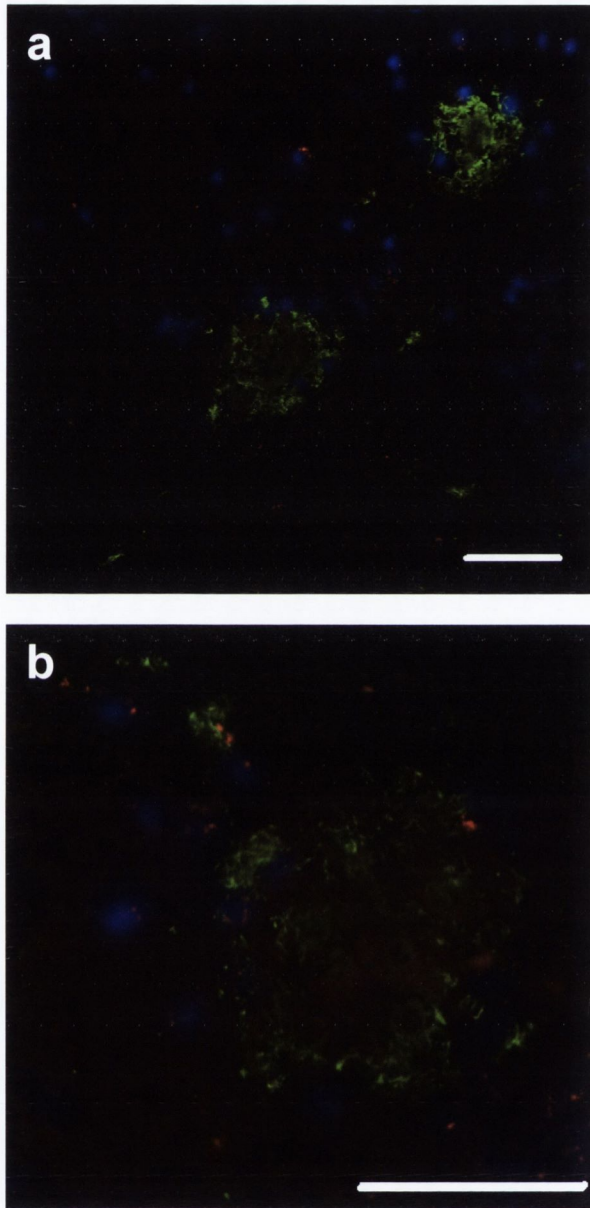


Figure 5.14. Cells proximal to amyloid plaques display MHCII immunofluorescence.

Representative amyloid immunofluorescent plaques (green), in a brain section prepared from a female APP/PS1 transgenic brain mouse, are encircled by cell nuclei (stained blue with DAPI). MHCII immunofluorescence (red) is expressed in cells proximal to the plaques. Upper micrograph (a) was taken at x40 magnification and the lower micrograph (b) taken at x63 magnification. Scale bar = 50 μm .

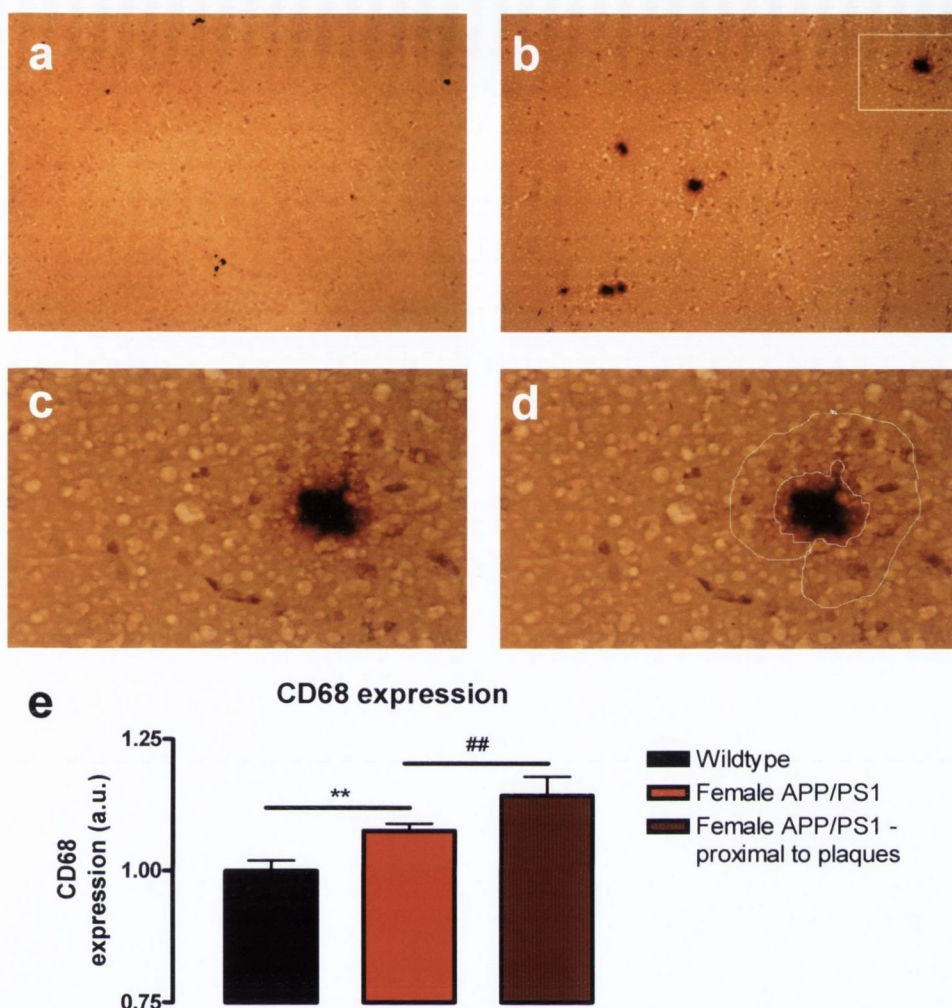


Figure 5.15. CD68 expression was increased in brain sections prepared from a female APP/PS1 mouse compared with brain sections prepared from a wildtype mouse.

Representative images indicating CD68 immunocytochemistry in sections obtained from wildtype (a) and female APP/PS1 transgenic (b) mice; the region of interest in (b) is enlarged in (c) and (d); magnification x40. Semi-quantitative analysis indicates significantly more CD68 staining in sections from female APP/PS1 transgenic mice compared with wildtype mice (** $p < 0.01$; Student's t-test for independent means). Areas proximal to plaques show greater CD68 staining (## $p < 0.01$; Student's t-test for independent means) and hence appear darker. The area outlined in (d) is representative of the area denoted as proximal to the plaque for CD68 presence analysis (note the plaque is excluded from the analysis).

All micrographs were taken at x20 magnification. Data were obtained from two brain sections of single animal in each group and are expressed in arbitrary units of the reciprocal of image pixel value and are means $\pm$ SEM.

CD68 staining performed by Belinda Grehan.

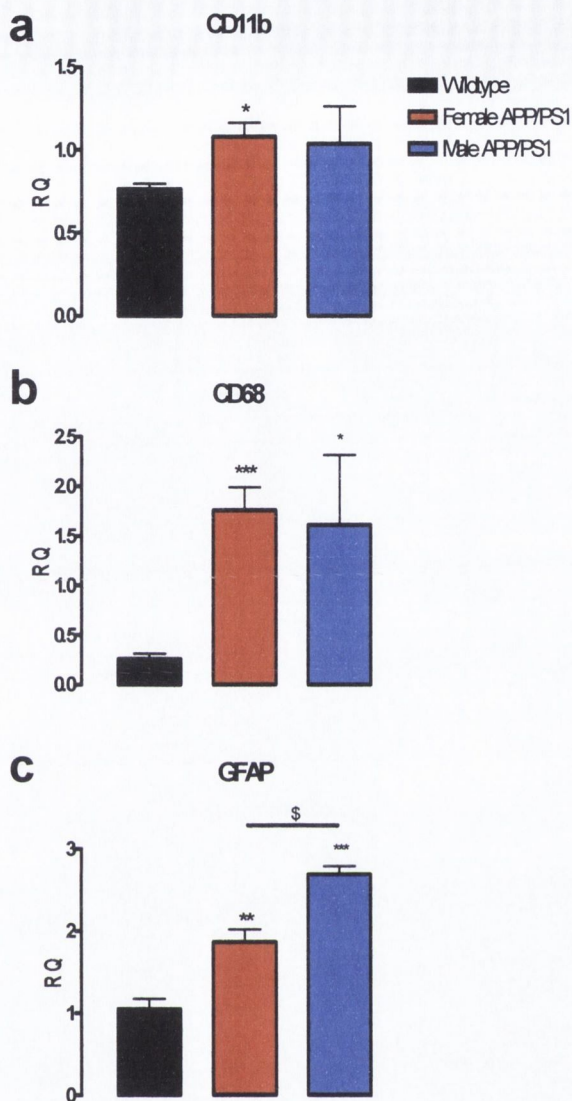
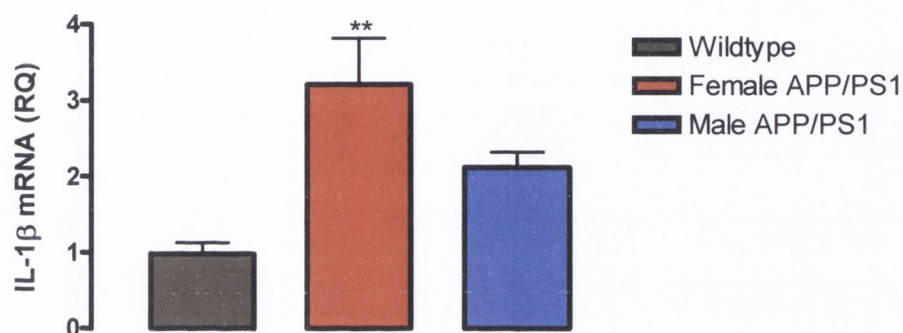


Figure 5.16. Expression of markers of microglial and astrocytic activation are upregulated in APP/PS1 transgenic mice.

CD68 mRNA (a) expression was significantly increased in brain tissue prepared from male and female APP/PS1 transgenic, compared with wildtype, mice (* $p < 0.05$; *** $p < 0.001$; ANOVA); a significant increase in CD11b (b) mRNA expression was observed only in brain tissue prepared from female APP/PS1 transgenic mice (* $p < 0.05$; ANOVA). GFAP mRNA (c) expression was significantly increased in brain tissue prepared from male and female APP/PS1 transgenic mice compared with wildtype mice (** $p < 0.01$; *** $p < 0.001$; ANOVA); GFAP mRNA expression was significantly increased in brain tissue prepared from male APP/PS1 transgenic mice compared with female APP/PS1 transgenic mice (§ $p < 0.05$; ANOVA).

Data are expressed as a ratio of CD11b, CD68 or GFAP mRNA:β-actin mRNA, and are means $\pm$ SEM (n = 5).

a



b

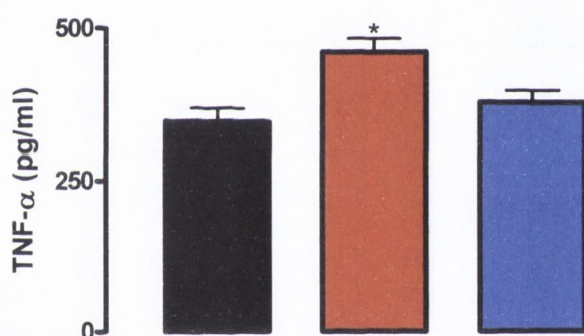


Figure 5.17. Upregulation of proinflammatory cytokines in brain tissue prepared from female APP/PS1 transgenic mice.

(a) IL-1 β mRNA expression was increased in brain tissue prepared from female APP/PS1 transgenic, compared with male APP/PS1 transgenic and wildtype, mice (* $p < 0.05$; ANOVA). Data are expressed as a ratio of IL-1 β mRNA: β -actin mRNA, and are means $\pm$ SEM ($n = 5$).

(b) TNF- α concentration was significantly increased in the brain tissue of female, compared with male APP/PS1 transgenic and wildtype mice (* $p < 0.05$; ANOVA). Data are expressed as pg/ml and are means $\pm$ SEM ($n = 5$).

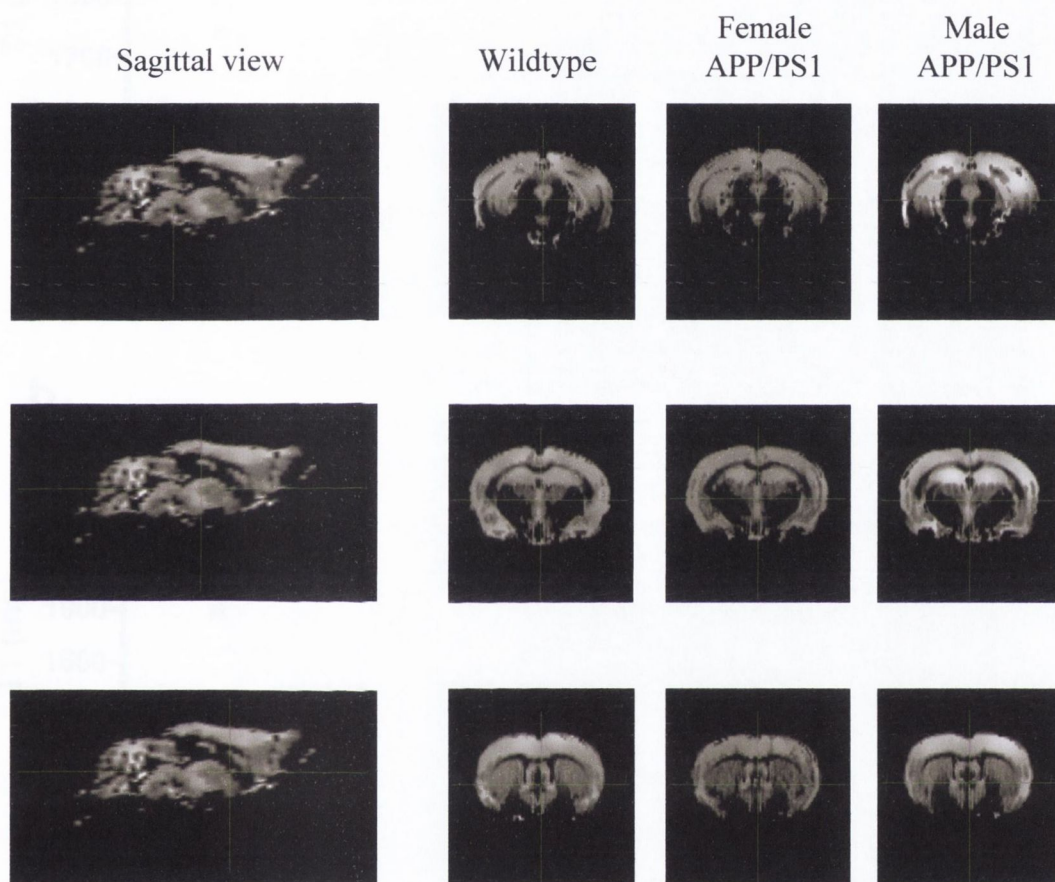


Figure 5.18. No age-related decline in gray matter volume was observed.

Changes in gray matter volume were assessed longitudinally within wildtype mice, female APP/PS1 transgenic mice and male APP/PS1 transgenic mice using VBM. Statistical parameter maps thresholded at $p < 0.001$, indicating a significant gray matter volumetric difference between 3-4 and 8-9 months of age, are overlaid onto group specific coronal templates at three different positions in the mouse fore-brain. The green lines in the sagittal view indicate position. The statistical parameter maps show no detail at $p < 0.001$ indicating no changes in gray matter volume.

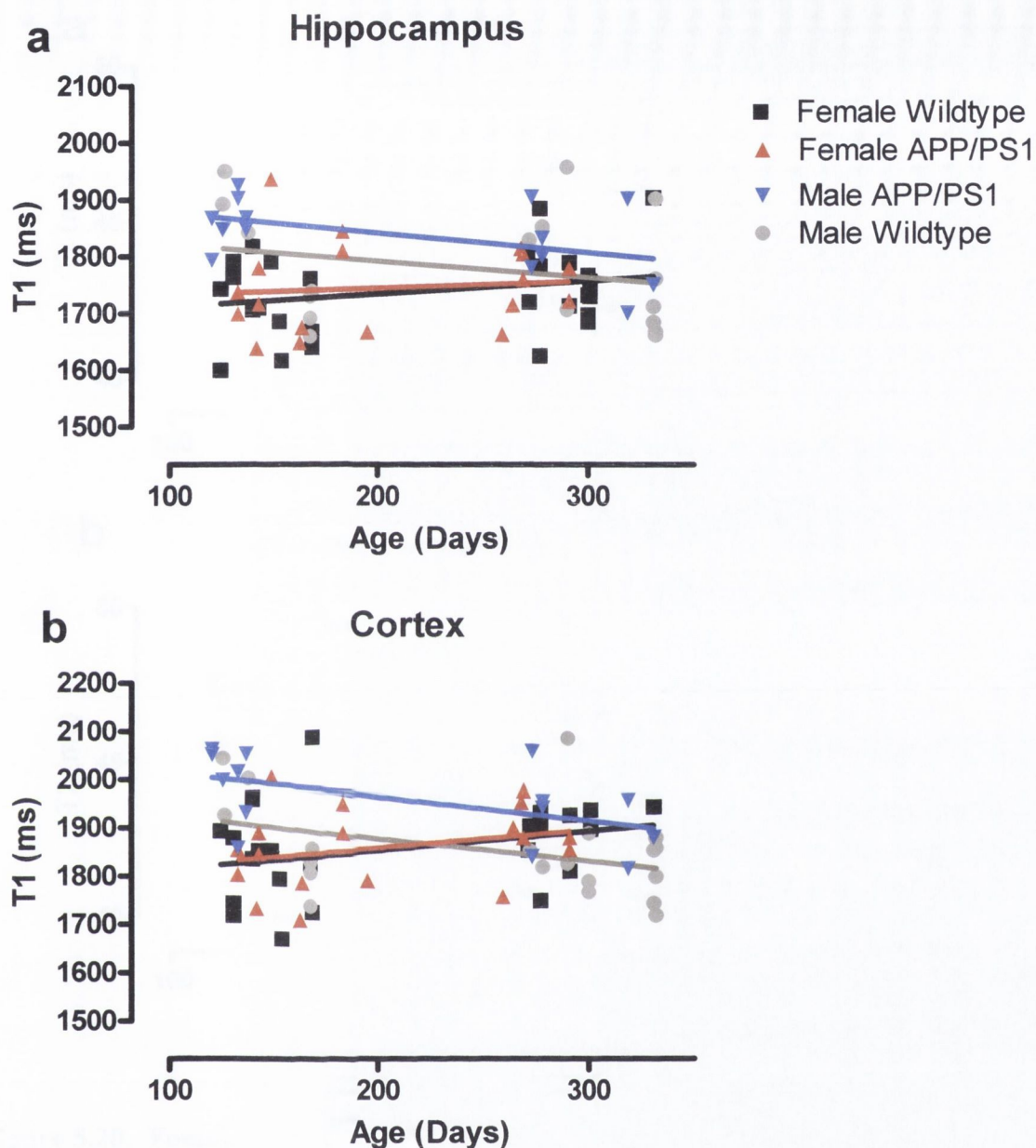


Figure 5.19. Male APP/PS1 mice displayed an increased cortical and hippocampal T1 relaxation time that declined with age.

