

Identifying the preventive cellular mechanisms of memory decline after surgery

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Thesis presented for the degree of Doctor of Philosophy
Imperial College London

November 2012

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Abstract

Memory and cognitive decline following surgery pose a major threat to the ageing population. Postoperative memory decline (POCD) has potentially devastating consequences due to its detrimental effects on quality of life and increased level of dependence. Acute neuroinflammatory changes after non-neurosurgical procedures have been linked to memory decline. Inflammation is the reflection of ongoing intracellular processes precipitated by stress following major insults such as surgery. I hypothesised that POCD is caused by an imbalance of cytoprotective mechanisms secondary to an inadequate physiological response to physiological stress.

I set out to identify: 1) how memory is impaired in murine surgical models, using wild type and two genetically modified mouse lines, involving membrane danger receptors, purinergic receptor, ion gated channel 7 (P2X7) gene knockouts and the cytoplasmic chaperone heat shockprotein-72 (Hsp72), Hsp72 overexpressors, and 2) the effect of xenon anaesthesia and atorvastatin on memory decline following surgery.

The inflammatory response was modelled by using multivariable analysis software (SIMCA) to identify a physiological signature, and the flow cytometry (FACS) technique of multiple cell-type responses within the hippocampus was optimised.

The lack of P2X7 receptors could not prevent the development of POCD after tibial fracture surgery under general anaesthesia. Over expression of Hsp72 proved to prevent POCD and its effect was associated with reduced microglia activation. Pretreatment with xenon and atorvastatin also prevented the development of POCD.

In conclusion, SIMCA and FACS are potentially useful complementary methods for assessment of inflammation. P2X7 could not be proven to be involved in the development of POCD while the role of Hsp72 in POCD was proved and the prevention of POCD by using xenon through this mechanisms and atorvastatin by ameliorating the inflammatory response.

To

Don Jesus, my grandfather Don Carlos Martín and Neil Soni

Table of Contents

ABSTRACT.....	3
DECLARATION	11
ACKNOWLEDGEMENTS.....	12
LIST OF FIGURES.....	15
LIST OF TABLES.....	19
ABBREVIATIONS AND ACRONYMS.....	20
THE HYPOTHESIS	23
THE AIMS.....	23
CHAPTER 1:	24
ROAD MAP	24
1.0 DEFINITION.....	25
1.2 THE PROBLEM.....	25
1.3 EPIDEMIOLOGY	25
1.4 OVERVIEW OF PROPOSED MECHANISMS.....	25
1.5 OVERVIEW OF PROPOSED INTERVENTIONS.....	26
1.6 HYPOTHESIS	26
1.7 THE THESIS WILL BE LAID OUT AS FOLLOWS	26
1.8 CHAPTERS.....	28
CHAPTER 2:	30
HISTORICAL BACKGROUND	30
2.1 DEFINITIONS.....	34
2.2 UNDERSTANDING MEMORY – PHYSIOLOGY AND ANATOMY	36
2.2.1 COGNITION, LEARNING AND MEMORY FORMATION IN HUMANS	36
2.2.1.a Cognition.....	36
2.2.1.b Learning.....	39
2.2.1.c Memory.....	39
2.3 ASSESSMENT OF MEMORY IN HUMANS.....	44
2.4 UNDERSTANDING OF MEMORY FORMATION IN ANIMALS.....	47
2.4.1 FEAR CONDITIONING.....	49
2.4.2 MORRIS WATER MAZE (MWM).....	51
2.5 THE EFFECT OF NEUROINFLAMMATION ON MEMORY.....	52
2.6 SICKNESS BEHAVIOUR.....	54
2.6.0 MECHANISM OF SPREAD INFLAMMATION	55
2.6.1 CYTOKINE INTERACTIONS WITH THE BBB	57
2.6.2 MECHANISMS OF INCREASED BBB PERMEABILITY	59
2.6.3 NEURAL PATHWAY BYPASSING THE BBB	61

2.7 NEUROINFLAMMATION AND SURGERY	63
2.8 SURGERY AND POSTOPERATIVE COGNITIVE DYSFUNCTION (POCD)	65
2.8.1 EPIDEMIOLOGY	66
2.8.2 POCD AND CARDIAC SURGERY	68
2.8.3 PCI VERSUS CABG AND POCD	69
2.8.4 OFF-PUMP CABG VERSUS ON-PUMP CABG AND POCD.....	69
2.8.5 PREDICTIVE RISK FACTORS	70
2.9 POCD FOLLOWING NON-CARDIAC SURGERY.....	72
2.10 RISK FACTORS ASSOCIATED WITH THE DEVELOPMENT OF POCD.....	73
2.10.1 PATIENT'S RELATED RISK FACTORS FOR THE DEVELOPMENT OF POCD	74
2.10.1.a Age.....	74
2.10.1.b Genetics	76
2.10.1.c Cognitive state of individual before surgery	77
2.10.1.d Alzheimer's disease (AD)	79
2.10.2 NON-PATIENT RELATED RISK FACTORS FOR DEVELOPMENT OF POCD	80
2.10.2.a Surgery.....	80
2.10.2.b Length of surgery.....	80
2.10.2.c Anaesthesia	81
2.11 POCD PATHOGENESIS	89
2.12 NEUROINFLAMMATORY CHANGES AND POCD	94
2.12.1 PRODUCTION OF PROINFLAMMATORY CYTOKINES	98
2.12.3 SIGNALLING THROUGH P2X7 RECEPTORS	101
2.12.4 HEAT SHOCK PROTEIN 72 (HSP72) AND POCD	104
2.12.5 REACTIVE OXYGEN SPECIES AND POCD	109
2.13 PROPOSED PREVENTATIVE MECHANISMS FOR DEVELOPMENT OF POCD ...	111
2.13.1 XENON AND POCD	113
2.13.2 STATIN AND POCD.....	115
2.14 STATEMENT OF THE PROBLEM	117
2.15 ANIMAL MODELS USED FOR ASSESSMENT OF POCD.....	118
2.15.1 THORACIC MODEL OF POCD.....	118
2.15.1.a Cardio-pulmonary bypass.....	118
2.15.2 ORTHOPAEDIC MODEL OF POCD	119
2.15.2.a Tibial fracture	119
2.15.3 ABDOMINAL SURGICAL MODELS OF POCD.....	120
2.15.3.a Splenectomy	120
2.15.3.b Hepatectomy.....	121
2.15.3.c Nephrectomy.....	121
2.16 HYPOTHESIS.....	122
2.17 AIMS	123
CHAPTER 3:	124
MATERIAL AND METHODS.....	124
3.1 GENERAL ANIMAL HANDLING.....	125
3.1.1 ANIMALS.....	125
3.1.1.a Wild type mouse.....	127
3.1.1.b P2X7 knock-out mouse.....	128
3.1.1.c Hsp72 Transgenic over expressing mouse	129
3.1.2 GENERAL MANAGEMENT	131
3.1.3 ANAESTHETIC PROCEDURES	132