Age was not a significant factor in hippocampal (a) and cortical (b) T1 relaxation times, as assessed by 2-way ANOVA. At 3-4 months of age, hippocampal and cortical T1 relaxation times in male APP/PS1 transgenic were significantly higher than in female APP/PS1 and wildtype mice ($p < 0.05$; cortex, $p < 0.01$; ANOVA Post-test Bonferroni). This difference was not evident at 8-9 months of age.

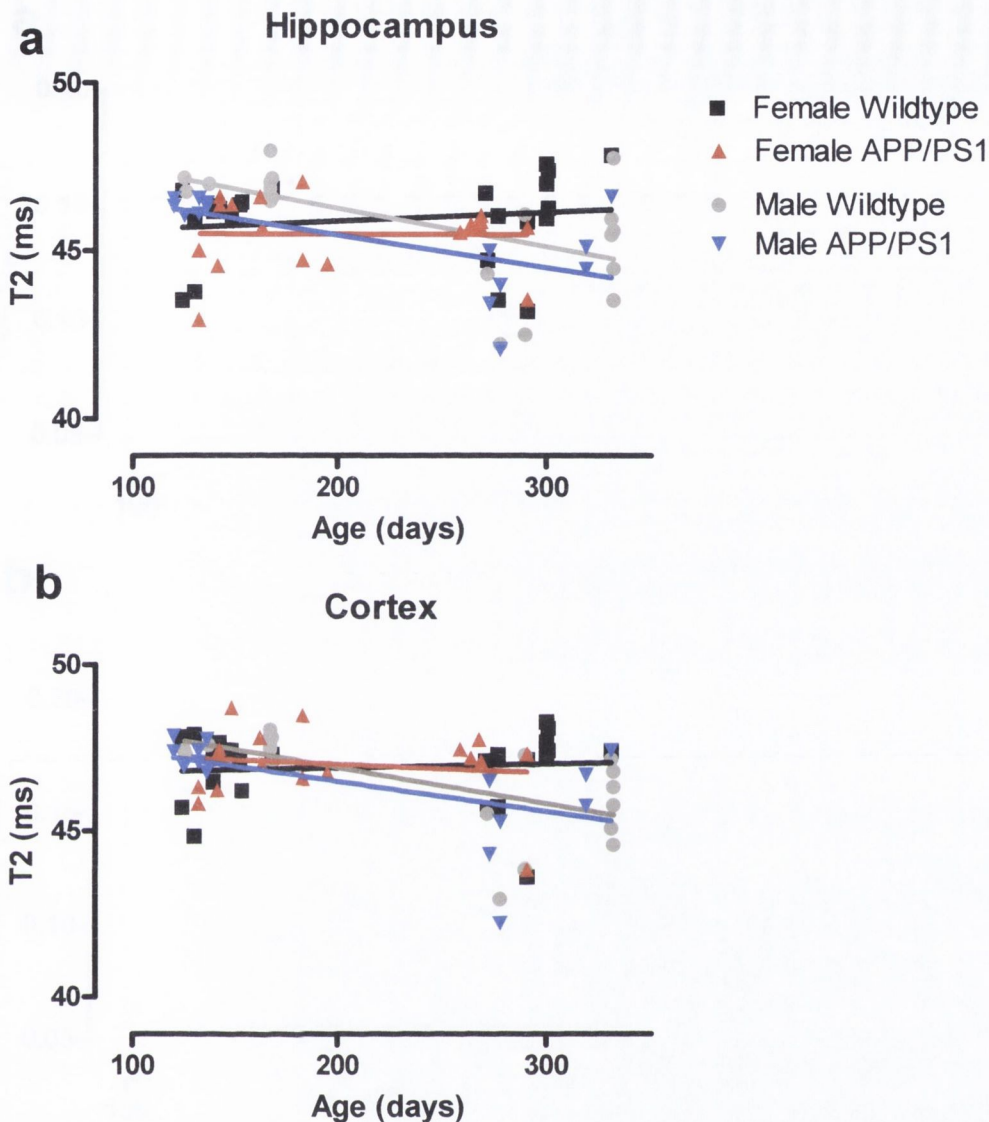


Figure 5.20. Female APP/PS1 and wildtype mice did not exhibit an age-related decline in T2 relaxation time in the hippocampus or cortex.

Age was a significant factor in hippocampal (a) and cortical (b) T2 relaxation times in male mice only (hippocampus, $p < 0.001$; $F(1, 29.74)$; cortex, $p < 0.001$; $F(1, 28.36)$; 2-way ANOVA comparing male experimental groups only). Male APP/PS1 transgenic and wildtype mice exhibited a significant age-related decline in T2 relaxation time in the hippocampus (transgenic, $p < 0.001$; wildtype, $p < 0.001$; Students t-test for independent means; values at 8-9 months versus 3-4 months) and cortex (transgenic, $p < 0.001$; wildtype, $p < 0.05$; Students t-test for independent means; values at 8-9 months versus 3-4 months). This decline was not evident in female T2 relaxation times.

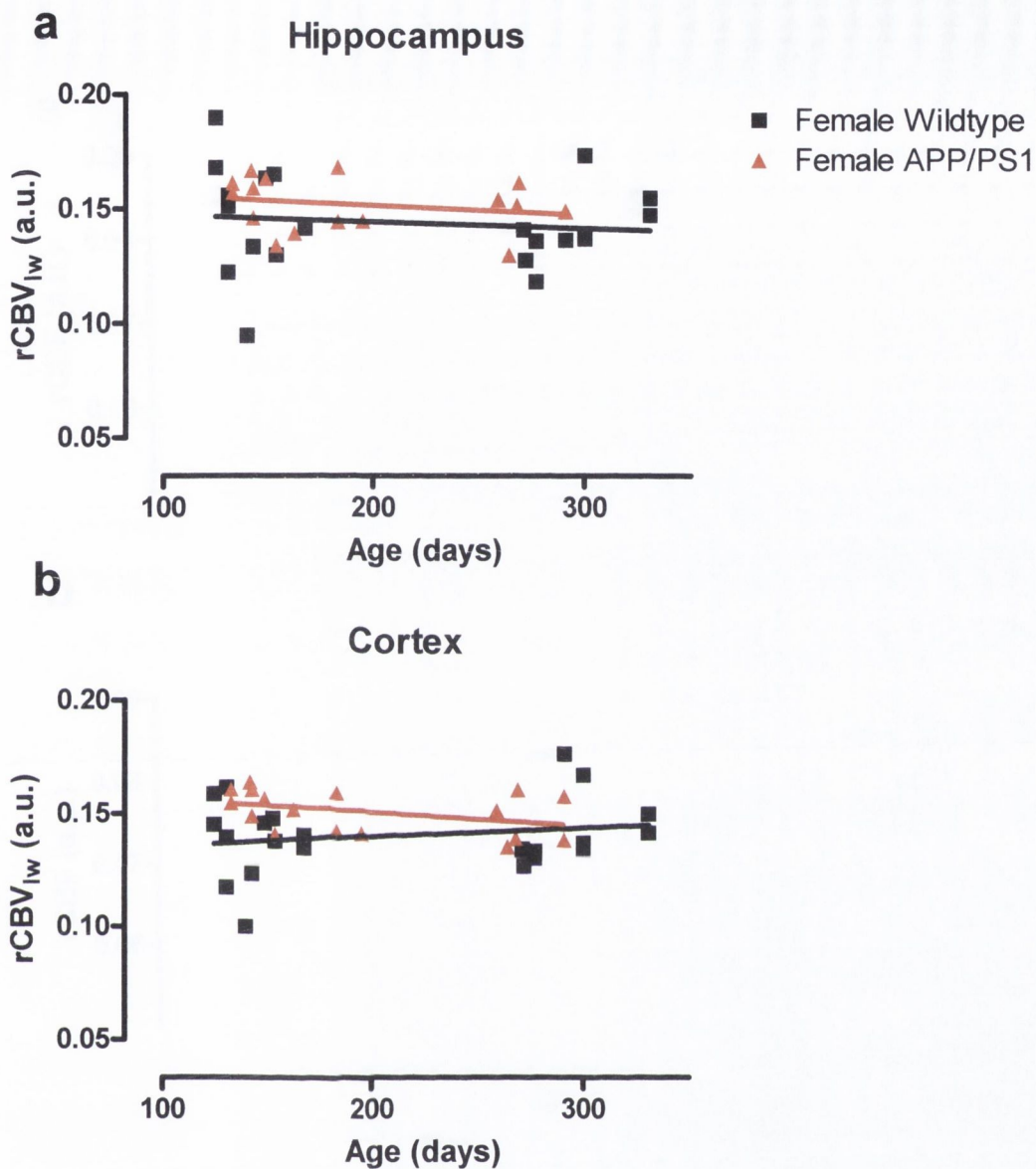


Figure 5.21. No age-related changes in $rCBV_{lw}$ were observed.

$rCBV_{lw}$ in the hippocampus (a) and cortex (b) of all groups is presented. No significant differences were observed in $rCBV_{lw}$ values at 3 – 4 months of age compared to 8 – 9 months of age, within each experimental group.

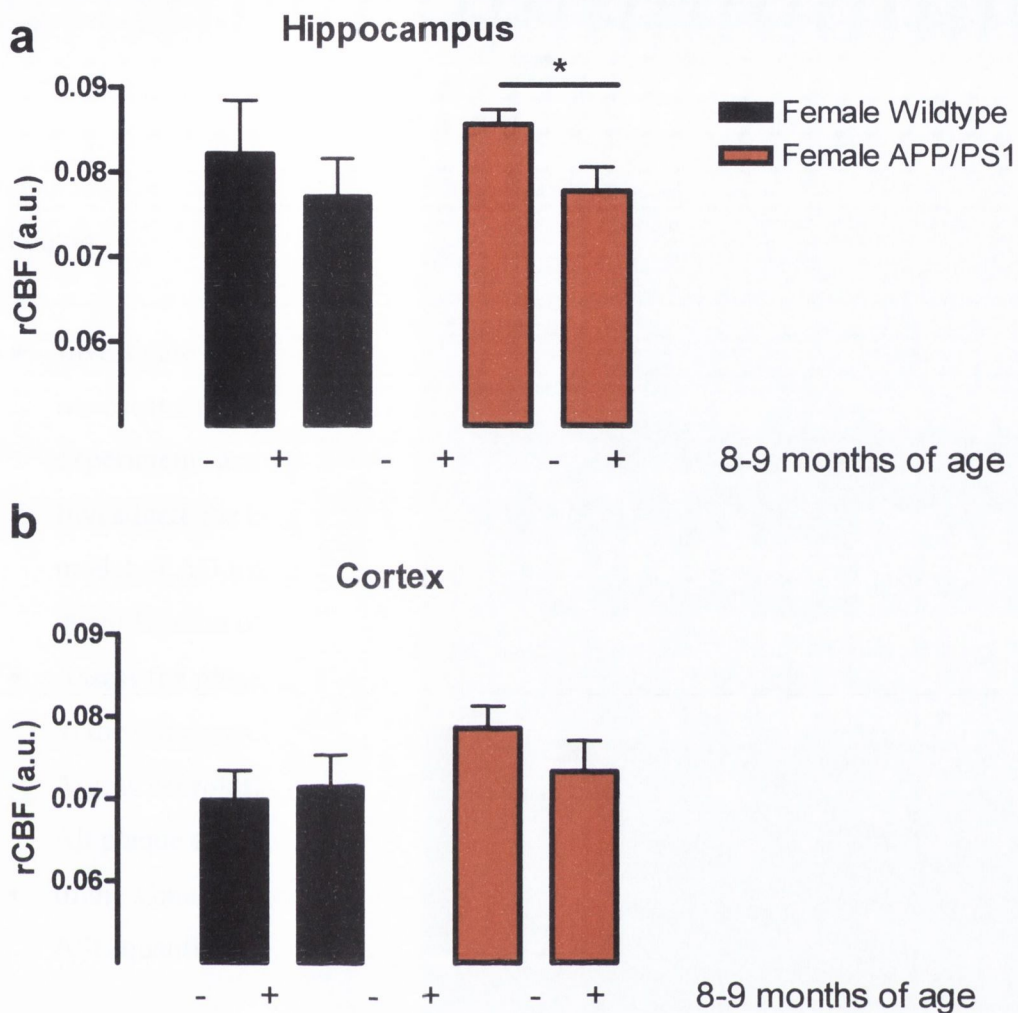


Figure 5.22. The rCBF in the hippocampus of female APP/PS1 transgenic mice decreased with age.

The rCBF of the hippocampus (a) and cortex (b) at 3 - 4 and 8 - 9 months of age is shown. rCBF was significantly decreased in the hippocampus of female APP/PS1 at 8 - 9 months of age ($p < 0.05$, Students t-test for independent means; values at 8 - 9 months versus 3 - 4 months). No further differences were observed.

Data are expressed in arbitrary units on the y-axis and are means $\pm$ SEM.

Chapter 6

Discussion

I set out to

- Investigate the interaction of mixed glial cells with a magnetic fluorescent nanostructure and evaluate the potential of this nanostructure in cell tracking experiments and as a basis for CNS drug delivery.
- Investigate the composition of A β plaques in an APP/PS1 transgenic mouse model of AD using a technique capable of accurate and simultaneous quantification of several metals from a single biological tissue specimen.
- Assess the presence of magnetite, an iron oxide implicated in ROS generation, in the same specimen.
- Assess neuroinflammation and its effect on spatial memory and early-stage A β plaque deposition in an APP/PS1 transgenic mouse model of AD.
- In the same cohort of animals, longitudinally assess MRI relaxometry and ASL-quantified cerebral blood perfusion.

The ability to track cells using multiple contrast-dependant imaging modalities is an attractive technique, especially in the neurosciences where cell migration from the PNS to the CNS is considered to be an important event. Similarly, the delivery of therapeutic agents to the CNS is an important step in developing treatments of CNS disorders. The nanostructure assessed in this study was developed with consideration to bimodal contrast generation and exhibited simultaneous magnetic and fluorescent contrast-generating capabilities. It is anticipated that the iron oxide core used to generate magnetic contrast could be replaced with a therapeutic agent and as such this nanostructure also represented a nano-sized drug delivery mechanism. Studies investigating the use of nanotechnology as CNS drug delivery platforms are widespread. However little is known about the potentially deleterious effects the nano-sized constituents of these platforms may have on CNS tissue cells, specifically

microglia, the immune-competent cell of the CNS. Assessment of the bimodal contrast generating capabilities of this nanostructure was carried out *in vitro* and *in vivo* and the results interpreted with consideration to the potential use of this nanostructure as a method of CNS drug delivery.

The nanostructure was rapidly internalized into mixed glial cells and the evidence indicated both phagocytosis and endocytosis as internalization mechanisms. The bimodal contrast-generating capability of the nanostructure was demonstrated using fluorescence-based detection methods, confocal microscopy and FACS, and magnetic-based detection methods, T2-weighted MRI. Significantly, the nanostructure exhibited no cytotoxic effects and the internalization of the nanostructure did not induce a robust microglial activation and no meaningful increase in cytokine production was measured, even though phagocytosis was observed. This suggests that the polyelectrolyte coatings used in the nanostructure do not induce microglial cytokine production, although microglia become phagocytic. This is a significant finding and such a property could be used to deliver therapeutic agents to CNS cells while minimising inflammation due to microglial internalization. Nanostructure uptake by astrocytes indicates that the nanostructure may require some additional design to enable cell-specific targeting.

As the nanostructure was seen to be phagocytosed by microglia it was hypothesized that macrophages would phagocytose the nanostructure if administered intravenously. Experiments were conducted to assess this and to determine if macrophage infiltration of the CNS, known to be ongoing in EAE mice 10 days post immunisation, could be visualized *in vivo* using MRI, and subsequently in brain sections using confocal microscopy. The results indicate poor macrophage uptake of the nanostructures and suggest their rapid clearance of the nanostructures from the bloodstream. These data, and results from experiments investigating the detachment of the fluorescent agent from the nanostructure surface, indicated that this nanostructure was not useful in experiments involving *in vivo* labelling of cells. However, studies investigating the migration of cells following administration to animals are also of great interest, most notably, stem cell migration studies (Schafer

et al., 2007, Chen et al., 2007, Kustermann et al., 2005) and T cell migration in EAE (Anderson et al., 2004). The nanostructure may be ideal for use in such experiments and could enable rapid analysis of the cells tracked, allowing confocal microscopy to be utilised for MRI correlation to preserve spatial information, or flow cytometric analysis for macroscopic evaluation.

The evidence validates the nanostructures as bimodal contrast agents and, the *in vivo* data indicate, their use exclusively in cell tracking experiments that involve administration of pre-labelled cells. The data also suggests the polyelectrolyte coating used in the nanostructure as potentially useful in CNS drug delivery systems, as evidenced by cellular internalization in the absence of an inflammatory response.