3.1.3.a Anaesthesia	132
3.1.3.b Xenon anaesthesia	135
3.1.3.c Regional anaesthesia technique.....	137
3.2 SURGICAL TECHNIQUES	140
3.2.1 SURGICAL EXPERIMENTAL MODELS.....	142
3.2.1.a Tibial fracture.....	142
3.2.1.b Unilateral nephrectomy.....	144
3.2.1.c Transient middle cerebral artery occlusion.....	146
3.3 OTHER TREATMENTS	148
3.3.1 LIPOPOLYSACCHARIDE (LPS) CHALLENGE.....	148
3.3.2 RETRIEVAL OF BRAIN TISSUE FROM NEWBORN MOUSE PUPS.....	150
3.4 BEHAVIOURAL STUDIES FOR ASSESSMENT OF POCD	150
3.4.1 PRE-INTERVENTION ASSESSMENT OF PHYSIOLOGICAL VARIABLES.....	150
3.4.1.a Hot plate.....	151
3.4.1.b Acoustic reflex.....	152
3.4.1.c Rotarod.....	153
3.4.1.d Spontaneous motor activity.....	154
3.4.2 OPTIMISATION OF BEHAVIOURAL TESTS FOR ASSESSMENT OF POCD.....	156
3.4.2.a Fear-conditioning.....	156
3.4.2.b Morris water maze.....	168
3.4.2.c Neurological assessment – Garcia Score	170
3.5 BIOLOGICAL ASSAYS.....	171
3.5.1 IN VITRO EXPERIMENTS	171
3.5.1.1 Cell culture.....	171
3.5.1.2 Calcium signalling for P2X7 identification	172
3.5.2 IMMUNOHISTOCHEMISTRY (IHC)	174
3.5.2.1 Sample preparation.....	177
3.5.2.2 Antibody labelling.....	178
3.5.2.3 Microscopy and image analysis.....	179
3.6 WESTERN BLOTTING (WB)	182
3.6.1 RETRIEVAL AND PREPARATION OF TISSUES.....	183
3.6.2 IMMUNOBLOTTING AND TISSUE LABELLING WITH ANTIBODIES	184
3.6.3 IMAGE REVELATION AND ANALYSIS.....	184
3.7 ENZYME-LINKED IMMUNOABSORBENT ASSAY (ELISA).....	185
3.7.1 SAMPLE PREPARATION FOR ELISA.....	186
3.8 FLUORESCENT-ACTIVATED CELL SORTING (FACS)	190
3.8.1 BASIC PRINCIPLES OF FACS.....	190
3.8.2 PREPARATION OF CELLS SUSPENSIONS.....	191
3.8.3 ANTIBODY STAINING OF CELL SUSPENSIONS.....	193
3.8.4 FACS DATA ANALYSIS.....	194
3.9 RENAL FUNCTION TESTS	195
3.10 DATA PRESENTATION AND ANALYSIS.....	196
3.10.1 SOFTWARES.....	196
3.10.1.1 GraphPad®	196
3.10.1.2 Statistical Isolinear Multicomponent Analysis (SIMCA®).....	196
3.10.1.3 Statistical Analysis	200
CHAPTER 4:	202
CHARACTERISATION OF THE INFLAMMATORY RESPONSE	202

4.1 METHODS.....	204
4.1.1 CYTOKINE ANALYSIS.....	204
4.2 RESULTS	207
4.2.1 PLASMA SAMPLES - DAY 1	207
4.2.2 HIPPOCAMPAL SAMPLES - DAY 1	211
4.2.3 PLASMA SAMPLES - DAY 7	215
4.2.4 HIPPOCAMPI SAMPLES - DAY 7	217
4.3 DISCUSSION	220
CHAPTER 5:	223
IDENTIFICATION OF THE CELL RESPONSE	223
5.1 METHODS.....	225
5.1.1 ANALYSIS OF MICROGLIA IN HIPPOCAMPUS SINGLE CELL SUSPENSIONS.....	227
5.1.2 DEVELOPMENT OF A FIXATION-HOMOGENISATION METHOD	227
5.1.3 DEVELOPMENT OF A DIRECT METHOD FOR REMOVAL OF MYELIN.....	228
5.1.4 MEASUREMENT OF CD11B EXPRESSION CHANGES AFTER SURGERY.....	229
5.2 RESULTS	230
5.2.1 ANALYSIS OF MICROGLIA IN HIPPOCAMPUS SINGLE CELL SUSPENSIONS.....	230
5.2.2 DEVELOPMENT OF A FIXATION-HOMOGENISATION METHOD	230
5.2.3 DEVELOPMENT OF A DIRECT METHOD FOR EXCLUSION OF MYELIN DEBRIS	232
5.2.4 MEASUREMENT OF CD11B EXPRESSION CHANGES AFTER SURGERY	233
5.3 DISCUSSION	234
CHAPTER 6:	239
THE ROLE OF P2X7 RECEPTOR IN THE DEVELOPMENT OF POCD	239
6.1 METHODS.....	241
6.1 <i>IN VIVO</i> EXPERIMENTS.....	242
6.1.1 <i>Characterization of the role of P2X7 receptor in sickness behaviour</i>	242
6.1.2 <i>Characterization of the role of P2X7 receptor in POCD</i>	243
6.1.3 CHARACTERIZATION OF THE ROLE OF P2X7 RECEPTOR IN ISCHAEMIC STROKE	244
6.2 RESULTS	245
6.2.1 <i>IN VITRO</i> EXPERIMENTS	245
6.2.1.a <i>Identification of P2X7 receptor in astrocytes and microglia</i>	245
6.2.1.b <i>Calcium signalling for identification of P2X7 activity after ATP challenge using confocal microscopy.....</i>	246
6.2.2 <i>IN VIVO</i> EXPERIMENTS.....	253
6.2.2.1 <i>Characterization of the role of P2X7 in sickness behaviour in mice</i>	253
6.2.3 CHARACTERIZATION OF THE ROLE OF P2X7 IN POCD	256
6.2.3.a <i>Learning abilities.....</i>	256
6.2.3.b <i>Hippocampal dependent memory</i>	257
6.2.3.c <i>Hippocampal independent memory</i>	259
6.2.4 ASSESSMENT OF THE SYSTEMIC INFLAMMATORY RESPONSE BY CYTOKINE VALUES.....	260
6.2.4.a <i>Plasma level of IL-1β after surgery.....</i>	260
6.2.4 CHARACTERIZATION OF THE ROLE OF P2X7 IN ISCHAEMIC STROKE	262
6.2.4.1 <i>Neurological assessment.....</i>	262
6.2.4.2 <i>Infarct size assessment.....</i>	262
6.3 DISCUSSION	263
CHAPTER 7:	266
THE EXPRESSION OF HSP72 AND POCD.....	266