The nanostructure generates MRI contrast due to the iron oxide in its core. Many studies have suggested endogenous iron modulation of MRI contrast as a potential biomarker for AD disease progression (Bartzokis et al., 2000) as increased brain concentrations of iron are associated with AD (Zecca et al., 2004). Iron, copper and zinc are also known to accelerate *in vitro* aggregation of A β (Khan et al., 2006). Investigation of the composition of A β plaques from human AD tissue has demonstrated increased levels of iron, copper and zinc in the plaques themselves (Lovell et al., 1998, Miller et al., 2006). It is important to emphasise that these findings relate to plaques from end-stage AD patients and it is reasonable to suggest, considering the complex and poorly understood nature of *in vivo* plaque formation and maturation, that A β plaque composition may change with disease progression.

The elemental composition of A β plaques from APP/PS1 transgenic mice displaying early-stage amyloid deposition was investigated in the present study. Studies investigating metal concentrations levels in biological tissue reliant on histochemical techniques are compromised by the non-specificity of results and the difficulty in translation to absolute concentration levels. XRF techniques originally developed for the investigation of elemental and atomic composition of materials are being adapted for use with biological tissue and here, the XRF analysis revealed an increase in iron and calcium in areas associated with A β plaques and in plaques

themselves; these findings concur with similar previous observations (Lovell et al., 1998, Miller et al., 2006, Leskovjan et al., 2009, Quintana et al., 2006, Dhenain et al., 2009). However, no consistent increase in copper or zinc was observed in A β plaques and these findings contrast with studies, utilising similar analytical techniques, which reported increases in these metals in human A β plaques (Miller et al., 2006). The concentrations of metals reported in human A β plaques are approximately 5 times greater than those observed in plaques in the APP/PS1 mouse model of AD used in this present study. Investigation of A β plaque composition in 13 – 14 month old APP/PS1 transgenics mice also reported metal concentration levels far lower than those reported in human A β plaques (Leskovjan et al., 2009). One possible explanation for these discrepancies is that metals colocalize to A β plaques over time. Another possible explanation is that the protein composition of A β plaques in mice are different from human A β plaques.

It has been reported that plaques in the brain parenchyma of mouse models of AD contain both transgene-derived human, and murine, A β . Reports indicate plaque cores of predominantly transgene-derived human A β are surrounded by murine A β (van Groen et al., 2006, Pype et al., 2003). Human A β has three histidine residues at position 6, 13 and 14, all of which act as iron binding sites (Atwood et al., 2000, Atwood et al., 1998), and histidine residue-bound iron has been shown to generate H₂O₂ by Fenton chemistry (Huang et al., 1999b, Huang et al., 1999a). However, murine A β lacks a histidine at position 13 and it's presence on the perimeter of plaques in APP/PS1 transgenic brain tissue may reduce metal colocalization to plaques (Radde et al., 2008). It has also been reported that zinc transporters, key to normal brain physiology, are colocalized with A β plaques (Zhang et al., 2008). If continual plaque colocalization of metals is occurring, the loss of zinc transporters and zinc in it's ionic form from cells proximal to A β plaques could possibly result in impaired physiological function of these cells (Choi and Koh, 1998).

Recent work investigating the type of iron present in A β plaques and brain tissue from AD patients has identified an iron oxide called magnetite (Collingwood et

al., 2008, Collingwood et al., 2005, Kirschvink et al., 1992). It has been implicated in the generation of ROS through its interaction with A β fibrils (Khan et al., 2006) and it was hypothesized that it would be present in areas of high iron concentration in brain tissue obtained from APP/PS1 transgenic mice.

The data indicated that magnetite and maghemite, its oxidative partner, were present in areas of high iron concentration in brain tissue prepared from an APP/PS1 transgenic mouse. It is possible that oxidation of magnetite to maghemite may have occurred post-mortem or during sample preparation, but it must also be considered that *in vivo* conversion of magnetite to maghemite could potentially lead to the generation of free radicals by Fenton chemistry. In any case, it is interesting that previous studies using similar techniques have also identified the presence of maghemite in AD brain tissue (Collingwood et al., 2005).

Analysis of metals by XRF techniques, modified for use with biological tissue, have confirmed the presence of iron, but not copper or zinc, in A β plaques from 8 – 9 month old APP/PS1 transgenic mice. Furthermore, the presence of magnetite, a mixed valence iron oxide implicated in the generation of ROS, was also confirmed in brain tissue prepared from 8 – 9 month old APP/PS1 transgenic mice. Future work using these X-ray-based techniques, now firmly established, could involve investigation of age-dependant changes in A β plaques, and surrounding tissue in mouse models of AD to further inform the debate surrounding continual metal aggregation to plaques. Work investigating the efficacy of metal chelation therapies in AD could also benefit from application of these techniques. A genetically-engineered mouse with murine APP knocked out, but expressing human APP, may present with more physiologically-accurate A β plaques and would reveal if murine A β is a limiting factor in the metal concentration levels of plaques.

Data from chapter 4 provided an insight into the elemental composition of A β plaques and although, a diagnostic hallmark of AD, their role in disease pathogenesis is still under debate. However, aggregated A β has been shown to exert neurotoxic effects *in vitro* and *in vivo*; this lends support to the amyloid hypothesis which

suggests that A β is the primary causative agent in AD (Walsh and Selkoe, 2007). Recent advances have led to the development of transgenic APP/PS1 mouse models of AD, in which transgene derived human A β concentration levels are increased through the expression of human APP, and A β ₁₋₄₂ production is favoured through the expression of the PS1 gene. Although A β plaque deposition increases with age, these mice do not exhibit neuronal loss (Lau et al., 2008, Maheswaran et al., 2009), thus providing the opportunity to assess changes due to a A β presence only.

Neuroinflammation, with a particular emphasis on microglial activation, was assessed in 8 – 9 month old APP/PS1 transgenic animals. Quantification of amyloid deposition in these mice indicated female APP/PS1 transgenic mice exhibited more A β , and accordingly more insoluble A β ₁₋₄₂ than male APP/PS1 transgenic mice. Data assessing microglia activation indicated a similar gender bias with transgenic mice exhibiting an increase in microglial activation but female transgenics displaying a more robust phenotype. This was further paralleled by an increases in TNF- α concentration and IL-1 β mRNA expression in female transgenic mice. There is considerable debate in the literature regarding the ability of A β to induce microglial activation *in vivo*. Several reports indicate that plaque formation precedes microglial activation (Gordon et al., 2002), and the data in this study suggests an A β -mediated microglial activation. Consistent with the negative impact proinflammatory cytokines can have on synaptic function, the data indicate that spatial learning was impaired in transgenic mice at 8-9 months of age. APP/PS1 transgenic mice exhibited an impairment in the reversal phase of a MWM task with more profound and persistent changes in female, compared with male, mice. No correlations between task performance and A β concentrations was observed, although it must be noted that the measure of A β concentration used here was not limited to the hippocampus. This may reflect the dynamic nature of A β plaque- or microglial- mediated changes in hippocampal function since the deposition of A β plaques varied between animals. Overall, the present data provide support for the proposal that activation of microglia, together with the upregulation of proinflammatory cytokines, mediate the impairment in hippocampal function and highlight the fact that female APP/PS1

transgenic mice are particularly affected by the ongoing neuropathology present in this mouse model of AD.

Longitudinal assessment of MRI relaxometry and cerebral blood perfusion was carried out in these animals, with initial assessment at 3 – 4 months of age and a further assessment at 8 – 9 months of age. Female wildtype and female APP/PS1 transgenic mice did not display an age-related decline in T2 relaxation time, although male APP/PS1 transgenic, and wildtype, mice did display this decline. No difference in iron levels, as determined by ICP-MS, in the brain tissue of female and male APP/PS1 mice was observed. This finding, in addition, to the reduced levels of amyloid plaque deposition and decreased expression of markers of neuroinflammation suggest the changes are not amyloid, or effects downstream of amyloid deposition, mediated. The consistency of the gender differential in wildtype, as well the transgenic, animals indicates the genetic manipulations required for the insertion of the APP^{swe} and PS1^{dE9} gene are not responsible for differences in T2 relaxation times.

The absence of a T2 relaxation time decrease in female APP/PS1 transgenic mice contrasts with reports which revealed that much older APP/PS1 transgenic mice displayed significant exacerbation of the age-related decline in T2 relaxation time due to the iron colocalized to A β plaques (Braakman et al., 2006). It is tempting to suggest that the age-related decline in T2 relaxation was masked due to ongoing decreases in cell density, which would lead to a T2 relaxation time increase, associated with amyloid plaque deposition. However, the similarity of the T2 relaxation time profile as exhibited by female wildtype mice suggests this is not the case. Furthermore the absence of volumetric changes, as assessed by VBM, in female APP/PS1 transgenic mice supports this conclusion.

MRI relaxometry data is often difficult to interpret in situations where changes are subtle and it would seem that T2 relaxometry, although useful in situations involving much greater A β plaque deposition, is not useful indicator in situations where there is a of low level of A β plaque deposition. The gender

differential in T2 relaxation time observed in this study has not been reported elsewhere. These results are difficult to interpret and appear distinct from any discussions relating to neuroinflammation, the findings require replication but may prove important should MR relaxometry evolve to use as a marker of subtle physiological changes in brain tissue.

Female APP/PS1 transgenic mice exhibited an age-related decline rCBF in the hippocampus but not the cortex. rCBF decline with age in similar animals has been reported but only in cortical areas and in a whole brain measure (Hooijmans et al., 2007, Dhenain et al., 2008, Weidensteiner et al., 2009). No observable decline in gray matter volume was observed in these animals with age. Therefore it might be concluded that the age-related decline in rCBF may be associated with CAA-mediated cerebrovasculature changes or A β -mediated neuronal impairment, but not neuronal loss. It is also possible that both of these factors contribute to the rCBF change observed. Although MRI assessment of rCBF provides clear indication of changes presumably due to CAA, a measure reflecting microglial activation would be highly advantageous in assessing AD onset and progression. Although, it must be acknowledged that the perfusion demands of inflammatory tissue compared with normal tissue are poorly understood.

The evidence presented indicates an increased level of neuroinflammation, characterized by microglial activation and proinflammatory cytokine production, in 8 – 9 month old APP/PS1 mice. These changes are postulated to contribute to changes in spatial memory and cerebral vasculature observed in the APP/PS1 mouse model of AD.

Chapter 7

References

- ABBOTT, N. J., RONNBACK, L. & HANSSON, E. 2006. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci*, 7, 41-53.
- ABRAHAM, H., LOSONCZY, A., CZECH, G. & LAZAR, G. 2001. Rapid activation of microglial cells by hypoxia, kainic acid, and potassium ions in slice preparations of the rat hippocampus. *Brain Res*, 906, 115-26.
- ALOISI, F. 2001. Immune function of microglia. *Glia*, 36, 165-79.
- ALONZO, N. C., HYMAN, B. T., REBECK, G. W. & GREENBERG, S. M. 1998. Progression of cerebral amyloid angiopathy: accumulation of amyloid-beta40 in affected vessels. *J Neuropathol Exp Neurol*, 57, 353-9.
- ALSOP, D. C., DETRE, J. A. & GROSSMAN, M. 2000. Assessment of cerebral blood flow in Alzheimer's disease by spin-labeled magnetic resonance imaging. *Ann Neurol*, 47, 93-100.
- ANDERSON, S. A., SHUKALIAK-QUANDT, J., JORDAN, E. K., ARBAB, A. S., MARTIN, R., MCFARLAND, H. & FRANK, J. A. 2004. Magnetic resonance imaging of labeled T-cells in a mouse model of multiple sclerosis. *Ann Neurol*, 55, 654-9.
- ANDREASEN, N., MINTHON, L., DAVIDSSON, P., VANMECHELEN, E., VANDERSTICHELE, H., WINBLAD, B. & BLENNOW, K. 2001. Evaluation of CSF-tau and CSF-Abeta42 as diagnostic markers for Alzheimer disease in clinical practice. *Arch Neurol*, 58, 373-9.
- APELT, J. & SCHLIEBS, R. 2001. Beta-amyloid-induced glial expression of both pro- and anti-inflammatory cytokines in cerebral cortex of aged transgenic Tg2576 mice with Alzheimer plaque pathology. *Brain Res*, 894, 21-30.
- ARD, M. D., COLE, G. M., WEI, J., MEHRLE, A. P. & FRATKIN, J. D. 1996. Scavenging of Alzheimer's amyloid beta-protein by microglia in culture. *J Neurosci Res*, 43, 190-202.
- ARENDASH, G. W., KING, D. L., GORDON, M. N., MORGAN, D., HATCHER, J. M., HOPE, C. E. & DIAMOND, D. M. 2001. Progressive, age-related behavioral impairments in transgenic mice carrying both mutant amyloid precursor protein and presenilin-1 transgenes. *Brain Res*, 891, 42-53.
- ARMSTRONG, R. A., CAIRNS, N. J. & LANTOS, P. L. 1998. Spatial distribution of diffuse, primitive, and classic amyloid-beta deposits and blood vessels in the upper laminae of the frontal cortex in Alzheimer disease. *Alzheimer Dis Assoc Disord*, 12, 378-83.
- ARRIAGADA, P. V., GROWDON, J. H., HEDLEY-WHITE, E. T. & HYMAN, B. T. 1992. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology*, 42, 631-9.
- ATWOOD, C. S., MARTINS, R. N., SMITH, M. A. & PERRY, G. 2002. Senile plaque composition and posttranslational modification of amyloid-beta peptide and associated proteins. *Peptides*, 23, 1343-50.
- ATWOOD, C. S., MOIR, R. D., HUANG, X., SCARPA, R. C., BACCARRA, N. M., ROMANO, D. M., HARTSHORN, M. A., TANZI, R. E. & BUSH, A. I. 1998. Dramatic aggregation of Alzheimer abeta by Cu(II) is induced by conditions representing physiological acidosis. *J Biol Chem*, 273, 12817-26.
- ATWOOD, C. S., SCARPA, R. C., HUANG, X., MOIR, R. D., JONES, W. D., FAIRLIE, D. P., TANZI, R. E. & BUSH, A. I. 2000. Characterization of copper interactions with Alzheimer amyloid beta peptides: identification of an

- attomolar-affinity copper binding site on amyloid beta1-42. *J Neurochem*, 75, 1219-33.
- BAETEN, K., HENDRIKS, J. J., HELTINGS, N., THEUNISSEN, E., VANDERLOCHT, J., RYCK, L. D., GELAN, J., STINISSEN, P. & ADRIAENSENS, P. 2008. Visualisation of the kinetics of macrophage infiltration during experimental autoimmune encephalomyelitis by magnetic resonance imaging. *J Neuroimmunol*, 195, 1-6.
- BALLATORE, C., LEE, V. M. & TROJANOWSKI, J. Q. 2007. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat Rev Neurosci*, 8, 663-72.
- BARD, F., CANNON, C., BARBOUR, R., BURKE, R. L., GAMES, D., GRAJEDA, H., GUIDO, T., HU, K., HUANG, J., JOHNSON-WOOD, K., KHAN, K., KHOLODENKO, D., LEE, M., LIEBERBURG, I., MOTTER, R., NGUYEN, M., SORIANO, F., VASQUEZ, N., WEISS, K., WELCH, B., SEUBERT, P., SCHENK, D. & YEDNOCK, T. 2000. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat Med*, 6, 916-9.
- BARNHAM, K. J., HAEFFNER, F., CICCOTOSTO, G. D., CURTAIN, C. C., TEW, D., MAVROS, C., BEYREUTHER, K., CARRINGTON, D., MASTERS, C. L., CHERNY, R. A., CAPPAL, R. & BUSH, A. I. 2004. Tyrosine gated electron transfer is key to the toxic mechanism of Alzheimer's disease beta-amyloid. *FASEB J*, 18, 1427-9.
- BARTZOKIS, G., SULTZER, D., CUMMINGS, J., HOLT, L. E., HANCE, D. B., HENDERSON, V. W. & MINTZ, J. 2000. In vivo evaluation of brain iron in Alzheimer disease using magnetic resonance imaging. *Arch Gen Psychiatry*, 57, 47-53.
- BECKER, C., HODENIUS, M., BLENDINGER, G., SECHI, A., HIERONYMUS, T., MÜLLER-SCHULTE, D., SCHMITZ-RODE, T. & ZENKE, M. 2007. Uptake of magnetic nanoparticles into cells for cell tracking. *Journal of Magnetism and Magnetic Materials*, 311, 234-237.
- BEHRENDT, M., SANDROS, M. G., MCKINNEY, R. A., MCDONALD, K., PRZYBYTKOWSKI, E., TABRIZIAN, M. & MAYSINGER, D. 2009. Imaging and organelle distribution of fluorescent InGaP/ZnS nanoparticles in glial cells. *Nanomed*, 4, 747-61.
- BENTAHIR, M., NYABI, O., VERHAMME, J., TOLIA, A., HORRE, K., WILTFANG, J., ESSELMANN, H. & DE STROOPER, B. 2006. Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. *J Neurochem*, 96, 732-42.
- BENVENISTE, H., EINSTEIN, G., KIM, K. R., HULETTE, C. & JOHNSON, G. A. 1999. Detection of neuritic plaques in Alzheimer's disease by magnetic resonance microscopy. *Proc Natl Acad Sci U S A*, 96, 14079-84.
- BERGER, C., HIESTAND, P., KINDLER-BAUMANN, D., RUDIN, M. & RAUSCH, M. 2006. Analysis of lesion development during acute inflammation and remission in a rat model of experimental autoimmune encephalomyelitis by visualization of macrophage infiltration, demyelination and blood-brain barrier damage. *NMR Biomed*, 19, 101-7.
- BRAAKMAN, N., MATYSIK, J., VAN DUINEN, S. G., VERBEEK, F., SCHLIEBS, R., DE GROOT, H. J. & ALIA, A. 2006. Longitudinal