7.1 METHODS.....	268
7.1.1 CHARACTERISATION OF THE ROLE OF HSP72 OVER EXPRESSION IN POCD	268
7.1.2 ASSESSMENT OF MICROGLIA ACTIVATION AFTER TIBIAL FRACTURE SURGERY UNDER GENERAL ANAESTHESIA.....	269
7.2 RESULTS	269
7.2.1 PHYSIOLOGICAL VARIABLES.....	269
7.2.2 CHARACTERIZATION OF THE ROLE OF HSP72 OVER EXPRESSION IN POCD	270
7.2.2.a <i>Learning abilities (acquisition)</i>	270
7.2.2.b <i>Hippocampal dependent memory</i>	271
7.2.2.c <i>Hippocampal independent memory</i>	273
7.2.3 ASSESSMENT OF MICROGLIA ACTIVATION AFTER TIBIAL FRACTURE SURGERY UNDER GENERAL ANAESTHESIA.....	274
7.3 DISCUSSION	278
CHAPTER 8:	283
THE ROLE OF XENON IN POCD	283
8.1 METHODS.....	285
8.1.1 CHARACTERISATION OF THE ROLE OF PRE-TREATMENT WITH XENON IN POCD	285
8.1.2 MEASUREMENT OF THE LEVEL OF PHOSPHOINOSITE KINASE-3 (PI3-KINASE)	286
8.1.3 ASSESSMENT OF THE SYSTEMIC INFLAMMATORY RESPONSE BY CYTOKINE VALUE.....	287
8.2 RESULTS	287
8.2.1 PHYSIOLOGICAL VARIABLES.....	287
8.2.2 CHARACTERIZATION OF THE EFFECT OF PRE-TREATMENT WITH XENON IN POCD	288
8.2.2.a <i>Learning abilities (acquisition)</i>	288
8.2.2.b <i>Hippocampal dependent memory</i>	289
8.2.2.c <i>Hippocampal independent memory</i>	291
8.2.3 MEASUREMENT OF LEVEL OF PI3-KINASE AND HSP72 IN THE HIPPOCAMPUS AFTER TIBIAL FRACTURE UNDER GENERAL ANAESTHESIA	292
8.2.3 ASSESSMENT OF THE SYSTEMIC INFLAMMATORY RESPONSE BY CYTOKINE VALUE.....	295
8.3 DISCUSSION	296
CHAPTER 9:	299
THE ROLE OF ATORVASTATIN IN POCD	299
9.1 METHODS.....	301
9.1.1 CHARACTERISATION OF THE ROLE OF ATORVASTATIN IN THE DEVELOPMENT OF POCD ...	302
9.1.2 MEASUREMENT OF THE LEVEL OF HSP72 AND ACTIVE NFkB	304
9.1.3 ASSESSMENT OF THE SYSTEMIC AND CNS INFLAMMATORY RESPONSE	304
9.1.4 ASSESSMENT OF MICROGLIA ACTIVATION	305
9.1.5 ASSESSMENT OF THE ENDOTHELIUM ACTIVATION	305
9.2 RESULTS	305
9.2.1 PHYSIOLOGICAL VARIABLES.....	305
9.2.2 CHARACTERISATION OF THE ROLE OF ATORVASTATIN IN THE DEVELOPMENT OF POCD ...	307
9.2.2.1 <i>Fear-conditioning</i>	307
9.2.2.2 <i>Morris water maze</i>	311
9.2.2.3 MEASUREMENT OF THE LEVEL OF HSP72 AND ACTIVE NFkB IN AREAS OF THE BRAIN WHERE MEMORY IS FORMED (HIPPOCAMPUS) AFTER UNILATERAL NEPHRECTOMY UNDER GENERAL ANAESTHESIA.....	313
9.2.2.4 ASSESSMENT OF THE SYSTEMIC AND CNS INFLAMMATORY RESPONSE.....	316
9.2.2.5 <i>Assessment of microglia activation in areas</i>	318
9.2.2.6 <i>Assessment of the endothelium activation</i>	321

9.3 DISCUSSION	323
CHAPTER 10:.....	327
MAIN DISCUSSION	327
10.1 ASSESSMENT OF THE INFLAMMATORY RESPONSE BY SIMCA AND FACS	329
10.2 P2X7 RECEPTORS	332
10.3 HEAT SHOCK PROTEIN 72	332
10.4 XENON	333
10.5 ATORVASTATIN.....	333
10.6 WHAT IS ALREADY KNOWN IN THIS AREA?	333
10.7 HOW THESE FINDINGS ARE LINKED TO THE LITERATURE?	335
10.7.1 ASSESSMENT OF THE INFLAMMATORY RESPONSE BY SIMCA AND FACS.....	335
10.7.2 P2X7 RECEPTORS	335
10.7.3 HEAT SHOCK PROTEIN 72	336
10.7.4 ISOFLURANE	338
10.7.5 XENON	340
10.7.6 ATORVASTATIN	340
11 REFERENCES	343
12 APPENDICES.....	370
12.1 APPENDIX 1	371
12.2 APPENDIX 2	373
12.3 APPENDIX 3	375
12.4 APPENDIX 4	376
11.4.1 ORIGINAL PUBLICATIONS.....	376
11.4.3 REVIEW ARTICLES.....	377
11.4.4 INVITED SPEAKER	377
11.4.4.a International meetings.....	377
11.4.4.b National meetings	378
11.4.4.c Regional meetings.....	378
11.5 POSTERS (POSTER PRESENTED BY STARRED AUTHORS).....	378
11.5.1 International meetings	378
11.5.2 National meetings.....	379

Declaration

I declare that hypothesis, aims and development of this thesis were conceived entirely by Marcela Paola Vizcaychipi at Chelsea and Westminster Campus, Imperial College London, UK.