- assessment of Alzheimer's beta-amyloid plaque development in transgenic mice monitored by in vivo magnetic resonance microimaging. *J Magn Reson Imaging*, 24, 530-6.
- BREITNER, J. C. 1996. The role of anti-inflammatory drugs in the prevention and treatment of Alzheimer's disease. *Annu Rev Med*, 47, 401-11.
- BROCHET, B., DELOIRE, M. S., TOUIL, T., ANNE, O., CAILLE, J. M., DOUSSET, V. & PETRY, K. G. 2006. Early macrophage MRI of inflammatory lesions predicts lesion severity and disease development in relapsing EAE. *Neuroimage*, 32, 266-74.
- CACABELOS, R., ALVAREZ, X. A., FERNANDEZ-NOVOA, L., FRANCO, A., MANGUES, R., PELLICER, A. & NISHIMURA, T. 1994. Brain interleukin-1 beta in Alzheimer's disease and vascular dementia. *Methods Find Exp Clin Pharmacol*, 16, 141-51.
- CAO, C., ARENDASH, G. W., DICKSON, A., MAMCARZ, M. B., LIN, X. & ETHELL, D. W. 2009. Abeta-specific Th2 cells provide cognitive and pathological benefits to Alzheimer's mice without infiltrating the CNS. *Neurobiol Dis*, 34, 63-70.
- CAO, D., LU, H., LEWIS, T. L. & LI, L. 2007. Intake of sucrose-sweetened water induces insulin resistance and exacerbates memory deficits and amyloidosis in a transgenic mouse model of Alzheimer disease. *J Biol Chem*, 282, 36275-82.
- CASTELLANI, R. J., MOREIRA, P. I., LIU, G., DOBSON, J., PERRY, G., SMITH, M. A. & ZHU, X. 2007. Iron: the Redox-active center of oxidative stress in Alzheimer disease. *Neurochem Res*, 32, 1640-5.
- CHALELA, J. A., ALSOP, D. C., GONZALEZ-ATAVALES, J. B., MALDJIAN, J. A., KASNER, S. E. & DETRE, J. A. 2000. Magnetic resonance perfusion imaging in acute ischemic stroke using continuous arterial spin labeling. *Stroke*, 31, 680-7.
- CHEN, G., CHEN, K. S., KNOX, J., INGLIS, J., BERNARD, A., MARTIN, S. J., JUSTICE, A., MCCONLOGUE, L., GAMES, D., FREEDMAN, S. B. & MORRIS, R. G. 2000. A learning deficit related to age and beta-amyloid plaques in a mouse model of Alzheimer's disease. *Nature*, 408, 975-9.
- CHEN, Z. C., XU, R. X., YANG, Z. J., FAN, J., XIU, J. G., DAI, G. H., JIANG, X. D., WEI, L. & LEI, H. 2007. [Sinerem labeling and MRI tracking of neural stem cells in vivo and in vitro]. *Nan Fang Yi Ke Da Xue Xue Bao*, 27, 611-5.
- CHERNY, R. A., LEGG, J. T., MCLEAN, C. A., FAIRLIE, D. P., HUANG, X., ATWOOD, C. S., BEYREUTHER, K., TANZI, R. E., MASTERS, C. L. & BUSH, A. I. 1999. Aqueous dissolution of Alzheimer's disease Abeta amyloid deposits by biometal depletion. *J Biol Chem*, 274, 23223-8.
- CHERTOK, B., MOFFAT, B. A., DAVID, A. E., YU, F., BERGEMANN, C., ROSS, B. D. & YANG, V. C. 2008. Iron oxide nanoparticles as a drug delivery vehicle for MRI monitored magnetic targeting of brain tumors. *Biomaterials*, 29, 487-96.
- CHEUNG, J. S., CHOW, A. M., HUI, E. S., YANG, J., TSE, H. F. & WU, E. X. 2006. Cell number quantification of USPIO-labeled stem cells by MRI: an in vitro study. *Conf Proc IEEE Eng Med Biol Soc*, 1, 476-9.
- CHIANG, P. K., LAM, M. A. & LUO, Y. 2008. The many faces of amyloid beta in Alzheimer's disease. *Curr Mol Med*, 8, 580-4.

- CHOI, D. W. & KOH, J. Y. 1998. Zinc and brain injury. *Annu Rev Neurosci*, 21, 347-75.
- CHOI, D. W., YOKOYAMA, M. & KOH, J. 1988. Zinc neurotoxicity in cortical cell culture. *Neuroscience*, 24, 67-79.
- CHOPRA, D., GULATI, M., SALUJA, V., PATHAK, P. & BANSAL, P. 2008. Brain permeable nanoparticles. *Recent Pat CNS Drug Discov*, 3, 216-25.
- CHUA-ANUSORN, W., WEBB, J., MACEY, D. J., POOTRAKUL, P. & ST PIERRE, T. G. 1997. The effect of histological processing on the form of iron in iron-loaded human tissues. *Biochim Biophys Acta*, 1360, 255-61.
- COLIN F. CHIGNELL, R. H. S. 1998. Effect of Magnetite Particles on Photoinduced and Nonphotoinduced Free Radical Processes in Human Erythrocytes. *Photochemistry and Photobiology*, 68, 598-601.
- COLLINGWOOD, J. & DOBSON, J. 2006. Mapping and characterization of iron compounds in Alzheimer's tissue. *J Alzheimers Dis*, 10, 215-22.
- COLLINGWOOD, J. F., CHONG, R. K., KASAMA, T., CERVERA-GONTARD, L., DUNIN-BORKOWSKI, R. E., PERRY, G., POSFAI, M., SIEDLAK, S. L., SIMPSON, E. T., SMITH, M. A. & DOBSON, J. 2008. Three-dimensional tomographic imaging and characterization of iron compounds within Alzheimer's plaque core material. *J Alzheimers Dis*, 14, 235-45.
- COLLINGWOOD, J. F., MIKHAYLOVA, A., DAVIDSON, M., BATICH, C., STREIT, W. J., TERRY, J. & DOBSON, J. 2005. In situ characterization and mapping of iron compounds in Alzheimer's disease tissue. *J Alzheimers Dis*, 7, 267-72.
- CONNOR, J. R., MENZIES, S. L., BURDO, J. R. & BOYER, P. J. 2001. Iron and iron management proteins in neurobiology. *Pediatr Neurol*, 25, 118-29.
- CONNOR, J. R., SNYDER, B. S., BEARD, J. L., FINE, R. E. & MUFSON, E. J. 1992. Regional distribution of iron and iron-regulatory proteins in the brain in aging and Alzheimer's disease. *J Neurosci Res*, 31, 327-35.
- CORR, S. A., BYRNE, S. J., TEKORIUTE, R., MELEDANDRI, C. J., BROUGHAM, D. F., LYNCH, M., KERSKENS, C., O'DWYER, L. & GUN'KO, Y. K. 2008. Linear assemblies of magnetic nanoparticles as MRI contrast agents. *J Am Chem Soc*, 130, 4214-5.
- CORR, S. A., O'BYRNE, A., GUN'KO, Y. K., GHOSH, S., BROUGHAM, D. F., MITCHELL, S., VOLKOV, Y. & PRINA-MELLO, A. 2006. Magnetic-fluorescent nanocomposites for biomedical multitasking. *Chem Commun (Camb)*, 4474-6.
- CUAJUNGCO, M. P., GOLDSTEIN, L. E., NUNOMURA, A., SMITH, M. A., LIM, J. T., ATWOOD, C. S., HUANG, X., FARRAG, Y. W., PERRY, G. & BUSH, A. I. 2000. Evidence that the beta-amyloid plaques of Alzheimer's disease represent the redox-silencing and entombment of abeta by zinc. *J Biol Chem*, 275, 19439-42.
- CUNNINGHAM, C. H., ARAI, T., YANG, P. C., MCCONNELL, M. V., PAULY, J. M. & CONOLLY, S. M. 2005. Positive contrast magnetic resonance imaging of cells labeled with magnetic nanoparticles. *Magn Reson Med*, 53, 999-1005.
- DAWSON, G. R., SEABROOK, G. R., ZHENG, H., SMITH, D. W., GRAHAM, S., O'DOWD, G., BOWERY, B. J., BOYCE, S., TRUMBAUER, M. E., CHEN, H. Y., VAN DER PLOEG, L. H. & SIRINATHSINGHJI, D. J. 1999. Age-related cognitive deficits, impaired long-term potentiation and reduction in

- synaptic marker density in mice lacking the beta-amyloid precursor protein. *Neuroscience*, 90, 1-13.
- DEIBEL, M. A., EHMANN, W. D. & MARKESBERY, W. R. 1996. Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress. *J Neurol Sci*, 143, 137-42.
- DEROUESNE, C. 2008. [Alzheimer and Alzheimer's disease: the present enlightened by the past. An historical approach]. *Psychol Neuropsychiatr Vieil*, 6, 115-28.
- DESESTRET, V., BRISSET, J. C., MOUCHARRAFIE, S., DEVILLARD, E., NATAF, S., HONNORAT, J., NIGHOGHOSSIAN, N., BERTHEZENE, Y. & WIART, M. 2009. Early-stage investigations of ultrasmall superparamagnetic iron oxide-induced signal change after permanent middle cerebral artery occlusion in mice. *Stroke*, 40, 1834-41.
- DEWITT, D. A., PERRY, G., COHEN, M., DOLLER, C. & SILVER, J. 1998. Astrocytes regulate microglial phagocytosis of senile plaque cores of Alzheimer's disease. *Exp Neurol*, 149, 329-40.
- DHENAIN, M., EL TANNIR EL TAYARA, N., WU, T. D., GUEGAN, M., VOLK, A., QUINTANA, C. & DELATOUR, B. 2009. Characterization of in vivo MRI detectable thalamic amyloid plaques from APP/PS1 mice. *Neurobiol Aging*, 30, 41-53.
- DHENAIN, M., EL TAYARA, N. E. T., COZZONE, P. J., VOLK, A., DELATOUR, B. & KOBER, F. 2008. P1-083: Longitudinal evaluation of cortical perfusion by quantitative arterial spin labeling MRI in a mouse model of Alzheimer's disease. *Alzheimer's and Dementia*, 4, T230-T231.
- DHENAIN, M., PRIVAT, N., DUYCKAERTS, C. & JACOBS, R. E. 2002. Senile plaques do not induce susceptibility effects in T2*-weighted MR microscopic images. *NMR Biomed*, 15, 197-203.
- DICARLO, G., WILCOCK, D., HENDERSON, D., GORDON, M. & MORGAN, D. 2001. Intrahippocampal LPS injections reduce Abeta load in APP+PS1 transgenic mice. *Neurobiol Aging*, 22, 1007-12.
- DIXON, W. T., DU, L. N., FAUL, D. D., GADO, M. & ROSSNICK, S. 1986. Projection angiograms of blood labeled by adiabatic fast passage. *Magn Reson Med*, 3, 454-62.
- DOBSON, J. 2001. Nanoscale biogenic iron oxides and neurodegenerative disease. *FEBS Lett*, 496, 1-5.
- DOBSON, J. 2002. Investigation of age-related variations in biogenic magnetite levels in the human hippocampus. *Exp Brain Res*, 144, 122-6.
- DOBSON, J. & GRASSI, P. 1996. Magnetic properties of human hippocampal tissue--evaluation of artefact and contamination sources. *Brain Res Bull*, 39, 255-9.
- DOUSSET, V., BROCHET, B., DELOIRE, M. S., LAGOARDE, L., BARROSO, B., CAILLE, J. M. & PETRY, K. G. 2006. MR imaging of relapsing multiple sclerosis patients using ultra-small-particle iron oxide and compared with gadolinium. *AJNR Am J Neuroradiol*, 27, 1000-5.
- DUDAL, S., KRZYWKOWSKI, P., PAQUETTE, J., MORISSETTE, C., LACOMBE, D., TREMBLAY, P. & GERVAIS, F. 2004. Inflammation occurs early during the Abeta deposition process in TgCRND8 mice. *Neurobiol Aging*, 25, 861-71.

- DUFF, K. 2006. Normal and abnormal tau neurobiology. *Alzheimer Dis Assoc Disord*, 20, 202-5.
- EDWARDS, R. H. 2001. Drug delivery via the blood-brain barrier. *Nat Neurosci*, 4, 221-2.
- EL TANNIR EL TAYARA, N., DELATOUR, B., LE CUDENNEC, C., GUEGAN, M., VOLK, A. & DHENAIN, M. 2006. Age-related evolution of amyloid burden, iron load, and MR relaxation times in a transgenic mouse model of Alzheimer's disease. *Neurobiol Dis*, 22, 199-208.
- EL TAYARA NEL, T., VOLK, A., DHENAIN, M. & DELATOUR, B. 2007. Transverse relaxation time reflects brain amyloidosis in young APP/PS1 transgenic mice. *Magn Reson Med*, 58, 179-84.
- ERIKSEN, J. L. & JANUS, C. G. 2007. Plaques, tangles, and memory loss in mouse models of neurodegeneration. *Behav Genet*, 37, 79-100.
- FALANGOLA, M. F., DYAKIN, V. V., LEE, S. P., BOGART, A., BABB, J. S., DUFF, K., NIXON, R. & HELPERN, J. A. 2007. Quantitative MRI reveals aging-associated T2 changes in mouse models of Alzheimer's disease. *NMR Biomed*, 20, 343-51.
- FALANGOLA, M. F., LEE, S. P., NIXON, R. A., DUFF, K. & HELPERN, J. A. 2005. Histological co-localization of iron in Abeta plaques of PS/APP transgenic mice. *Neurochem Res*, 30, 201-5.
- FIACCO, T. A., AGULHON, C. & MCCARTHY, K. D. 2009. Sorting out astrocyte physiology from pharmacology. *Annu Rev Pharmacol Toxicol*, 49, 151-74.
- FILALI, M. & LALONDE, R. 2009. Age-related cognitive decline and nesting behavior in an APP^{swe}/PS1^{bigenic} model of Alzheimer's disease. *Brain Res*, 1292, 93-9.
- FLODEN, A. M. & COMBS, C. K. 2006. Beta-amyloid stimulates murine postnatal and adult microglia cultures in a unique manner. *J Neurosci*, 26, 4644-8.
- FRAUTSCHY, S. A., COLE, G. M. & BAIRD, A. 1992. Phagocytosis and deposition of vascular beta-amyloid in rat brains injected with Alzheimer beta-amyloid. *Am J Pathol*, 140, 1389-99.
- FRIEDLICH, A. L., LEE, J. Y., VAN GROEN, T., CHERNY, R. A., VOLITAKIS, I., COLE, T. B., PALMITER, R. D., KOH, J. Y. & BUSH, A. I. 2004. Neuronal zinc exchange with the blood vessel wall promotes cerebral amyloid angiopathy in an animal model of Alzheimer's disease. *J Neurosci*, 24, 3453-9.
- GAMES, D., ADAMS, D., ALESSANDRINI, R., BARBOUR, R., BERTHELETTE, P., BLACKWELL, C., CARR, T., CLEMENS, J., DONALDSON, T., GILLESPIE, F. & ET AL. 1995. Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature*, 373, 523-7.
- GAMES, D., KHAN, K. M., SORIANO, F. G., KEIM, P. S., DAVIS, D. L., BRYANT, K. & LIEBERBURG, I. 1992. Lack of Alzheimer pathology after beta-amyloid protein injections in rat brain. *Neurobiol Aging*, 13, 569-76.
- GARCAO, P., OLIVEIRA, C. R. & AGOSTINHO, P. 2006. Comparative study of microglia activation induced by amyloid-beta and prion peptides: role in neurodegeneration. *J Neurosci Res*, 84, 182-93.
- GARCIA-ALLOZA, M., ROBBINS, E. M., ZHANG-NUNES, S. X., PURCELL, S. M., BETENSKY, R. A., RAJU, S., PRADA, C., GREENBERG, S. M.,

- BACSKAI, B. J. & FROSCH, M. P. 2006. Characterization of amyloid deposition in the APP^{swe}/PS1^{ΔE9} mouse model of Alzheimer disease. *Neurobiol Dis*, 24, 516-24.
- GARDEN, G. A. & MOLLER, T. 2006. Microglia biology in health and disease. *J Neuroimmune Pharmacol*, 1, 127-37.
- GARZON-RODRIGUEZ, W., YATSIMIRSKY, A. K. & GLABE, C. G. 1999. Binding of Zn(II), Cu(II), and Fe(II) ions to Alzheimer's A beta peptide studied by fluorescence. *Bioorg Med Chem Lett*, 9, 2243-8.
- GODOY, M. C., TARELLI, R., FERRARI, C. C., SARCHI, M. I. & PITOSI, F. J. 2008. Central and systemic IL-1 exacerbates neurodegeneration and motor symptoms in a model of Parkinson's disease. *Brain*, 131, 1880-94.
- GONZALEZ-AT, J. B., ALSOP, D. C. & DETRE, J. A. 2000. Cerebral perfusion and arterial transit time changes during task activation determined with continuous arterial spin labeling. *Magn Reson Med*, 43, 739-46.
- GONZALEZ-SCARANO, F. & MARTIN-GARCIA, J. 2005. The neuropathogenesis of AIDS. *Nat Rev Immunol*, 5, 69-81.
- GORDON, M. N., HOLCOMB, L. A., JANTZEN, P. T., DICARLO, G., WILCOCK, D., BOYETT, K. W., CONNOR, K., MELACHRINO, J., O'CALLAGHAN, J. P. & MORGAN, D. 2002. Time course of the development of Alzheimer-like pathology in the doubly transgenic PS1+APP mouse. *Exp Neurol*, 173, 183-95.
- GOTZ, J., CHEN, F., VAN DORPE, J. & NITSCH, R. M. 2001. Formation of neurofibrillary tangles in P301 τ transgenic mice induced by A β 42 fibrils. *Science*, 293, 1491-5.
- GRIFFIN, W. S., SHENG, J. G., ROBERTS, G. W. & MRAK, R. E. 1995. Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution. *J Neuropathol Exp Neurol*, 54, 276-81.
- GRIFFIN, W. S., STANLEY, L. C., LING, C., WHITE, L., MACLEOD, V., PERROT, L. J., WHITE, C. L., 3RD & ARAOZ, C. 1989. Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *Proc Natl Acad Sci U S A*, 86, 7611-5.
- HAASS, C., HUNG, A. Y. & SELKOE, D. J. 1991. Processing of beta-amyloid precursor protein in microglia and astrocytes favors an internal localization over constitutive secretion. *J Neurosci*, 11, 3783-93.
- HARDY, J. & ALLSOP, D. 1991. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol Sci*, 12, 383-8.
- HAUTOT, D., PANKHURST, Q. A. & DOBSON, J. 2005. Superconducting quantum interference device measurements of dilute magnetic materials in biological samples. *Review of Scientific Instruments*, 76, 045101.
- HAUTOT, D., PANKHURST, Q. A., KHAN, N. & DOBSON, J. 2003. Preliminary evaluation of nanoscale biogenic magnetite in Alzheimer's disease brain tissue. *Proc Biol Sci*, 270 Suppl 1, S62-4.
- HEBER, S., HERMS, J., GAJIC, V., HAINFELLNER, J., AGUZZI, A., RULICKE, T., VON KRETZSCHMAR, H., VON KOCH, C., SISODIA, S., TREMML, P., LIPP, H. P., WOLFER, D. P. & MULLER, U. 2000. Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members. *J Neurosci*, 20, 7951-63.

- HELPERN, J. A., LEE, S. P., FALANGOLA, M. F., DYAKIN, V. V., BOGART, A., ARDEKANI, B., DUFF, K., BRANCH, C., WISNIEWSKI, T., DE LEON, M. J., WOLF, O., O'SHEA, J. & NIXON, R. A. 2004. MRI assessment of neuropathology in a transgenic mouse model of Alzheimer's disease. *Magn Reson Med*, 51, 794-8.
- HERBER, D. L., MERCER, M., ROTH, L. M., SYMMONDS, K., MALONEY, J., WILSON, N., FREEMAN, M. J., MORGAN, D. & GORDON, M. N. 2007. Microglial activation is required for Abeta clearance after intracranial injection of lipopolysaccharide in APP transgenic mice. *J Neuroimmune Pharmacol*, 2, 222-31.
- HERBER, D. L., ROTH, L. M., WILSON, D., WILSON, N., MASON, J. E., MORGAN, D. & GORDON, M. N. 2004. Time-dependent reduction in Abeta levels after intracranial LPS administration in APP transgenic mice. *Exp Neurol*, 190, 245-53.
- HERMS, J., ANLIKER, B., HEBER, S., RING, S., FUHRMANN, M., KRETZSCHMAR, H., SISODIA, S. & MULLER, U. 2004. Cortical dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family members. *EMBO J*, 23, 4106-15.
- HOLCOMB, L., GORDON, M. N., MCGOWAN, E., YU, X., BENKOVIC, S., JANTZEN, P., WRIGHT, K., SAAD, I., MUELLER, R., MORGAN, D., SANDERS, S., ZEHR, C., O'CAMPO, K., HARDY, J., PRADA, C. M., ECKMAN, C., YOUNKIN, S., HSIAO, K. & DUFF, K. 1998. Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nat Med*, 4, 97-100.
- HOLSINGER, A. E. & RIEDERER, S. J. 1990. The importance of phase-encoding order in ultra-short TR snapshot MR imaging. *Magn Reson Med*, 16, 481-8.
- HOOIJMANS, C. R., RUTTERS, F., DEDEREN, P. J., GAMBAROTA, G., VELTIEN, A., VAN GROEN, T., BROERSEN, L. M., LUTJOHANN, D., HEERSCHAP, A., TANILA, H. & KILIAAN, A. J. 2007. Changes in cerebral blood volume and amyloid pathology in aged Alzheimer APP/PS1 mice on a docosahexaenoic acid (DHA) diet or cholesterol enriched Typical Western Diet (TWD). *Neurobiol Dis*, 28, 16-29.
- HOSSMANN, K. A. 1994. Viability thresholds and the penumbra of focal ischemia. *Ann Neurol*, 36, 557-65.
- HSIAO, K., CHAPMAN, P., NILSEN, S., ECKMAN, C., HARIGAYA, Y., YOUNKIN, S., YANG, F. & COLE, G. 1996. Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science*, 274, 99-102.
- HUANG, X., ATWOOD, C. S., HARTSHORN, M. A., MULTHAUP, G., GOLDSTEIN, L. E., SCARPA, R. C., CUAJUNGCO, M. P., GRAY, D. N., LIM, J., MOIR, R. D., TANZI, R. E. & BUSH, A. I. 1999a. The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry*, 38, 7609-16.
- HUANG, X., CUAJUNGCO, M. P., ATWOOD, C. S., HARTSHORN, M. A., TYNDALL, J. D., HANSON, G. R., STOKES, K. C., LEOPOLD, M., MULTHAUP, G., GOLDSTEIN, L. E., SCARPA, R. C., SAUNDERS, A. J., LIM, J., MOIR, R. D., GLABE, C., BOWDEN, E. F., MASTERS, C. L., FAIRLIE, D. P., TANZI, R. E. & BUSH, A. I. 1999b. Cu(II) potentiation of

- alzheimer abeta neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction. *J Biol Chem*, 274, 37111-6.
- HUGHES, E. G., ELMARIAH, S. B. & BALICE-GORDON, R. J. 2009. Astrocyte secreted proteins selectively increase hippocampal GABAergic axon length, branching, and synaptogenesis. *Mol Cell Neurosci*.
- HULET, S. W., POWERS, S. & CONNOR, J. R. 1999. Distribution of transferrin and ferritin binding in normal and multiple sclerotic human brains. *J Neurol Sci*, 165, 48-55.
- IDE-EKTESSABI, A. & RABIONET, M. 2005. The role of trace metallic elements in neurodegenerative disorders: quantitative analysis using XRF and XANES spectroscopy. *Anal Sci*, 21, 885-92.
- II, M., SUNAMOTO, M., OHNISHI, K. & ICHIMORI, Y. 1996. beta-Amyloid protein-dependent nitric oxide production from microglial cells and neurotoxicity. *Brain Res*, 720, 93-100.
- IRIZARRY, M. C., MCNAMARA, M., FEDORCHAK, K., HSIAO, K. & HYMAN, B. T. 1997. APPSw transgenic mice develop age-related A beta deposits and neuropil abnormalities, but no neuronal loss in CA1. *J Neuropathol Exp Neurol*, 56, 965-73.
- ISHIHARA, T., GONDO, T., TAKAHASHI, M., UCHINO, F., IKEDA, S., ALLSOP, D. & IMAI, K. 1991. Immunohistochemical and immunoelectron microscopical characterization of cerebrovascular and senile plaque amyloid in aged dogs' brains. *Brain Res*, 548, 196-205.
- ITAGAKI, S., MCGEER, P. L., AKIYAMA, H., ZHU, S. & SELKOE, D. 1989. Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. *J Neuroimmunol*, 24, 173-82.
- J. C. SCAIANO, S. M. J. R. 1997. Dramatic Effect of Magnetite Particles on the Dynamics of Photogenerated Free Radicals. *Photochemistry and Photobiology*, 65, 759-762.
- JACK, C. R., JR., GARWOOD, M., WENGENACK, T. M., BOROWSKI, B., CURRAN, G. L., LIN, J., ADRIANY, G., GROHN, O. H., GRIMM, R. & PODUSLO, J. F. 2004. In vivo visualization of Alzheimer's amyloid plaques by magnetic resonance imaging in transgenic mice without a contrast agent. *Magn Reson Med*, 52, 1263-71.
- JACK, C. R., JR., WENGENACK, T. M., REYES, D. A., GARWOOD, M., CURRAN, G. L., BOROWSKI, B. J., LIN, J., PREBOSKE, G. M., HOLASEK, S. S., ADRIANY, G. & PODUSLO, J. F. 2005. In vivo magnetic resonance microimaging of individual amyloid plaques in Alzheimer's transgenic mice. *J Neurosci*, 25, 10041-8.
- JANKOWSKY, J. L., SLUNT, H. H., RATOVITSKI, T., JENKINS, N. A., COPELAND, N. G. & BORCHELT, D. R. 2001. Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. *Biomol Eng*, 17, 157-65.
- JEWELL, C. M. & LYNN, D. M. 2008. Multilayered polyelectrolyte assemblies as platforms for the delivery of DNA and other nucleic acid-based therapeutics. *Advanced Drug Delivery Reviews*, 60, 979-999.
- JOHNSON, N. A., JAHNG, G.-H., WEINER, M. W., MILLER, B. L., CHUI, H. C., JAGUST, W. J., GORNO-TEMPINI, M. L. & SCHUFF, N. 2006. Pattern of cerebral hypoperfusion in Alzheimer's disease and mild cognitive impairment

- measured with arterial spin-labeling MR imaging: Initial experience. *International Congress Series*, 1290, 108-122.
- JOHNSON, N. A., JAHNG, G. H., WEINER, M. W., MILLER, B. L., CHUI, H. C., JAGUST, W. J., GORNO-TEMPINI, M. L. & SCHUFF, N. 2005. Pattern of cerebral hypoperfusion in Alzheimer disease and mild cognitive impairment measured with arterial spin-labeling MR imaging: initial experience. *Radiology*, 234, 851-9.
- KATO, S., GONDO, T., HOSHII, Y., TAKAHASHI, M., YAMADA, M. & ISHIHARA, T. 1998. Confocal observation of senile plaques in Alzheimer's disease: senile plaque morphology and relationship between senile plaques and astrocytes. *Pathol Int*, 48, 332-40.
- KE, Y. & MING QIAN, Z. 2003. Iron misregulation in the brain: a primary cause of neurodegenerative disorders. *Lancet Neurol*, 2, 246-53.
- KELLER, J. N. 2006. Age-related neuropathology, cognitive decline, and Alzheimer's disease. *Ageing Res Rev*, 5, 1-13.
- KELLY, M. E., BLAU, C. W. & KERSEKENS, C. M. 2009. Bolus-tracking arterial spin labelling: theoretical and experimental results. *Phys Med Biol*, 54, 1235-51.
- KERSEKENS, C. M., HOEHN-BERLAGE, M., SCHMITZ, B., BUSCH, E., BOCK, C., GYNGELL, M. L. & HOSSMANN, K. A. 1996. Ultrafast perfusion-weighted MRI of functional brain activation in rats during forepaw stimulation: comparison with T2-weighted MRI. *NMR Biomed*, 9, 20-3.
- KHAN, A., DOBSON, J. P. & EXLEY, C. 2006. Redox cycling of iron by Abeta42. *Free Radic Biol Med*, 40, 557-69.
- KIELIAN, T. 2004. Microglia and chemokines in infectious diseases of the nervous system: views and reviews. *Front Biosci*, 9, 732-50.
- KIM, Y. S. & JOH, T. H. 2006. Microglia, major player in the brain inflammation: their roles in the pathogenesis of Parkinson's disease. *Exp Mol Med*, 38, 333-47.
- KIRSCH, S. J., JACOBS, R. W., BUTCHER, L. L. & BEATTY, J. 1992. Prolongation of magnetic resonance T2 time in hippocampus of human patients marks the presence and severity of Alzheimer's disease. *Neurosci Lett*, 134, 187-90.
- KIRSCHVINK, J. L., KOBAYASHI-KIRSCHVINK, A. & WOODFORD, B. J. 1992. Magnetite biomineralization in the human brain. *Proc Natl Acad Sci U S A*, 89, 7683-7.
- KOEPPEN, A. H. 1995. The history of iron in the brain. *J Neurol Sci*, 134 Suppl, 1-9.
- KREUTER, J. 2001. Nanoparticulate systems for brain delivery of drugs. *Adv Drug Deliv Rev*, 47, 65-81.
- KREUTER, J. 2004. Influence of the surface properties on nanoparticle-mediated transport of drugs to the brain. *J Nanosci Nanotechnol*, 4, 484-8.
- KREUTER, J., HEKMATARA, T., DREIS, S., VOGEL, T., GELPERINA, S. & LANGER, K. 2007. Covalent attachment of apolipoprotein A-I and apolipoprotein B-100 to albumin nanoparticles enables drug transport into the brain. *J Control Release*, 118, 54-8.
- KUCHIBHOTLA, K. V., GOLDMAN, S. T., LATTARULO, C. R., WU, H. Y., HYMAN, B. T. & BACSKAI, B. J. 2008. Abeta plaques lead to aberrant

- regulation of calcium homeostasis in vivo resulting in structural and functional disruption of neuronal networks. *Neuron*, 59, 214-25.
- KUMAR-SINGH, S., THEUNS, J., VAN BROECK, B., PIRICI, D., VENNEKENS, K., CORSMIT, E., CRUTS, M., DERMAUT, B., WANG, R. & VAN BROECKHOVEN, C. 2006. Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40. *Hum Mutat*, 27, 686-95.
- KUSTERMANN, E., ROELL, W., BREITBACH, M., WECKER, S., WIEDERMANN, D., BUEHRLE, C., WELZ, A., HESCHELER, J., FLEISCHMANN, B. K. & HOEHN, M. 2005. Stem cell implantation in ischemic mouse heart: a high-resolution magnetic resonance imaging investigation. *NMR Biomed*, 18, 362-70.
- LANDRETH, G. E. & REED-GEAGHAN, E. G. 2009. Toll-like receptors in Alzheimer's disease. *Curr Top Microbiol Immunol*, 336, 137-53.
- LAU, J. C., LERCH, J. P., SLED, J. G., HENKELMAN, R. M., EVANS, A. C. & BEDELL, B. J. 2008. Longitudinal neuroanatomical changes determined by deformation-based morphometry in a mouse model of Alzheimer's disease. *Neuroimage*, 42, 19-27.
- LEE, S. P., FALANGOLA, M. F., NIXON, R. A., DUFF, K. & HELPERN, J. A. 2004. Visualization of beta-amyloid plaques in a transgenic mouse model of Alzheimer's disease using MR microscopy without contrast reagents. *Magn Reson Med*, 52, 538-44.
- LEMERE, C. A., LOPERA, F., KOSIK, K. S., LENDON, C. L., OSSA, J., SAIDO, T. C., YAMAGUCHI, H., RUIZ, A., MARTINEZ, A., MADRIGAL, L., HINCAPIE, L., ARANGO, J. C., ANTHONY, D. C., KOO, E. H., GOATE, A. M. & SELKOE, D. J. 1996. The E280A presenilin 1 Alzheimer mutation produces increased A beta 42 deposition and severe cerebellar pathology. *Nat Med*, 2, 1146-50.
- LESKOVJAN, A. C., LANZIROTTI, A. & MILLER, L. M. 2009. Amyloid plaques in PSAPP mice bind less metal than plaques in human Alzheimer's disease. *Neuroimage*, 47, 1215-20.
- LEVITAN, D., LEE, J., SONG, L., MANNING, R., WONG, G., PARKER, E. & ZHANG, L. 2001. PS1 N- and C-terminal fragments form a complex that functions in APP processing and Notch signaling. *Proc Natl Acad Sci U S A*, 98, 12186-90.
- LEWIS, J., DICKSON, D. W., LIN, W. L., CHISHOLM, L., CORRAL, A., JONES, G., YEN, S. H., SAHARA, N., SKIPPER, L., YAGER, D., ECKMAN, C., HARDY, J., HUTTON, M. & MCGOWAN, E. 2001. Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science*, 293, 1487-91.
- LOCKMAN, P. R., KOZIARA, J. M., MUMPER, R. J. & ALLEN, D. D. 2004. Nanoparticle surface charges alter blood-brain barrier integrity and permeability. *J Drug Target*, 12, 635-41.
- LOCKMAN, P. R., OYEWUMI, M. O., KOZIARA, J. M., RODER, K. E., MUMPER, R. J. & ALLEN, D. D. 2003. Brain uptake of thiamine-coated nanoparticles. *J Control Release*, 93, 271-82.
- LONG, T. C., SALEH, N., TILTON, R. D., LOWRY, G. V. & VERONESI, B. 2006. Titanium dioxide (P25) produces reactive oxygen species in immortalized

- brain microglia (BV2): implications for nanoparticle neurotoxicity. *Environ Sci Technol*, 40, 4346-52.
- LONG, T. C., TAJUBA, J., SAMA, P., SALEH, N., SWARTZ, C., PARKER, J., HESTER, S., LOWRY, G. V. & VERONESI, B. 2007. Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons in vitro. *Environ Health Perspect*, 115, 1631-7.
- LOPES, K. O., SPARKS, D. L. & STREIT, W. J. 2008. Microglial dystrophy in the aged and Alzheimer's disease brain is associated with ferritin immunoreactivity. *Glia*, 56, 1048-60.
- LOPEZ, O. L., BECKER, J. T., KLUNK, W., SAXTON, J., HAMILTON, R. L., KAUFER, D. I., SWEET, R. A., CIDIS MELTZER, C., WISNIEWSKI, S., KAMBOH, M. I. & DEKOSKY, S. T. 2000. Research evaluation and diagnosis of probable Alzheimer's disease over the last two decades: I. *Neurology*, 55, 1854-62.
- LOPEZ, O. L. & DEKOSKY, S. T. 2003. [Neuropathology of Alzheimer's disease and mild cognitive impairment]. *Rev Neurol*, 37, 155-63.
- LOVELL, M. A., ROBERTSON, J. D., TEESDALE, W. J., CAMPBELL, J. L. & MARKESBERY, W. R. 1998. Copper, iron and zinc in Alzheimer's disease senile plaques. *J Neurol Sci*, 158, 47-52.
- LOVELL, M. A., SMITH, J. L. & MARKESBERY, W. R. 2006. Elevated zinc transporter-6 in mild cognitive impairment, Alzheimer disease, and pick disease. *J Neuropathol Exp Neurol*, 65, 489-98.
- LUBER-NAROD, J. & ROGERS, J. 1988. Immune system associated antigens expressed by cells of the human central nervous system. *Neurosci Lett*, 94, 17-22.
- LUE, L. F., KUO, Y. M., ROHER, A. E., BRACHOVA, L., SHEN, Y., SUE, L., BEACH, T., KURTH, J. H., RYDEL, R. E. & ROGERS, J. 1999. Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am J Pathol*, 155, 853-62.
- LUE, L. F., WALKER, D. G. & ROGERS, J. 2001. Modeling microglial activation in Alzheimer's disease with human postmortem microglial cultures. *Neurobiol Aging*, 22, 945-56.
- MA, Y., HOF, P. R., GRANT, S. C., BLACKBAND, S. J., BENNETT, R., SLATEST, L., MCGUIGAN, M. D. & BENVENISTE, H. 2005. A three-dimensional digital atlas database of the adult C57BL/6J mouse brain by magnetic resonance microscopy. *Neuroscience*, 135, 1203-15.
- MAHESWARAN, S., BARJAT, H., RUECKERT, D., BATE, S. T., HOWLETT, D. R., TILLING, L., SMART, S. C., POHLMANN, A., RICHARDSON, J. C., HARTKENS, T., HILL, D. L., UPTON, N., HAJNAL, J. V. & JAMES, M. F. 2009. Longitudinal regional brain volume changes quantified in normal aging and Alzheimer's APP x PS1 mice using MRI. *Brain Res*, 1270, 19-32.
- MAYNARD, C. J., CAPPAL, R., VOLITAKIS, I., CHERNY, R. A., WHITE, A. R., BEYREUTHER, K., MASTERS, C. L., BUSH, A. I. & LI, Q. X. 2002. Overexpression of Alzheimer's disease amyloid-beta opposes the age-dependent elevations of brain copper and iron. *J Biol Chem*, 277, 44670-6.
- MCATEER, M. A., SIBSON, N. R., VON ZUR MUHLEN, C., SCHNEIDER, J. E., LOWE, A. S., WARRICK, N., CHANNON, K. M., ANTHONY, D. C. &