All experiments were executed by Marcela Paola Vizcaychipi. Techniques taught by scientific colleagues are the following:

1. Introduction to surgical techniques:
 - Tibial fracture surgery: Dr M. Cibelli.
 - Middle cerebral artery occlusion: Dr L. Xu.
 - Nephrectomy: Dr D. Ma.
2. Introduction to behavioural tests:
 - Fear-Conditioning and Morris water maze: Dr A. Rei-Fidalgo.
 - Smart cage: Dr. Xingmin (Simon) Xie.
 - Acoustic sensorial activity: Dr M. Shamloo.
3. Laboratory techniques:
 - Western blotting: Ms L. Benzonana.
 - Immunostaining: Dr M. Hussain.
 - Fluorescence immunostaining: Dr G. Barreto.
 - Flowcytometry: Dr K. O'Dea.
 - Cryo-sectioning: Mr T. Pheby.
 - Daily laboratory mentoring: F. Noormohamed.
4. Statistical analysis: SIMCA: Bernard North.
5. Scientific mentoring: Rona Giffard and Kieran O'Dea.
6. Animal donations:
 - P2X7: GSK-Glaxo Smith Kline, Harlow, Essex.
 - Hsp72 overexpressors: Professor R. Giffard.
 - C57BL6 brain pups: Mr M. Johnson.

Dr C. Pac-Soo provided insights on statin and neurodegenerative diseases. I taught BSc students, MREs students laboratory techniques through out my thesis (Jack Penn, Anthony Fung) who worked along side my project and helped me in the execution of western blotting, ELISAs and immunostainings).

MPV

Acknowledgements

I wish to thank all the people who have helped me throughout my studies. The body of work would have never been possible without them.

Through the years, I have met the finest clinicians and scientist that inspired me on my studies. My thanks and gratitude to Drs Anita Holdcroft and Charles Schmulian who, without hesitation introduced me to the world of research in United Kingdom. Dr Schmulian helped me to convert my dreams into reality and Dr Holdcroft pooled the right streams to direct the flow to the right fountain.

I am eternally thankful to my supervisors, Dr Daqing Ma and Professor Mervyn Maze who gave me the opportunity to work in their laboratory and guided through my studies. I also want to thank Dr Mark Palazzo for his support over the last few years.

I am especially thankful to Dr Patrick Doyle who trusted in me, supported me and had the courage to change the system to support clinical research from the clinical arena. Mr Faruq Noormohamed has expedited all the necessary paper work making the process smooth and nice for what I am grateful too.

I would also like to thank Professor Rona Giffard and her team especially, Lijun Xu who taught me the necessary techniques to execute my ideas and Rona for helping me to define and re-define my process of thinking and help me to learn how to canalize them and focus before moving forward.

I wish to thank Dr Kieran O'Dea with whom I spent hours on scientific discussions and made me feel we have the power to make the difference if we focus on what we believe and work towards and answer by developing techniques and questioning ourselves continuously.

Dr Dafydd Lloyd confidence and courage to commit his career under my supervision made me strong and boost my needs for perfection and develop strategies to complete targets and at the same time has given me the opportunity to convey my ideas and continue searching for an answer to an evolving clinical and social problem. So, I am thankful to him too.

I am also thankful to all my laboratory colleagues and especially Laura Benzonana for her kindness friendship and support.

I wish to thank Professor Takata for supporting me and also for his critical mind and needs to see me as a finisher. Professor Takata taught me by example and encouraged me to work hard and focus on this study. He proved to me it is possible and the power is in one so I am eternally thanked to him as well.

I would also like to thank all my clinical colleagues at Chelsea and Westminster Hospital, especially, Drs Neil Soni, Michelle Hayes and Rick Keays. Dr Keays made sure I had enough time to continue working in the laboratory while I looked after the sickest patients in the intensive care unit.

My gratitude is also towards to the National Health System especially, Charing Cross Hospital and Chelsea and Westminster Hospital which supported my academic career while working in the clinical field. I wish to thank the Joint Research Committee at Chelsea and Westminster Hospital, the Intensive Care Society for supporting my scholarship at Stanford University in California. I also wish to thank the Alzheimer Society for its award to support the future of my ongoing research.

I am perpetually thankful to Dr Eduardo M. Svoren who I admire for his passion to knowledge and inquisitive mind. I will be always filled with gratitude towards him.

I am also thankful to Doris Doberenz for her genuine interest to see me moving to the next level helped me go through the last phase of my studies.

Last, but far from least, I am eternally thankful to all and especially to Simon M. Greenfield who is part of my life and his care and affection has encouraged me to continue exploring new avenues and make me feel that I can do it all again. *God bless and protect you.*