- CHOUDHURY, R. P. 2007. In vivo magnetic resonance imaging of acute brain inflammation using microparticles of iron oxide. *Nat Med*, 13, 1253-8.
- MCGRAW, J., HIEBERT, G. W. & STEEVES, J. D. 2001. Modulating astrogliosis after neurotrauma. *J Neurosci Res*, 63, 109-15.
- MCQUILLAN, K., LYNCH, M. A. & MILLS, K. H. G. 2009. A[beta]-specific Th1 cells exacerbate plaque formation and induce memory impairments in APPswe/pS1dE9 mice. *Alzheimer's and Dementia*, 5, P323-P323.
- MEADOWCROFT, M. D., CONNOR, J. R., SMITH, M. B. & YANG, Q. X. 2009. MRI and histological analysis of beta-amyloid plaques in both human Alzheimer's disease and APP/PS1 transgenic mice. *J Magn Reson Imaging*, 29, 997-1007.
- MEIER, P. & ZIERLER, K. L. 1954. On the theory of the indicator-dilution method for measurement of blood flow and volume. *J Appl Physiol*, 6, 731-44.
- MEYER-LUEHMANN, M., SPIRES-JONES, T. L., PRADA, C., GARCIA-ALLOZA, M., DE CALIGNON, A., ROZKALNE, A., KOENIGSKNECHT-TALBOO, J., HOLTZMAN, D. M., BACSKAI, B. J. & HYMAN, B. T. 2008. Rapid appearance and local toxicity of amyloid-beta plaques in a mouse model of Alzheimer's disease. *Nature*, 451, 720-4.
- MILLER, L. M., WANG, Q., TELIVALA, T. P., SMITH, R. J., LANZIROTTI, A. & MIKLOSSY, J. 2006. Synchrotron-based infrared and X-ray imaging shows focalized accumulation of Cu and Zn co-localized with beta-amyloid deposits in Alzheimer's disease. *J Struct Biol*, 155, 30-7.
- MONTESANO, R., PEPPER, M. S., MOHLE-STEINLEIN, U., RISAU, W., WAGNER, E. F. & ORCI, L. 1990. Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. *Cell*, 62, 435-45.
- MOSELEY, M. E., COHEN, Y., MINTOROVITCH, J., CHILEUITT, L., SHIMIZU, H., KUCHARCZYK, J., WENDLAND, M. F. & WEINSTEIN, P. R. 1990. Early detection of regional cerebral ischemia in cats: comparison of diffusion- and T2-weighted MRI and spectroscopy. *Magn Reson Med*, 14, 330-46.
- MOTT, R. T. & HULETTE, C. M. 2005. Neuropathology of Alzheimer's disease. *Neuroimaging Clin N Am*, 15, 755-65, ix.
- MULLER, U., CRISTINA, N., LI, Z. W., WOLFER, D. P., LIPP, H. P., RULICKE, T., BRANDNER, S., AGUZZI, A. & WEISSMANN, C. 1994. Behavioral and anatomical deficits in mice homozygous for a modified beta-amyloid precursor protein gene. *Cell*, 79, 755-65.
- NAKAMURA, S., MURAYAMA, N., NOSHITA, T., ANNOURA, H. & OHNO, T. 2001. Progressive brain dysfunction following intracerebroventricular infusion of beta(1-42)-amyloid peptide. *Brain Res*, 912, 128-36.
- NASLUND, J., HAROUTUNIAN, V., MOHS, R., DAVIS, K. L., DAVIES, P., GREENGARD, P. & BUXBAUM, J. D. 2000. Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. *JAMA*, 283, 1571-7.
- NITIN, N., LACONTE, L. E., ZURKIYA, O., HU, X. & BAO, G. 2004. Functionalization and peptide-based delivery of magnetic nanoparticles as an intracellular MRI contrast agent. *J Biol Inorg Chem*, 9, 706-12.

- NIWA, K., CARLSON, G. A. & IADECOLA, C. 2000. Exogenous A beta1-40 reproduces cerebrovascular alterations resulting from amyloid precursor protein overexpression in mice. *J Cereb Blood Flow Metab*, 20, 1659-68.
- NIWA, K., KAZAMA, K., YOUNKIN, L., YOUNKIN, S. G., CARLSON, G. A. & IADECOLA, C. 2002. Cerebrovascular autoregulation is profoundly impaired in mice overexpressing amyloid precursor protein. *Am J Physiol Heart Circ Physiol*, 283, H315-23.
- NIWA, K., PORTER, V. A., KAZAMA, K., CORNFIELD, D., CARLSON, G. A. & IADECOLA, C. 2001. A beta-peptides enhance vasoconstriction in cerebral circulation. *Am J Physiol Heart Circ Physiol*, 281, H2417-24.
- O'LEARY, T. P. & BROWN, R. E. 2009. Visuo-spatial learning and memory deficits on the Barnes maze in the 16-month-old APPswe/PS1dE9 mouse model of Alzheimer's disease. *Behav Brain Res*, 201, 120-7.
- OAKLEY, A. E., COLLINGWOOD, J. F., DOBSON, J., LOVE, G., PERROTT, H. R., EDWARDSON, J. A., ELSTNER, M. & MORRIS, C. M. 2007. Individual dopaminergic neurons show raised iron levels in Parkinson disease. *Neurology*, 68, 1820-5.
- ODDO, S., CACCAMO, A., CHENG, D., JOULEH, B., TORP, R. & LAFERLA, F. M. 2007. Genetically augmenting tau levels does not modulate the onset or progression of Abeta pathology in transgenic mice. *J Neurochem*, 102, 1053-63.
- OPAZO, C., HUANG, X., CHERNY, R. A., MOIR, R. D., ROHER, A. E., WHITE, A. R., CAPPAL, R., MASTERS, C. L., TANZI, R. E., INESTROSA, N. C. & BUSH, A. I. 2002. Metalloenzyme-like activity of Alzheimer's disease beta-amyloid. Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H(2)O(2). *J Biol Chem*, 277, 40302-8.
- PANKHURST, Q., HAUTOT, D., KHAN, N. & DOBSON, J. 2008. Increased levels of magnetic iron compounds in Alzheimer's disease. *J Alzheimers Dis*, 13, 49-52.
- PARESCE, D. M., GHOSH, R. N. & MAXFIELD, F. R. 1996. Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. *Neuron*, 17, 553-65.
- PARKES, L. M., RASHID, W., CHARD, D. T. & TOFTS, P. S. 2004. Normal cerebral perfusion measurements using arterial spin labeling: reproducibility, stability, and age and gender effects. *Magn Reson Med*, 51, 736-43.
- PERLMUTTER, L. S., SCOTT, S. A., BARRON, E. & CHUI, H. C. 1992. MHC class II-positive microglia in human brain: association with Alzheimer lesions. *J Neurosci Res*, 33, 549-58.
- PERRY, V. H., CUNNINGHAM, C. & BOCHE, D. 2002. Atypical inflammation in the central nervous system in prion disease. *Curr Opin Neurol*, 15, 349-54.
- PIGINO, G., MORFINI, G., ATAGI, Y., DESHPANDE, A., YU, C., JUNGBAUER, L., LADU, M., BUSCIGLIO, J. & BRADY, S. 2009. Disruption of fast axonal transport is a pathogenic mechanism for intraneuronal amyloid beta. *Proc Natl Acad Sci U S A*, 106, 5907-12.
- PIVNEVA, T. A. 2008. Microglia in normal condition and pathology. *Fiziol Zh*, 54, 81-9.

- POPESCU, B. F., ROBINSON, C. A., RAJPUT, A., RAJPUT, A. H., HARDER, S. L. & NICHOL, H. 2009. Iron, copper, and zinc distribution of the cerebellum. *Cerebellum*, 8, 74-9.
- PRATICO, D., URYU, K., LEIGHT, S., TROJANOSWIKI, J. Q. & LEE, V. M. 2001. Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis. *J Neurosci*, 21, 4183-7.
- PUOLIVALI, J., WANG, J., HEIKKINEN, T., HEIKKILA, M., TAPIOLA, T., VAN GROEN, T. & TANILA, H. 2002. Hippocampal A beta 42 levels correlate with spatial memory deficit in APP and PS1 double transgenic mice. *Neurobiol Dis*, 9, 339-47.
- PYPE, S., MOECHARS, D., DILLEN, L. & MERCKEN, M. 2003. Characterization of amyloid beta peptides from brain extracts of transgenic mice overexpressing the London mutant of human amyloid precursor protein. *J Neurochem*, 84, 602-9.
- QUINTANA, C., BELLEFQIH, S., LAVAL, J. Y., GUERQUIN-KERN, J. L., WU, T. D., AVILA, J., FERRER, I., ARRANZ, R. & PATINO, C. 2006. Study of the localization of iron, ferritin, and hemosiderin in Alzheimer's disease hippocampus by analytical microscopy at the subcellular level. *J Struct Biol*, 153, 42-54.
- RADDE, R., DUMA, C., GOEDERT, M. & JUCKER, M. 2008. The value of incomplete mouse models of Alzheimer's disease. *Eur J Nucl Med Mol Imaging*, 35 Suppl 1, S70-4.
- RAUSCH, M., HIESTAND, P., BAUMANN, D., CANNET, C. & RUDIN, M. 2003. MRI-based monitoring of inflammation and tissue damage in acute and chronic relapsing EAE. *Magn Reson Med*, 50, 309-14.
- RAUSCH, M., HIESTAND, P., FOSTER, C. A., BAUMANN, D. R., CANNET, C. & RUDIN, M. 2004. Predictability of FTY720 efficacy in experimental autoimmune encephalomyelitis by in vivo macrophage tracking: clinical implications for ultrasmall superparamagnetic iron oxide-enhanced magnetic resonance imaging. *J Magn Reson Imaging*, 20, 16-24.
- RAVEL, B. & NEWVILLE, M. 2005. ATHENA, ARTEMIS, HEPHAESTUS: data analysis for X-ray absorption spectroscopy using IFEFFIT. *J Synchrotron Radiat*, 12, 537-41.
- REISERER, R. S., HARRISON, F. E., SYVERUD, D. C. & MCDONALD, M. P. 2007. Impaired spatial learning in the APPSwe + PSEN1DeltaE9 bigenic mouse model of Alzheimer's disease. *Genes Brain Behav*, 6, 54-65.
- RODRIGUEZ, O., FRICKE, S., CHIEN, C., DETTIN, L., VANMETER, J., SHAPIRO, E., DAI, H. N., CASIMIRO, M., ILEVA, L., DAGATA, J., JOHNSON, M. D., LISANTI, M. P., KORETSKY, A. & ALBANESE, C. 2006. Contrast-enhanced in vivo imaging of breast and prostate cancer cells by MRI. *Cell Cycle*, 5, 113-9.
- ROGERS, J., LUBER-NAROD, J., STYREN, S. D. & CIVIN, W. H. 1988. Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. *Neurobiol Aging*, 9, 339-49.
- ROGERS, J., STROHMEYER, R., KOVELOWSKI, C. J. & LI, R. 2002a. Microglia and inflammatory mechanisms in the clearance of amyloid beta peptide. *Glia*, 40, 260-9.

- ROGERS, J. T., RANDALL, J. D., CAHILL, C. M., EDER, P. S., HUANG, X., GUNSHIN, H., LEITER, L., MCPHEE, J., SARANG, S. S., UTSUKI, T., GREIG, N. H., LAHIRI, D. K., TANZI, R. E., BUSH, A. I., GIORDANO, T. & GULLANS, S. R. 2002b. An iron-responsive element type II in the 5'-untranslated region of the Alzheimer's amyloid precursor protein transcript. *J Biol Chem*, 277, 45518-28.
- ROSSI, D. & VOLTERRA, A. 2009. Astrocytic dysfunction: insights on the role in neurodegeneration. *Brain Res Bull*, 80, 224-32.
- ROTTKAMP, C. A., RAINA, A. K., ZHU, X., GAIER, E., BUSH, A. I., ATWOOD, C. S., CHEVION, M., PERRY, G. & SMITH, M. A. 2001. Redox-active iron mediates amyloid-beta toxicity. *Free Radic Biol Med*, 30, 447-50.
- ROVELET-LECRUX, A., HANNEQUIN, D., RAUX, G., LE MEUR, N., LAQUERRIERE, A., VITAL, A., DUMANCHIN, C., FEUILLETTE, S., BRICE, A., VERCELLETTO, M., DUBAS, F., FREBOURG, T. & CAMPION, D. 2006. APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. *Nat Genet*, 38, 24-6.
- SAHOO, Y., GOODARZI, A., SWIHART, M. T., OHULCHANSKY, T. Y., KAUR, N., FURLANI, E. P. & PRASAD, P. N. 2005. Aqueous ferrofluid of magnetite nanoparticles: Fluorescence labeling and magnetophoretic control. *J Phys Chem B*, 109, 3879-85.
- SAYRE, L. M., PERRY, G. & SMITH, M. A. 1999. Redox metals and neurodegenerative disease. *Curr Opin Chem Biol*, 3, 220-5.
- SCHAFER, R., KEHLBACH, R., WISKIRCHEN, J., BANTLEON, R., PINTASKE, J., BREHM, B. R., GERBER, A., WOLBURG, H., CLAUSSEN, C. D. & NORTHOFF, H. 2007. Transferrin receptor upregulation: in vitro labeling of rat mesenchymal stem cells with superparamagnetic iron oxide. *Radiology*, 244, 514-23.
- SCHULTHEISS-GRASSI, P. P. & DOBSON, J. 1999. Magnetic analysis of human brain tissue. *Biometals*, 12, 67-72.
- SCHULTHEISS-GRASSI, P. P., WESSIKEN, R. & DOBSON, J. 1999. TEM investigations of biogenic magnetite extracted from the human hippocampus. *Biochim Biophys Acta*, 1426, 212-6.
- SEABROOK, G. R., SMITH, D. W., BOWERY, B. J., EASTER, A., REYNOLDS, T., FITZJOHN, S. M., MORTON, R. A., ZHENG, H., DAWSON, G. R., SIRINATHSINGHI, D. J., DAVIES, C. H., COLLINGRIDGE, G. L. & HILL, R. G. 1999. Mechanisms contributing to the deficits in hippocampal synaptic plasticity in mice lacking amyloid precursor protein. *Neuropharmacology*, 38, 349-59.
- SENSI, S. L., PAOLETTI, P., BUSH, A. I. & SEKLER, I. 2009. Zinc in the physiology and pathology of the CNS. *Nat Rev Neurosci*, 10, 780-91.
- SHAFTEL, S. S., KYRKANIDES, S., OLSCHOWKA, J. A., MILLER, J. N., JOHNSON, R. E. & O'BANION, M. K. 2007. Sustained hippocampal IL-1 beta overexpression mediates chronic neuroinflammation and ameliorates Alzheimer plaque pathology. *J Clin Invest*, 117, 1595-604.
- SHEPHERD, C., MCCANN, H. & HALLIDAY, G. M. 2009. Variations in the neuropathology of familial Alzheimer's disease. *Acta Neuropathol*, 118, 37-52.

- SIBSON, N. R., LOWE, J. P., BLAMIRE, A. M., MARTIN, M. J., OBRENOVITCH, T. P. & ANTHONY, D. C. 2008. Acute astrocyte activation in brain detected by MRI: new insights into T(1) hypointensity. *J Cereb Blood Flow Metab*, 28, 621-32.
- SILVA, A. C., ZHANG, W., WILLIAMS, D. S. & KORETSKY, A. P. 1997. Estimation of water extraction fractions in rat brain using magnetic resonance measurement of perfusion with arterial spin labeling. *Magn Reson Med*, 37, 58-68.
- SIMARD, A. R., SOULET, D., GOWING, G., JULIEN, J. P. & RIVEST, S. 2006. Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron*, 49, 489-502.
- SMITH, J. L., XIONG, S., MARKESBERY, W. R. & LOVELL, M. A. 2006. Altered expression of zinc transporters-4 and -6 in mild cognitive impairment, early and late Alzheimer's disease brain. *Neuroscience*, 140, 879-88.
- SMITH, M. A., HARRIS, P. L., SAYRE, L. M. & PERRY, G. 1997. Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc Natl Acad Sci U S A*, 94, 9866-8.
- SMITH, M. A., HIRAI, K., HSIAO, K., PAPPOLLA, M. A., HARRIS, P. L., SIEDLAK, S. L., TABATON, M. & PERRY, G. 1998. Amyloid-beta deposition in Alzheimer transgenic mice is associated with oxidative stress. *J Neurochem*, 70, 2212-5.
- SMITH, S. M., JENKINSON, M., WOOLRICH, M. W., BECKMANN, C. F., BEHRENS, T. E., JOHANSEN-BERG, H., BANNISTER, P. R., DE LUCA, M., DROBNJAK, I., FLITNEY, D. E., NIAZY, R. K., SAUNDERS, J., VICKERS, J., ZHANG, Y., DE STEFANO, N., BRADY, J. M. & MATTHEWS, P. M. 2004. Advances in functional and structural MR image analysis and implementation as FSL. *Neuroimage*, 23 Suppl 1, S208-19.
- SMITH, S. M., ZHANG, Y., JENKINSON, M., CHEN, J., MATTHEWS, P. M., FEDERICO, A. & DE STEFANO, N. 2002. Accurate, robust, and automated longitudinal and cross-sectional brain change analysis. *Neuroimage*, 17, 479-89.
- SNOW, A. D., SEKIGUCHI, R., NOCHLIN, D., FRASER, P., KIMATA, K., MIZUTANI, A., ARAI, M., SCHREIER, W. A. & MORGAN, D. G. 1994. An important role of heparan sulfate proteoglycan (Perlecan) in a model system for the deposition and persistence of fibrillar A beta-amyloid in rat brain. *Neuron*, 12, 219-34.
- SOLÉ, V. A., PAPILLON, E., COTTE, M., WALTER, P. & SUSINI, J. 2007. A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra. *Spectrochimica Acta Part B: Atomic Spectroscopy*, 62, 63-68.
- SORBI, S., FORLEO, P., TEDDE, A., CELLINI, E., CIANTELLI, M., BAGNOLI, S. & NACMIAS, B. 2001. Genetic risk factors in familial Alzheimer's disease. *Mech Ageing Dev*, 122, 1951-60.
- STEINER, H., WINKLER, E., EDBAUER, D., PROKOP, S., BASSET, G., YAMASAKI, A., KOSTKA, M. & HAASS, C. 2002. PEN-2 is an integral component of the gamma-secretase complex required for coordinated expression of presenilin and nicastrin. *J Biol Chem*, 277, 39062-5.