List of Figures

FIGURE 2.1: SCHEMATIC REPRESENTATION OF THE STRUCTURE OF COGNITION.	37
FIGURE 2.2: THIS FLOWCHART ILLUSTRATE THE STAGES OF MEMORY PROCESSING.	40
FIGURE 2.3: SCHEMATIC FLOWCHART REPRESENTING DIFFERENT TYPE OF MEMORIES.	42
FIGURE 2.4: REPRESENTATIVE IMAGE OF RATS' NAVIGATION PATH	52
FIGURE 2.5: SCHEMATIC REPRESENTATION OF THE BLOOD BRAIN BARRIER	56
FIGURE 2.6: THIS FLOWCHART ILLUSTRATES THE COMMONEST SURGERIES.....	68
FIGURE 2.7: SCHEMATIC ILLUSTRATION OF RISK FACTORS	74
FIGURE 2.8: SCHEMATIC ILLUSTRATION ON THE PRODUCTION OF IL-1B.....	97
FIGURE 2.9: SCHEMATIC REPRESENTATION OF THE INFLAMMATORY RESPONSE	111
FIGURE 3.1: SCHEMATIC REPRESENTATION OF THE ANAESTHETIC EQUIPMENT	133
FIGURE 3.2: THIS IS A SCHEMATIC REPRESENTATION OF THE EXPERIMENTS	136
FIGURE 3.3: SCHEMATIC REPRESENTATION OF THE CLOSED CIRCUIT.....	137
FIGURE 3.4: THIS FIGURE ILLUSTRATES THE HOME-MADE PHYSICAL RESTRAINER.....	139
FIGURE 3.5: THIS PHOTOGRAPHY ILLUSTRATES THE SURGICAL ENVIRONMENT	142
FIGURE 3.6: THIS IS AN ILLUSTRATION OF THE INTERNAL FIXATION OF THE TIBIA	143
FIGURE 3.7: THIS FIGURE SHOWS THE ISOLATION OF THE LEFT KIDNEY.....	145
FIGURE 3.8: THIS IS AN SCHEMATIC ILLUSTRATION OF THE CEREBRAL ARTERIES.....	147
FIGURE 3.9: THIS SCATTER HISTOGRAM ILLUSTRATES THE NEGATIVE EFFECT OF LPS.....	149
FIGURE 3.10: THIS PICTURE ILLUSTRATES THE HOT PLATE.	152
FIGURE 3.11: MOUSE STARTLE REFLEX CHAMBER	153
FIGURE 3.12: THIS PICTURE SHOWS THE BOXES WHERE MOTION WAS ASSESSED IN MICE.....	154
FIGURE 3.13: THIS FIGURE ILLUSTRATES HOW MOTION ACTIVITY WAS ASSESSED	155
FIGURE 3.14: THIS IS A REPRESENTATION OF THE DIFFERENT PHASES OF	156
FIGURE 3.15: THIS SCATTER HISTOGRAM ILLUSTRATES THE DEVELOPMENT	158
Imperial College London	15

FIGURE 3.16: THIS HISTOGRAM ILLUSTRATES THE EFFECT OF DIFFERENT CLEANING	159
FIGURE 3.17: SCHEMATIC REPRESENTATION OF THE FEAR-CONDITIONING TRAINING.....	160
FIGURE 3.18: THIS SNAPSHOT ILLUSTRATES THE ASSESSMENT OF MEMORY.....	165
FIGURE 3.19: THIS FIGURE ILLUSTRATES HOW A FREEZING THRESHOLD IS CHOSEN.	167
FIGURE 3.20: SCHEMATIC REPRESENTATION OF THE MORRIS WATER MAZE SET UP.....	168
FIGURE 3.21: THIS FIGURE ILLUSTRATES BASELINE CELL NETWORKING.	174
FIGURE 3.22: THESE PANELS ILLUSTRATE DIFFERENT STAGES OF MICROGLIA ACTIVATION	175
FIGURE 3.23: SCHEMATIC ILLUSTRATION AND LIST OF STEPS REQUIRED FOR.....	176
FIGURE 3.24: THIS FIGURE ILLUSTRATES HOW IMAGES WERE ANALYSED	181
FIGURE 3.25: THIS FIGURE ILLUSTRATES THE WESTERN BLOT STEPS.....	183
FIGURE 3.26: THIS SCHEMATIC REPRESENTATION ILLUSTRATES THE ELISA STEPS.....	185
FIGURE 3.27: THIS FIGURE ILLUSTRATES HOW THE RAW DATA IS CAPTURE.....	188
FIGURE 3.28: THIS FIGURE ILLUSTRATES A) THE IDENTIFICATION OF CELLS	194
FIGURE 4.1: SCORES FOR PCA TO IDENTIFY OUTLIERS.	208
FIGURE 4.2: SCORES FOR PCA..	209
FIGURE 4.3: PLOT OF CROSS-VALIDATED DISCRIMINATORY SCORES.	210
FIGURE 4.4: SCORES FOR PCA TO IDENTIFY OUTLIERS..	211
FIGURE 4.5: SCORES FOR IDENTIFICATION OF CLASS SEPARATION.....	212
FIGURE 4.6: LOADING COLUMN PLOT FOR DISCRIMINATORY CYTOKINES	213
FIGURE 4.7: SCORES FOR PCA TO IDENTIFY OUTLIERS.	215
FIGURE 4.8: SCORES FOR PCA TO IDENTIFY OUTLIERS.	217
FIGURE 4.9: LOADING COLUMN PLOT FOR DISCRIMINATORY CYTOKINES.	218
FIGURE 4.10: PLOT OF DISCRIMINATORY SCORES.....	219
FIGURE 5.1: A SINGLE CELL SUSPENSION WAS PREPARED BY COLLAGENASE	230
FIGURE 5.2: CELL IDENTIFICATION IN FIXED-HOMOGENISED MOUSE HIPPOCAMPI.....	231

FIGURE 5.3: CELLS IN FIXED-HOMOGENISED HIPPOCAMPI SUSPENSIONS.....	233
FIGURE 5.4: MICROGLIA EXPRESSION OF CD11B AND CD68 AT 24H POST-SURGERY.....	234
FIGURE 6.1: ILLUSTRATIVE WESTERN BLOTTING IMAGES.....	245
FIGURE 6.2: REPRESENTATIVE ILLUSTRATION OF MICROGLIA (BV2 CELLS).....	246
FIGURE 6.3: REPRESENTATIVE IMAGES OF 20D OLD CORTICAL MOUSE ASTROCYTE	247
FIGURE 6.4: REPRESENTATIVE IMAGES OF IMMORTALIZED MICROGLIA CELLS (BV2)	248
FIGURE 6.5: THIS FIGURE ILLUSTRATES THE RESPONSE OF HIPPOCAMPUS ASTROCYTES.....	249
FIGURE 6.6: REPRESENTATIVE IMAGES OF MOUSE CORTICAL ASTROCYTES AT BASELINE AND AT DIFFERENT TIME POINTS UP TO 30M AFTER ATP STIMULATION.....	250
FIGURE 6.7: ILLUSTRATION OF MICROVESICLES RELEASED BY CORTICAL ASTROCYTES.....	251
FIGURE 6.8: REPRESENTATIVE IMAGES OF CASPASE-1 ACTIVITY IN BV2 CELLS	252
FIGURE 6.9 THIS SCATTER HISTOGRAM REPRESENTS ACQUISITION AFTER TRAINING.	254
FIGURE 6.10: THIS SCATTER HISTOGRAM REPRESENTS HIPPOCAMPAL DEPENDENT MEMORY AFTER TREATMENT.	255
FIGURE 6.11: THIS HISTOGRAM REPRESENTS HIPPOCAMPAL INDEPENDENT MEMORY AFTER TREATMENT.	256
FIGURE 6.12: ACQUISITION OF MEMORY DURING THE TRAINING	257
FIGURE 6.13: HIPPOCAMPAL DEPENDENT MEMORY	258
FIGURE 6.14: HIPPOCAMPAL DEPENDENT MEMORY ASSESSED BY CONDITIONAL CONTEXTUAL.	259
FIGURE 6.15: HIPPOCAMPAL INDEPENDENT MEMORY.....	260
FIGURE 6.16: THIS FIGURE ILLUSTRATES THE PLASMA LEVEL OF IL-1B.....	262
FIGURE 6.17: REPRESENTATIVE ILLUSTRATION OF THE ISCHAEMIC INFARCTION.....	263
FIGURE 7.1: THIS BAR HISTOGRAM ILLUSTRATES THE ACQUISITION OF MEMORY	270
FIGURE 7.2: HIPPOCAMPAL DEPENDENT MEMORY WAS IMPAIRED FOLLOWING ANAESTHESIA OR SURGERY.....	272

FIGURE 7.3: HIPPOCAMPAL DEPENDENT MEMORY WAS IMPAIRED	272
FIGURE 7.4: HIPPOCAMPAL INDEPENDENT MEMORY	274
FIGURE 7.5: MICROGLIA ACTIVATION WAS ASSESSED BY IMMUNOREACTIVITY FOR CD68	276
FIGURE 8.1: THIS BAR HISTOGRAM ILLUSTRATES THE ACQUISITION OF MEMORY	288
FIGURE 8.2: HIPPOCAMPAL DEPENDENT MEMORY WAS IMPAIRED FOLLOWING TREATMENT. ...	291
FIGURE 8.3: THIS FIGURE ILLUSTRATES THE WESTERN BLOTTING ANALYSIS.	293
FIGURE 8.4: THIS FIGURE ILLUSTRATES A SCATTER HISTOGRAM.....	294
FIGURE 8.5: THIS FIGURE ILLUSTRATES A SCATTER HISTOGRAM OF THE ELISA	296
FIGURE 9.1: THIS IS A SCHEMATIC REPRESENTATION OF THE TIMELINE.....	303
FIGURE 9.2: THIS BAR HISTOGRAM ILLUSTRATES THE ACQUISITION.....	308
FIGURE 9.3: THIS SCATTER HISTOGRAM ILLUSTRATES THE RESULT OF	309
FIGURE 9.4: THIS SCATTER HISTOGRAM ILLUSTRATES.....	311
FIGURE 9.5: THIS IMAGE SUMMARISES THE MWM RESULTS OVER TIME.....	312
FIGURE 9.6: THIS FIGURE ILLUSTRATES A SCATTER HISTOGRAM.....	314
FIGURE 9.7: THIS FIGURE IS A SCATTER HISTOGRAM OF THE WESTERN BLOTTING	315
FIGURE 9.8: THIS FIGURE IS A SCATTER HISTOGRAM OF THE ELISA	316
FIGURE 9.9: THIS FIGURE ILLUSTRATES A SCATTER HISTOGRAM OF THE ELISA	318
FIGURE 9.10: MICROGLIA ACTIVATION	320
FIGURE 9.11: ATORVASTATIN SUPPRESSES THE EXPRESSION OF COX-2 ON ENDOTHELIUM.....	322

List of tables

TABLE 2.1: SUMMARY OF MECHANISMS ASSOCIATED WITH SPREAD OF INFLAMMATION.....	57
TABLE 3.2: THIS TABLE SUMMARISE THE TYPE OF ANIMALS AND RATIONALE.....	127
TABLE 3.3: PHYSIOLOGICAL VARIABLES UTILISED FOR ASSESSMENT.....	135
TABLE 3.4: THIS TABLE SHOWS THE NUMBER OF SURGICAL PROCEDURES.....	141
TABLE 3.6: THIS TABLE ILLUSTRATES THE HIPPOCAMPAL DEPENDENT	164
TABLE 3.9: THIS TABLE IS A SNAPSHOT OF THE TABLE GENERATED.....	189
TABLE 4.2: THIS TABLE SUMMARISES THE DISCRIMINATORY	220
TABLE 5.1: THIS TABLE ILLUSTRATES THE STEPS TAKEN FOR OPTIMIZATION.....	226
TABLE 6.1: THIS TABLE SUMMARISES THE EXPERIMENTS PERFORMED	242
TABLE 6.2: THIS TABLE SUMMARISES THE 2-WAY ANOVA.....	261
TABLE 7.1: NUMBER OF CD68 IMMUNOPOSITIVE CELLS IN CA1 AND CA,	276
TABLE 7.2: NUMBER OF CD68 IMMUNOPOSITIVE CELLS IN CA2 AND HILUS.....	278
TABLE 8.1: THIS TABLE ILLUSTRATES THE EFFECT OF DIFFERENT TREATMENTS.....	290
TABLE 9.1: THIS DATA (MEAN $\pm$ SD) (N=9-10 PER GROUP) SUMMARISES	306
TABLE 9.2: THIS TABLE SUMMARISES THE MORPHOLOGICAL RESPONSES.....	320
TABLE 3.1: PHYSIOLOGICAL SCORING SYSTEM USED FOR ASSESSMENT OF ANIMALS.....	371
TABLE 3.5: BASELINE ASSESSMENT OF C57BL6-Wt, P2X7-KO AND Hsp72-Tg MICE.....	372
TABLE 3.7: SOLUTIONS AND CORRESPONDING COMPONENTS USED FOR IHC STUDIES.....	373
TABLE 3.8: SUMMARY OF ANTIBODIES USED FOR WB ON MY STUDIES.	374