- STOLL, G. & BENDSZUS, M. 2009. Imaging of inflammation in the peripheral and central nervous system by magnetic resonance imaging. *Neuroscience*, 158, 1151-60.
- STOLTENBERG, M., BRUHN, M., SONDERGAARD, C., DOERING, P., WEST, M. J., LARSEN, A., TRONCOSO, J. C. & DANSCHER, G. 2005. Immersion autometallographic tracing of zinc ions in Alzheimer beta-amyloid plaques. *Histochem Cell Biol*, 123, 605-11.
- STOLTENBERG, M., BUSH, A. I., BACH, G., SMIDT, K., LARSEN, A., RUNGBY, J., LUND, S., DOERING, P. & DANSCHER, G. 2007. Amyloid plaques arise from zinc-enriched cortical layers in APP/PS1 transgenic mice and are paradoxically enlarged with dietary zinc deficiency. *Neuroscience*, 150, 357-69.
- STOZICKA, Z., ZILKA, N. & NOVAK, M. 2007. Risk and protective factors for sporadic Alzheimer's disease. *Acta Virol*, 51, 205-22.
- STREIT, W. J. 2004. Microglia and Alzheimer's disease pathogenesis. *J Neurosci Res*, 77, 1-8.
- STROMNES, I. M. & GOVERMAN, J. M. 2006. Active induction of experimental allergic encephalomyelitis. *Nat Protoc*, 1, 1810-9.
- SUTTON, C., BRERETON, C., KEOGH, B., MILLS, K. H. & LAVELLE, E. C. 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med*, 203, 1685-91.
- THOMAS, T., THOMAS, G., MCLENDON, C., SUTTON, T. & MULLAN, M. 1996. beta-Amyloid-mediated vasoactivity and vascular endothelial damage. *Nature*, 380, 168-71.
- THOMPSON, K., MENZIES, S., MUCKENTHALER, M., TORTI, F. M., WOOD, T., TORTI, S. V., HENTZE, M. W., BEARD, J. & CONNOR, J. 2003. Mouse brains deficient in H-ferritin have normal iron concentration but a protein profile of iron deficiency and increased evidence of oxidative stress. *J Neurosci Res*, 71, 46-63.
- THOMPSON, K. J., SHOHAM, S. & CONNOR, J. R. 2001. Iron and neurodegenerative disorders. *Brain Res Bull*, 55, 155-64.
- TREHIN, R., FIGUEIREDO, J. L., PITTET, M. J., WEISSLEDER, R., JOSEPHSON, L. & MAHMOOD, U. 2006. Fluorescent nanoparticle uptake for brain tumor visualization. *Neoplasia*, 8, 302-11.
- VAN BROECKHOVEN, C. 1998. Alzheimer's disease: identification of genes and genetic risk factors. *Prog Brain Res*, 117, 315-25.
- VAN GROEN, T., KILIAAN, A. J. & KADISH, I. 2006. Deposition of mouse amyloid beta in human APP/PS1 double and single AD model transgenic mice. *Neurobiol Dis*, 23, 653-62.
- VAN KASTEREN, S. I., CAMPBELL, S. J., SERRES, S., ANTHONY, D. C., SIBSON, N. R. & DAVIS, B. G. 2009. Glyconanoparticles allow pre-symptomatic in vivo imaging of brain disease. *Proc Natl Acad Sci U S A*, 106, 18-23.
- VAN WALDERVEEN, M. A., BARKHOF, F., POUWELS, P. J., VAN SCHIJNDEL, R. A., POLMAN, C. H. & CASTELIJS, J. A. 1999. Neuronal damage in T1-hypointense multiple sclerosis lesions demonstrated in vivo using proton magnetic resonance spectroscopy. *Ann Neurol*, 46, 79-87.

- VANHOUTTE, G., DEWACHTER, I., BORGHGRAEF, P., VAN LEUVEN, F. & VAN DER LINDEN, A. 2005. Noninvasive in vivo MRI detection of neuritic plaques associated with iron in APP[V717I] transgenic mice, a model for Alzheimer's disease. *Magn Reson Med*, 53, 607-13.
- VOLTERRA, A. & MELDOLESI, J. 2005. Astrocytes, from brain glue to communication elements: the revolution continues. *Nat Rev Neurosci*, 6, 626-640.
- WALSH, D. M., KLYUBIN, I., FADEEVA, J. V., CULLEN, W. K., ANWYL, R., WOLFE, M. S., ROWAN, M. J. & SELKOE, D. J. 2002. Naturally secreted oligomers of amyloid beta protein potentially inhibit hippocampal long-term potentiation in vivo. *Nature*, 416, 535-9.
- WALSH, D. M. & SELKOE, D. J. 2007. A beta oligomers - a decade of discovery. *J Neurochem*, 101, 1172-84.
- WANG, J., TANILA, H., PUOLIVALI, J., KADISH, I. & VAN GROEN, T. 2003. Gender differences in the amount and deposition of amyloidbeta in APPswe and PS1 double transgenic mice. *Neurobiol Dis*, 14, 318-27.
- WEBER, R., WEGENER, S., RAMOS-CABRER, P., WIEDERMANN, D. & HOEHN, M. 2005. MRI detection of macrophage activity after experimental stroke in rats: new indicators for late appearance of vascular degradation? *Magn Reson Med*, 54, 59-66.
- WEIDENSTEINER, C., METZGER, F., BRUNS, A., BOHRMANN, B., KUENNECKE, B. & VON KIENLIN, M. 2009. Cortical hypoperfusion in the B6.PS2APP mouse model for Alzheimer's disease: comprehensive phenotyping of vascular and tissular parameters by MRI. *Magn Reson Med*, 62, 35-45.
- WEISSLEDER, R., KELLY, K., SUN, E. Y., SHTATLAND, T. & JOSEPHSON, L. 2005. Cell-specific targeting of nanoparticles by multivalent attachment of small molecules. *Nat Biotechnol*, 23, 1418-23.
- WELLER, R. O., BOCHE, D. & NICOLL, J. A. 2009. Microvasculature changes and cerebral amyloid angiopathy in Alzheimer's disease and their potential impact on therapy. *Acta Neuropathol*, 118, 87-102.
- WHITE, A. R., REYES, R., MERCER, J. F., CAMAKARIS, J., ZHENG, H., BUSH, A. I., MÜLTHAUP, G., BEYREUTHER, K., MASTERS, C. L. & CAPPAL, R. 1999. Copper levels are increased in the cerebral cortex and liver of APP and APLP2 knockout mice. *Brain Res*, 842, 439-44.
- WILCOCK, D. M., GORDON, M. N. & MORGAN, D. 2006. Quantification of cerebral amyloid angiopathy and parenchymal amyloid plaques with Congo red histochemical stain. *Nat Protoc*, 1, 1591-5.
- WOLFE, M. S., XIA, W., OSTASZEWSKI, B. L., DIEHL, T. S., KIMBERLY, W. T. & SELKOE, D. J. 1999. Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature*, 398, 513-7.
- WOOD, J. A., WOOD, P. L., RYAN, R., GRAFF-RADFORD, N. R., PILAPIL, C., ROBITAILLE, Y. & QUIRION, R. 1993. Cytokine indices in Alzheimer's temporal cortex: no changes in mature IL-1 beta or IL-1RA but increases in the associated acute phase proteins IL-6, alpha 2-macroglobulin and C-reactive protein. *Brain Res*, 629, 245-52.

- WU, E. X., TANG, H., ASAI, T. & YAN, S. D. 2004. Regional cerebral blood volume reduction in transgenic mutant APP (V717F, K670N/M671L) mice. *Neurosci Lett*, 365, 223-7.
- YOSHIKAWA, K. 1993. [Molecular neurobiology of Alzheimer amyloid protein precursor]. *Seikagaku*, 65, 1149-59.
- YOSHIYAMA, Y., HIGUCHI, M., ZHANG, B., HUANG, S. M., IWATA, N., SAIDO, T. C., MAEDA, J., SUHARA, T., TROJANOWSKI, J. Q. & LEE, V. M. 2007. Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. *Neuron*, 53, 337-51.
- YOUNG-PEARSE, T. L., BAI, J., CHANG, R., ZHENG, J. B., LOTURCO, J. J. & SELKOE, D. J. 2007. A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. *J Neurosci*, 27, 14459-69.
- ZECCA, L., YODIM, M. B., RIEDERER, P., CONNOR, J. R. & CRICHTON, R. R. 2004. Iron, brain ageing and neurodegenerative disorders. *Nat Rev Neurosci*, 5, 863-73.
- ZHANG, J., YAROWSKY, P., GORDON, M. N., DI CARLO, G., MUNIREDDY, S., VAN ZIJL, P. C. & MORI, S. 2004. Detection of amyloid plaques in mouse models of Alzheimer's disease by magnetic resonance imaging. *Magn Reson Med*, 51, 452-7.
- ZHANG, L., YU, F., COLE, A. J., CHERTOK, B., DAVID, A. E., WANG, J. & YANG, V. C. 2009a. Gum Arabic-Coated Magnetic Nanoparticles for Potential Application in Simultaneous Magnetic Targeting and Tumor Imaging. *AAPS J*.
- ZHANG, L. H., WANG, X., STOLTENBERG, M., DANSCHER, G., HUANG, L. & WANG, Z. Y. 2008. Abundant expression of zinc transporters in the amyloid plaques of Alzheimer's disease brain. *Brain Res Bull*, 77, 55-60.
- ZHANG, X., LUHRS, K. J., RYFF, K. A., MALIK, W. T., DRISCOLL, M. J. & CULVER, B. 2009b. Suppression of nuclear factor kappa B ameliorates astrogliosis but not amyloid burden in APP^{swe}/PS1^{dE9} mice. *Neuroscience*, 161, 53-8.
- ZHANG, Z., DHARMAKUMAR, R., MASCHERI, N., FAN, Z., WU, S. & LI, D. 2009c. Comparison of superparamagnetic and ultrasmall superparamagnetic iron oxide cell labeling for tracking green fluorescent protein gene marker with negative and positive contrast magnetic resonance imaging. *Mol Imaging*, 8, 148-55.
- ZHUO, J. M., PRAKASAM, A., MURRAY, M. E., ZHANG, H. Y., BAXTER, M. G., SAMBAMURTI, K. & NICOLLE, M. M. 2008. An increase in Aβ₄₂ in the prefrontal cortex is associated with a reversal-learning impairment in Alzheimer's disease model Tg2576 APP^{sw} mice. *Curr Alzheimer Res*, 5, 385-91.

Appendix I

ASL theory

A theoretical model to facilitate the quantification of cerebral perfusion with ASL has been described in detail previously (Kelly *et al*, 2009). Briefly, the model is based on the following Fokker-Planck equation for the concentration of labeled spins, c , within the entire volume, V , from the labeling plane to the image plane:

$$\frac{\partial c}{\partial t} = - \underbrace{F \frac{\partial c}{\partial V}}_{\text{Flow}} + \underbrace{P \frac{\partial^2 c}{\partial V^2}}_{\text{Diffusion}} - \underbrace{\frac{c}{T_1}}_{\text{Relaxation}} .$$

(1)

This equation describes the variation of c within V over time using three terms. The first term incorporates transport of labeled spins due to bulk flow, F . The second term, which includes the perfusion coefficient, P , takes into account random effects such as pseudo-diffusion within the microvasculature (Kim and Kim, 2006) and the exchange of labeled spins between the capillary bed and extravascular water. These effects result in the dispersion of the labelled spins on traveling from the labeling plane to the image plane. The third term represents the T_1 relaxation of the labelled spins.

The bolus-tracking ASL (btASL) sequence (described below) can be described by a rectangular input function or bolus, $c_0(t)$, defined by $c_0(t) = C_0(\Theta(t) - \Theta(t - \tau))$ where C_0 is the initial concentration of inflowing spins at the labeling plane and $\Theta(t)$ is a rectangular input function defined as $\Theta(t) = 1$ for $t \geq 0$ and $\Theta(t) = 0$ for $t < 0$. The solution to equation (1) for a rectangular input function has been found (Kelly *et al*, 2009a):

$$c(V,t) = \int_0^t \underbrace{c_0(t)}_{\text{AIF}} \underbrace{\exp\left(\frac{-(t-\tau)}{T_1}\right)}_{m(t-\tau)} \underbrace{\frac{V}{\sqrt{4\pi P(t-\tau)^3}} \exp\left(-\frac{(V-F(t-\tau))^2}{4P(t-\tau)}\right)}_{r(V,t-\tau)} d\tau$$

(2)

From this it can be seen that the solution has the conventional structure of a residue detection experiment with bolus dispersion (Petersen *et al*, 2006) and is equivalent to the convolution of an arterial input function (AIF) with a relaxation function, $m(t)$, and residue function, $r(V,t)$. The MTT and CTT can be calculated from the first and second moments of $r(V,t)$ respectively. The MTT is calculated from the first moment of $r(V,t)$ as:

$$MTT = \frac{\int_0^\infty t \cdot r(V,t) dt}{\int_0^\infty r(V,t) dt} = \frac{V}{F} \quad (3)$$

which is defined as the average time it takes a particle to traverse the vasculature (Meier and Zierler 1954). The CTT is calculated from the second moment of $r(V,t)$ as:

$$CTT = \frac{\frac{1}{2} \int_0^\infty (t - \bar{t}_1)^2 \cdot r(V,t) dt}{\int_0^\infty t \cdot r(V,t) dt} = \frac{P}{F^2}$$

(4)

which has been interpreted as the time taken for labeled arterial water to be distributed at the region of interest (ROI) (Kelly *et al*, 2009).

Appendix II

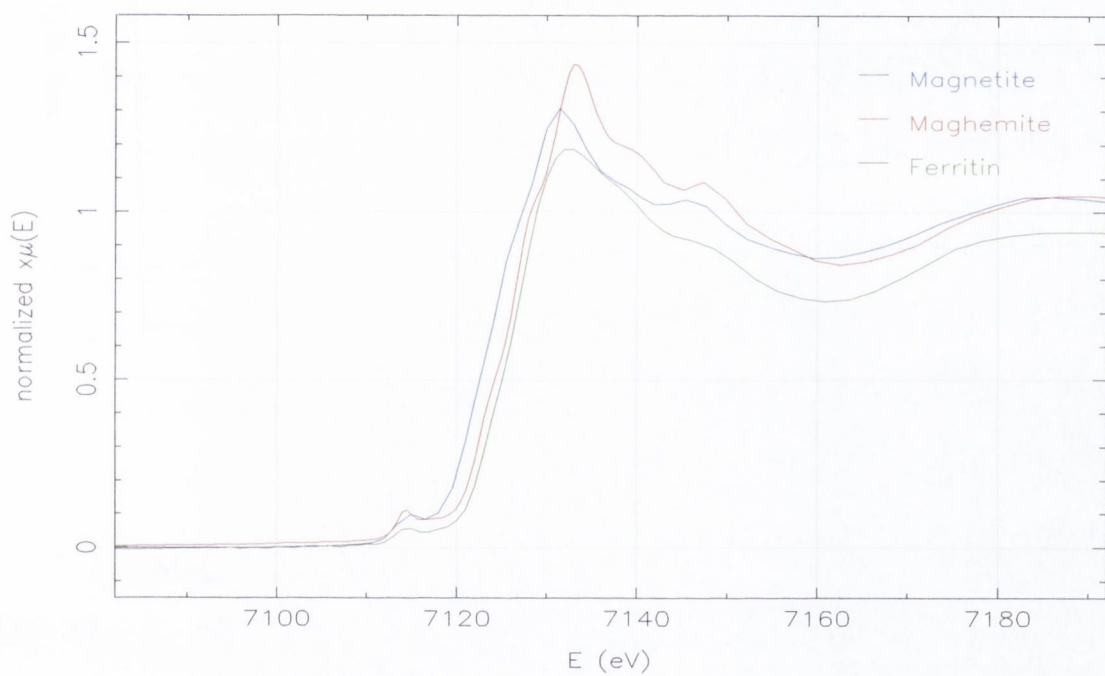
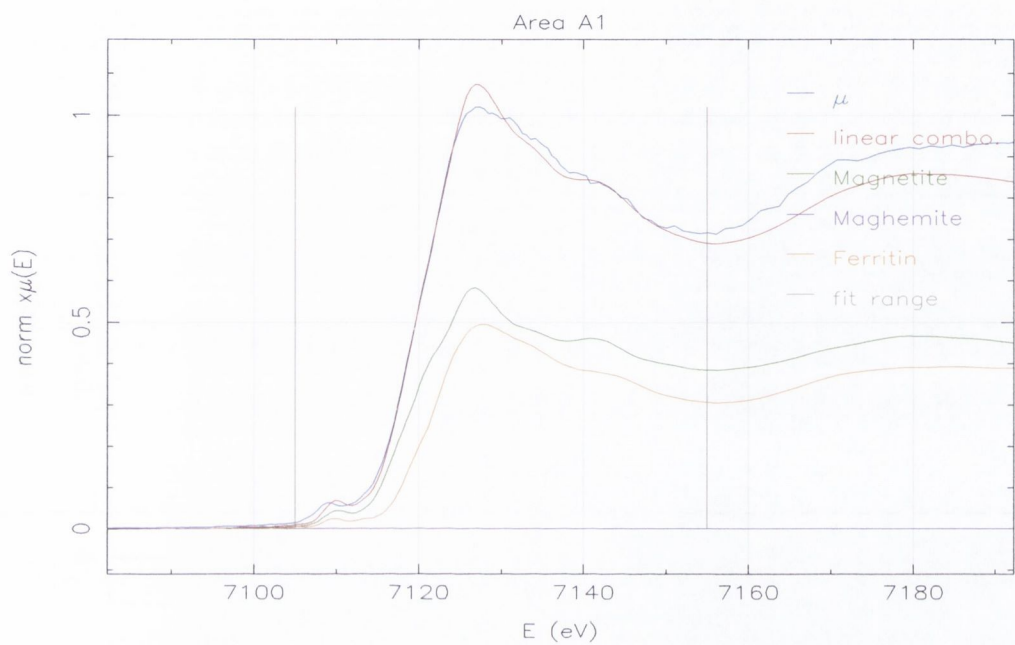


Figure 1. XANES spectra of standards.