Abbreviations and acronyms

AD: Alzheimer disease

ANOVA: Analysis of variance

ApoE: Apolipoprotein E

APP: Amyloid precursor protein

ATP: Adenosine triphosphate

A β : Amyloid beta

BB-G: Brilliant Blue-G

BBB: Blood brain barrier

Bcl-2: B-cell lymphoma-2

C57BL6/Wt: Jax mice inbred strain - Wild type

C8-B4: Immortalized microglia cell line of 8-day postnatal mouse cerebellum

CA: Cornu ammonis

cAMP: Cyclic adenosine monophosphate

CCR: Contextual conditional response

CD11b: Cluster of differentiation molecule 11b

CNS: Central nervous system

COX-2: Cyclooxygenase-2

CR: Conditional response

DAMPs: Damage associated molecular pattern mechanisms

DG: Dentate gyrus

DNA: Deoxyribonucleic acid

ELISA: Enzyme-linked immunoabsorbent assay

ER: Endoplasmic reticulum

FACS: Fluorescent-activated cell sorting

FC: Fear-conditioning

FiO₂: Fraction of inspired oxygen

GA: General anaesthesia

GABA: gamma-aminobutyric acid

GWAS: Genome-wide association studies

HIF-1 α : Hypoxia-inducible factor-1 α

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A

HMGB-1: High mobility group protein B-1
HPA: Hypothalamic-pituitary-adrenal axis
Hsc70: Heat shock cognate 70
Hsp72-Tg: Hsp72 transgenic
Hsp72: Heat shock protein 72
i.p.: Intraperitoneal
i.v.: Intravenous
IFN γ : Interferon gamma
IL-1 β : Interleukin-1 beta
IL-6: Interleukin-6
IL-10: Interleukin-10
IL-18: Interleukin-18
iNOS: Inducible nitric oxide synthetase
ISPOCD: International study for postoperative cognitive dysfunction
KO: Knockout
LOAD: Late Onset Alzheimer's disease
LPS: Lipopolysaccharide
MCAO: Middle cerebral artery occlusion
MCP-1: Monocyte chemoattractant protein-1
MMPs: Matrix metalloproteinases
NALP3: Nucleotide-binding domain-leucine-rich-repeat- and PYD containing protein 3
NF κ B: Nuclear factor kappa light-chain-enhancer of activated B cells
NMDA: N-Methyl-D aspartic acid
OPLS-DA: Orthogonal partial least squares-discriminative analysis
OSC: Orthogonal signalling correction
oxLDL: Oxidised low density lipoprotein
P2: Purinergic receptor
P2X7-KO: Purinergic receptor X7 knock out
P2X7: Purinergic ionotropic X7 nucleotide receptor
pAkt: Phospho-protein kinase
PAMPs: Pathogen associated molecular pattern mechanisms
PBS: Phosphate buffer saline
PCA: Principal component analysis

pCREB: Phosphorilated cAMP responsive element-binding protein

PCs: Principal components

PD: Postoperative delirium

PI3-kinase: Phosphoinositide 3 kinase

PLS-DA: Partial least squares-discriminative analysis

POCD: Postoperative cognitive decline or dysfunction

POMD: Postoperative memory decline

PFA: Paraformaldehyde

Q2: Cross validation correlative coefficient

RA: Rheumatoid arthritis

RAGE: Receptor for advanced glycation end products

ROS: Reactive oxygen species

S-P: Surgery-Placebo

s.c.: Subcutaneous

SD: Standard deviation

SEM: Standard error of the mean

SIMCA: Soft independent modelling of class analogy

SpO₂: Oxygen saturation

SUS-plot: Share unique structure plot

TBS: Tris buffered saline

Tg: Transgenic

TLR: Toll-like receptor

TLR4: Toll-like receptor

TNF- α : Tumour necrosis factor alpha

UCR: Unconditional response

WB: Western blotting

Wt: Wild type

The hypothesis

POCD is mediated by endothelial cell activation which leads to a disruption of signalling through Hsp72 chaperone and purinergic nucleotide receptors (P2X7). This is a consequence of surgery and anaesthesia. Interventions with xenon and statins targeting these will be shown to produce benefit.

The aims

The aims of this PhD thesis are to use existing animal models for assessment of memory changes and inflammatory response after surgery. This thesis will also attempt to identify techniques to study the systemic and central nervous system inflammatory response to surgery and anaesthesia and develop a model to predict the development of POCD based on the inflammatory response. Then it will elucidate the role of P2X7 receptors and Hsp72 in POCD and finally it will attempt to identify preventative mechanisms through up regulation of Hsp72 to complete the thesis by testing the effect of xenon and statin on POCD.

Chapter 1:

Road Map

1.0 Definition

Postoperative cognitive decline (POCD): is an acute deterioration of cognition and memory following surgery which can last for months and years.

1.2 The problem

It is recognized that surgical procedures may be followed by a decline in cognitive functional status (POCD). This is of critical importance in relation to independent living, need for care, personal and economic cost, and quality of life and so has significant ramifications.

With an ageing patient population and enhanced anaesthetic and surgical capabilities and hence expectations more high-risk patients are undergoing surgical procedures so this problem is likely to increase (Alexander, Newby et al. 2007).

1.3 Epidemiology

POCD occurs in 62% of patients undergoing major surgery (Young and Inouye 2007; Deiner and Silverstein 2009); the percentage is significantly higher following cardiac or hip fracture surgery (Siegel, Agranoff et al. 1999).

1.4 Overview of proposed mechanisms

Neuroinflammation has been postulated as one of the key players in memory decline after surgery. Chaperones such as heat shock proteins have a pivotal role in crosstalk between the innate and adaptive immune system. It has been shown

that heat shock proteins especially, Hsp72 is cytoprotective and prevents inflammatory changes via deactivation of the transcription factor NFκB.

Furthermore, dangerous signalling through purinergic receptors, especially P2X7, and an increase in endothelial cell activity has been implicated in the development of neurocognitive changes after neurological insults.

1.5 Overview of proposed interventions

Amelioration of the inflammatory response by antagonising the inflammatory cascade has been attempted to control the development of POCD. However, to my knowledge, no one has tried to control the humoral response by interfering with the expression of Hsp72 and reduction of cellular activity at endothelial level.

1.6 Hypothesis

POCD is mediated by endothelial cell activation which leads to a disruption of signalling through Hsp72 chaperone and purinergic nucleotide receptors (P2X7). This is a consequence of surgery and anaesthesia. Interventions with Xenon and statins targeting these will be shown to produce benefit.

1.7 The thesis will be laid out as follows

To address this question the thesis will review what is known of the subject to date and a synopsis of what has been done. This introduction will thereby provide the background and the rationale for the hypothesis.

To test the hypothesis requires the development of an animal model. This will be described and the methods used to validate the model and also look at its limitations.

Techniques for assessing the model, both in terms of memory but also the inflammatory response, will be required and I will describe the rationale for the techniques, their development and a thorough description and validation of the techniques. This will include the limitations of the techniques and also place in context other potential techniques.