XANES spectra of magnetite, maghemite and ferritin standards.

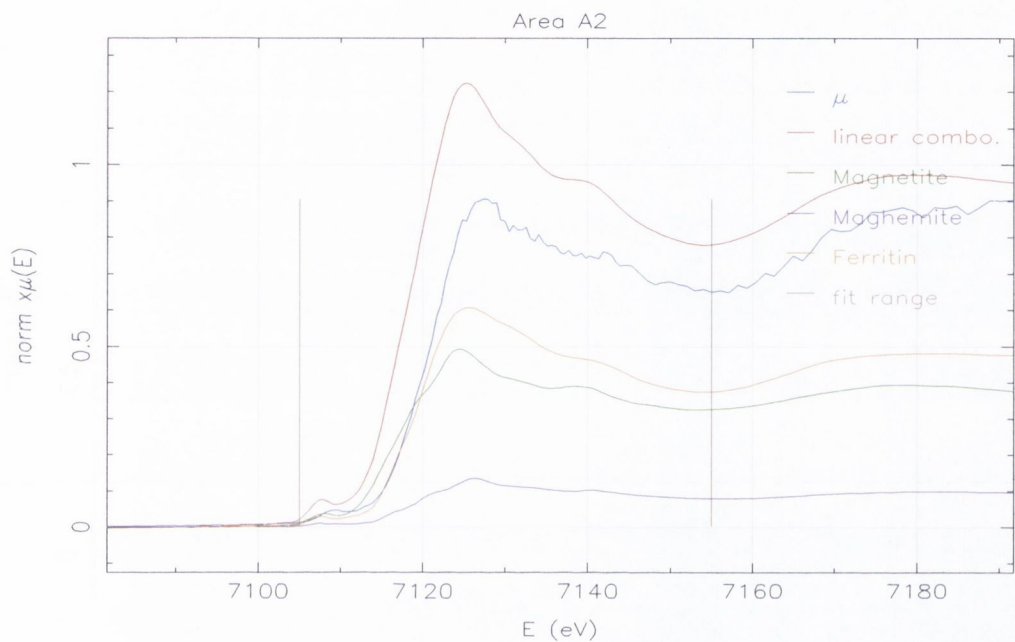
Standards and XANES spectra courtesy of
Dr. Joanna Collingwood.



	Magnetite	Maghemite	Ferritin	Fitting Parameter (χ^2)	R (mean square sum of data misfit)
Area A1	45%	0%	42%	0.03363	0.000732

Figure 2. Linear-combination fits components of Area A1.

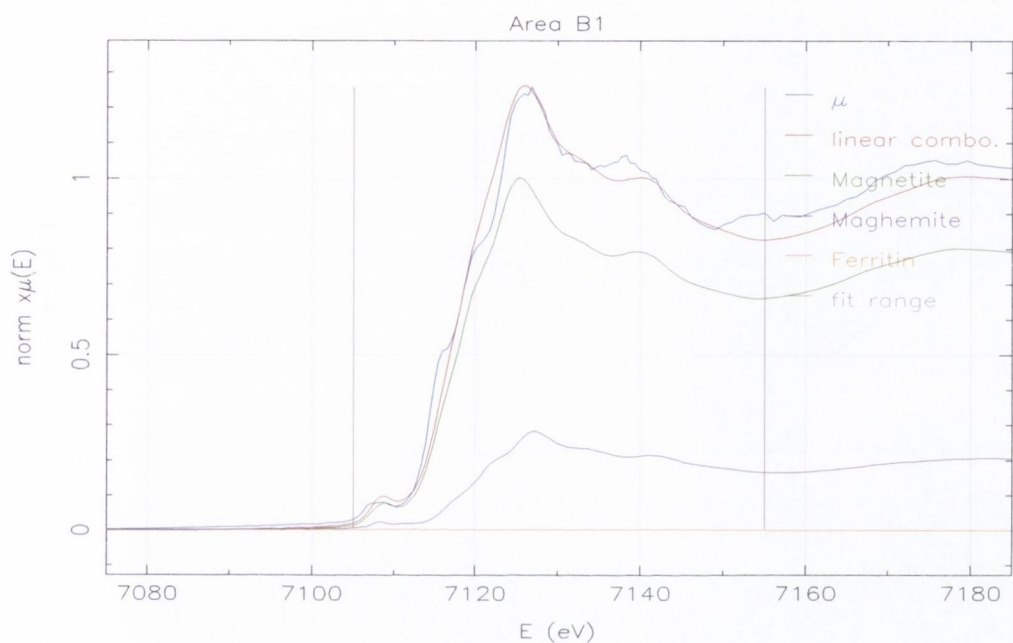
Components plot of linear-combination fits of Area A1 XANES spectra fitted to XANES spectra of magnetite, maghemite and ferritin standards.



	Magnetite	Maghemite	Ferritin	Fitting Parameter (χ^2)	R (mean square sum of data misfit)
Area A2	52%	0%	22%	0.03744	0.001056

Figure 3. Linear-combination fits components of Area A2.

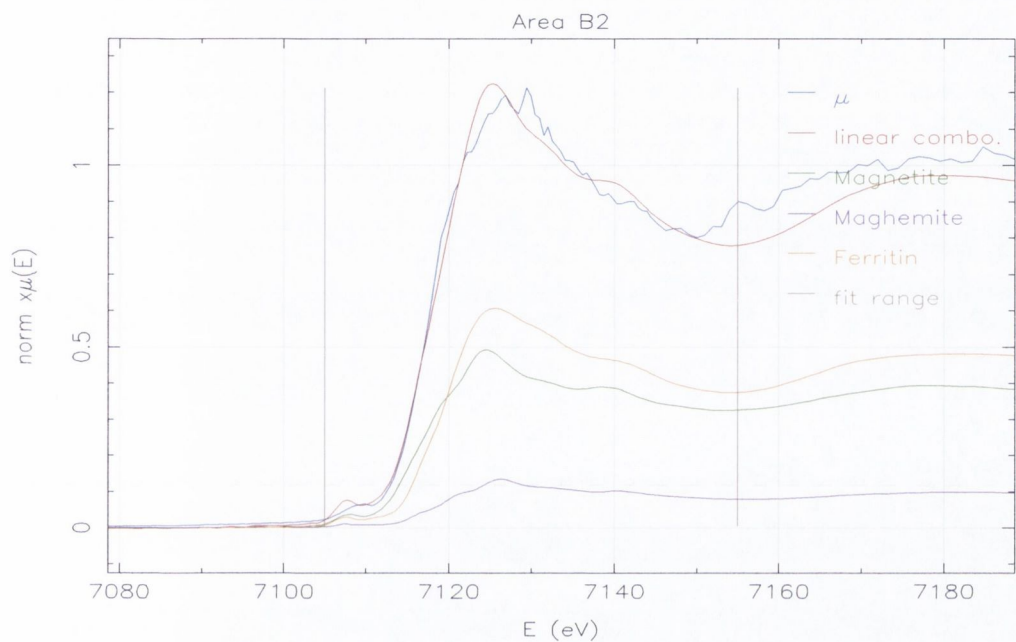
Components plot of linear-combination fits of Area A2 XANES spectra fitted to XANES spectra of magnetite, maghemite and ferritin standards.



	Magnetite	Maghemite	Ferritin	Fitting Parameter (χ^2)	R (mean square sum of data misfit)
Area B1	77%	20%	0%	0.18669	0.002785

Figure 4. Linear-combination fits components of Area B1.

Components plot of linear-combination fits of Area B1 XANES spectra fitted to XANES spectra of magnetite, maghemite and ferritin standards.



	Magnetite	Maghemite	Ferritin	Fitting Parameter (χ^2)	R (mean square sum of data misfit)
Area B2	38%	9%	51%	0.15782	0.002342

Figure 5. Linear-combination fits components of Area B2.

Components plot of linear-combination fits of Area B2 XANES spectra fitted to XANES spectra of magnetite, maghemite and ferritin standards.

Appendix III

Cerebral MTT (s)		
	Female Wildtype	Female APP/PS1
Cortex		
3 – 4 months of age	1.98 ± 0.244	1.95 ± 0.19
8 – 9 months of age	2.03 ± 0.234	2.01 ± 0.11
Hippocampus		
3 – 4 months of age	1.81 ± 0.199	1.79 ± 0.14
8 – 9 months of age	1.87 ± 0.282	1.92 ± 0.11
Cerebral CTT (s)		
Cortex		
3 – 4 months of age	1.82 ± 0.222	1.94 ± 0.16
8 – 9 months of age	1.95 ± 0.250	1.88 ± 0.09
Hippocampus		
3 – 4 months of age	1.901 ± 0.237	1.82 ± 0.18
8 – 9 months of age	1.791 ± 0.152	1.92 ± 0.11

Table 1 MTT and CTT data

Appendix IV

Company addresses

Ambion Inc.,
Lingley House
120 Birchwood Boulevard
Warrington WA3 7QH
UK
www.ambion.com

Beckman Coulter Ireland Inc.
Mervue Business Park,
Galway
www.beckman.com

Bio-imaging Systems USA,
1495 NE 33rd Ave #101
Homestead FL, 33033
www.bioamerica-inc.com

Biotium, Inc.
3159 corporate place, Suite 8
Hayward, CA 94545
www.biotium.com

Cambridge BioScience Ltd
24-25 Signet Court
Newmarket Road

Applied Biosystems,
Frankfurter Straße 129 B
64293 Darmstadt
Germany
www.appliedbiosystems.com

Becton Dickinson & Companies,
The Danby Building,
Edmund Halley Road,
Oxford Science Park
Oxford, OX4 4DQ
United Kingdom
www.becton.com

Biosciences Ireland,
3 Charlemont Terrace
Crofton Road
Dun Laoghaire
www.biosciences.ie

Bruker BioSpin MRI GmbH
Rudolf-Plank-Str. 23
76275 Ettlingen
www.bruker-biospin.com

Carl Stuart Limited
Tallaght Business Park
Whitestown

Cambridge CB5 8LA
United Kingdom
www.bioscience.co.uk

Dublin 24
Ireland
www.carlstuart.com

Crafty Devils Paper Craft
22/23 Taw Mill Business Park
Howard Avenue
Barnstaple
Devon
EX32 8QA
United Kingdom
www.craftydevilspapercraft.co.uk

Dako Denmark A/S
Produktionsvej 42
DK-2600 Glostrup
Denmark
www.dako.com

Difco Laboratories Ltd.,
West Molesey,
Surrey, KT8 2SE

Edwards BOC,
Manor Royal,
Crawley,
West Sussex,
RH10 9LW,
United Kingdom
www.edwardsvacuum.com

Electron Microscopy Services,
1560 Industry Rd.,
Box 550 Hatfield,
PA 19440
www.emsdiasum.com

GIBCO,
c/o Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley, UK. PA4 9RF
www.invitrogen.com/gibco

Greiner Bio-One GmbH.
Maybachstrasse 2
72636 Frickenhausen,

Harlan Ltd., UK,
www.harlan.com

Germany

www.greinerbioone.com

HVS Image

www.hvsimage.com

Jencons

Unit 15, The Birches

Willard Way

Imberhorne Industrial Estate

East Grinstead, West Sussex

RH19 1XZ UK

www.jencons.co.uk

Tocris Bioscience

16144 Westwoods Business Park

Ellisville, Missouri 63021

USA

www.tocris.com

Labsystems Ltd.

Oy Tiilitie 3

FI-01720 VANTAA

Finland

www.anilabsystems.com

MatTek Corporation,

200 Homer Avenue,

Ashland, MA 01721

The Jackson Laboratory

600 Main Street

Bar Harbor, Maine 04609 USA

www.jax.org

Jeol Ltd.,

1-2, Musashino 3-chome Akishima

Tokyo 196-8558, Japan

www.jeol.com

KINEMATICA AG

Luzernerstrasse 147a

CH-6014 Littau-Lucerne

Switzerland.

www.kinematica.ch

Leica Microsystems Inc.

2345 Waukegan Road

Bannockburn, 60015 United States

www.leica-microsystems.com

Merial Animal Health,

CM19 5TG, UK

www.uk.merial.com

USA

www.mattek.com

Meso Scale Discovery

9238 Gaither Road

Gaithersburg,

Maryland 20877

www.mesoscale.com

Millipore Headquarters:

290 Concord Road

Billerica, MA 01821

www.millipore.com

NanoDrop Technologies

3411 Silverside Rd

Bancroft Building

Wilmington, DE 19810

USA

www.nanodrop.com

Olympus Life Science,

Japan.

www.olympus.com

Promega Corporation

2800 Woods Hollow Road

Madison, WI 53711 USA

Phone 608.274.4330

Milestone Scientific Inc.

45 Knightsbridge Road

Piscataway, NJ 08854

www.milestonescientific.com

MWG Biotech,

Anzinger Str. 7a

D-85560, Ebersberg

www.mwg-biotech.com

Ohaus World Headquarters

P.O. Box 2033

19A Chapin Road

Pine Brook

NJ 07058

USA

www.ohaus.com

Pierce Perbio Science

Postbus 32

4870 AA Etten-Leu

www.piercenet.com

Quantum Design,

6325 Lusk Boulevard,

San Diego, CA 92121-3733

www.dqusa.com

www.promega.com

Razel Scientific Instruments,
P.O. Box 111
St. Albans, VT 05478, UK
www.razelscientific.com

SARSTEDT Ltd.
Sinnottstown Lane
Drinagh - Wexford
Ireland
www.sarstedt.com

Tissue-Tek,
p/a Electron Microscopy Services

Varian Medical Systems, Inc.
3100 Hansen Way
Palo Alto, CA 94304-1038
www.varian.com

Carl Zeiss Jena GmbH
Carl-Zeiss-Promenade 10
07745 Jena, Germany
www.zeiss.com

SA Instruments
65 Main Street
Stony Brook, NY 11790, USA
www.i4sa.com

Sigma-Aldrich Company Ltd.
The Old Brickyard
New Road
Gillingham
Dorset
SP8 4XT
www.sigmaaldrich.com

Ugo Basile
Biological research apparatus
Via G. Borghi 43
21025 Comerio VA – Italy
www.ugobasile.com

VECTOR LABORATORIES LTD.
UNITED KINGDOM
3, Accent Park, Bakewell Road
Orton Southgate, Peterborough,
PE2 6XS, United Kingdom
www.vectorlabs.com

Appendix V

Solutions

Phosphate-buffered saline (PBS)

137 mM sodium chloride (NaCl)

2.7 mM potassium chloride (KCl)

8.1 mM di-sodium hydrogen phosphate (Na_2HPO_4)

1.5 mM potassium di-hydrogen phosphate (KH_2PO_4)

In dH_2O , pH 7.3.

PHEM buffer

60mM 1,4-Piperazinediethanesulfonic acid (PIPES)

25mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)

10mM Ethylene-bis(oxyethylenitrilo)tetraacetic acid (EGTA)

2mM Magnesium Chloride MgCl_2

In deionized H_2O , pH 6.9.

Cell Culture Media

Dulbecco's Modified Eagle Media (DMEM; GIBCO, UK) supplemented with filter-sterilized 10% heat-inactivated foetal calf serum (Sigma, UK), streptomycin (100U/ml; GIBCO, UK) and penicillin (100U/ml; GIBCO, UK).

Fluorescence-activated cell sorting (FACS) buffer

500 ml PBS

0.5 g sodium azide (NaN_3)

Prussian blue reagent

4% Potassium ferrocyanide

4% hydrochloric acid (1.2 mmol/l)

1:1, freshly prepared.

Homogenising buffer

0.292 g sodium chloride (NaCl)

1 g sodium dodecyl sulphate (SDS)

Dissolve in 100 ml distilled H₂O, pH 10.0.

Add: 10 µl/ml protease inhibitor cocktail

5 µl/ml phosphatase inhibitor I cocktail

5 µl/ml phosphatase inhibitor II cocktail

MSD Kit buffer

200 ml 1X tris wash buffer (TWB, Mesoscale Discovery, USA)

Add: 10 µl/ml protease inhibitor cocktail

5 µl/ml phosphatase inhibitor I cocktail

5 µl/ml phosphatase inhibitor II cocktail

Guanidine Buffer

31.25 ml Guanidine (5.0 M)

18.75 ml ddH₂O

25 mM HEPES

pH 7.4.

KREBS buffer

136 mM sodium chloride (NaCl)

2.55 mM potassium chloride (KCl)

1.175 mM potassium di-hydrogen phosphate (KH₂PO₄)

2.24 mM magnesium sulphate (MgSO₄)

15.95 mM sodium bicarbonate (NaHCO_3)

10 mM Glucose

In dH_2O , pH 7.3.

ELISA wash buffer

500 ml PBS

500 μl Tween 20

Saturated sodium chloride solution

50 g sodium chloride (NaCl)

500 ml 80% ethanol.

NaCl should form a thick layer on the bottom the container, leave stirring overnight.

Congo red staining solution

2.5 g congo red

500 ml saturated NaCl solution

Stir overnight, filter through 0.22 μm membrane filter before use.