The purinergic receptors described in the introduction will be examined. When the techniques have been validated I will use both the animal model and these techniques to try to identify the role of these purinergic receptors; mainly P2X7 on POCD. Some work exists that suggests that an animal model may be useful. This will be done by assessing sickness behaviour, POCD and motor activity after CNS insult (stroke).

As Hsp72 is reputed to be helpful in preventing POCD I will use these following techniques to test preventative mechanisms through up regulation of Hsp72.

Xenon has been mooted as having properties of cerebral protection, these methods can be used to test the effect of xenon on POCD as preventative strategy to ameliorate the effect of surgery/anaesthesia on memory. Statins have also been suggested as having potential protective activity and these will be tested.

It is hoped that by doing this an effective model will be developed as will techniques that can be used to evaluate this area. I will have investigated areas of current interest that may be involved in terms of mechanism but also looked at potential preventive strategies that may provide further insight into causation and mechanism but also potentially open the door to preventative interventions.

1.8 Chapters

1.8.1 Chapter 1: Road map of the thesis

The introduction provides an overview of POCD and a rationale for the hypothesis.

1.8.2 Chapter 2: Background and review of the literature to set the basis for the subject and the rationale for the hypothesis.

1.8.3 Chapter 3: Material & Methods

To test the hypothesis will require the development of an animal model therefore, the pros and cons in developing an animal model will be argued and then why there is a need for different surgical models. How to develop an animal model which is to be able to test and assess memory changes and inflammatory response after surgery. Model validation and how this was done.

Identification of techniques to study the systemic and central nervous system inflammatory response to stress.

1.8.4 Chapter 4: Characterisation of the inflammatory response by using multiple-cytokines analysis in plasma and CNS by SIMCA.

1.8.5 Chapter 5: Identification of cells in the hippocampus and their activation by FACS. Here is the rationale for the decision to focus on the hippocampus and not the cortex.

1.8.6 Chapter 6: Signalling through P2X7. Why this model did not work?

1.8.7 Chapter 7: Over expression of Hsp72

1.8.8 Chapter 8: Preventative mechanisms: Xenon

1.8.9 Chapter 9: Preventative mechanisms: Statin

1.8.10 Chapter 10: Main discussion and conclusion

1.8.10.1 The conclusion: This will pull these threads together and show how they contribute to an understanding of the mechanism of POCD. It will describe the model and its usefulness. It will describe interventions and will enable some speculation on where this research has brought us and therefore where it should lead on to.

Chapter 2:

Historical Background

Circa 400 BC, Hippocrates described in the book of epidemics a patient named “Erasinus”, who became “crazed at night” and died within 5 days. Perhaps, this is one of the first cases of acute mental disorder associated with an acute medical condition described in the literature.

It was not until 1885 when at the Sorbonne in Paris in 1885, Armand Ribot under the influence of Ernest Renan described the progressive dysfunction of memory in disease. Ribot was well known for his law of regression. He postulated that recent memories are affected first, secondly personal memories disappear as they “go downwards to the past” and thirdly, things that are acquired intellectually are lost bit by bit and the last memories to disappear are related to emotions and habits. What is most fascinating is that Ribot postulated that memory depends on permanent organisation and modifications of neurones and “the neurones disorganisation leads to memory impairment” (Ribot 1887).

Whilst at Saint Thomas Hospital in London, England in 1887, George H Savage reported the first observational study on memory decline after anaesthesia entitled “Insanity following the use of anaesthetics and operations”. His narrative highlighted patients pre-morbid conditions, ageing, major surgeries and anaesthesia (Savage 1887). His observations were remarkable and formed the foundations for the development and understanding of memory decline after surgery. It is fascinating how after such a clear description of the problem Savage concluded, “*All writers and observers have noticed that it is very rarely that one cause alone is efficient for the production of any attack of insanity*” (Savage 1887).

In the 1950s, Bedford conducted a large observational study looking at memory changes after 1193 surgical procedures under general anaesthesia and used individual case review to analyse the findings (Bedford 1955).

Seven years later Boshes *et al* discussed in details the neurological, psychiatric and psychological aspects of patients undergoing cardiac surgery and developed more comprehensive quantitative methods for assessment of cognitive changes after surgery (Boshes, Priest *et al.* 1957).

During the 1960s and 1970s, there was a more systematic approach to try to achieve a better understanding of memory impairment and a considerable debate was generated as to whether there was a problem with memory storage or retrieval.

In the meantime, Branthwaite reported a survey carried out on all cases requiring open cardiac surgery at the Royal Brompton Hospital in 1972 and once again her findings agreed with those in the original paper published by Bedford in 1950 (Branthwaite 1972).

As a consequence of these findings the research community focused on developing quantitative methods for the assessment of memory decline after surgery. The first structured battery of behavioural tests used after surgery was introduced by Savegeau *et al* in 1982 (Savageau, Stanton *et al.* 1982). In the same decade, Shaw *et al* from Newcastle, UK used 10 different

neuropsychological tests to assess early memory changes after cardiac surgery (Shaw 1986). The clinical community was increasingly aware of the neurocognitive changes after surgery and by the end of the 1980s clinicians used several different tools to assess cognition (Squire 1987) in the perioperative period. Clinicians were also aware of the predisposing risk factors for development of memory impairment after surgery under general anaesthesia.

Even though there were some hypotheses relating to potential mechanisms leading to memory disturbances after surgery this remained neither defined nor characterized from the cellular point of view until the earliest 1980 when Aberg and collaborators started looking at markers of neuronal cell injury (Aberg, Ronquist et al. 1982). Initially they identified high levels of adenylate kinase (AK) in cerebrospinal fluid (CSF) after cardiac surgery but not after non-cardiac surgery. It took almost 10 years for the same group to try to characterize the type of cells injured during surgery and they found that S-100 β , a dimeric protein which consists of 2 subunits (α and β) was increased in CSF after open-heart-surgery. The S-100 β is highly brain specific and it is localized mainly in the glial cells (Aberg 1995).

In parallel to physiological developments in the understanding of memory, Brenda Milner in 1957 reported the dramatic effect on memory of bilateral medial temporal lobe resection performed to treat refractory epilepsy. The patient was named Henry Molaison and became known by neuroscientists and psychologists as H.M (1926-2008) (Scoville and Milner 1957). These initial findings established key principles about the organisation of memory and

identified the hippocampus, amygdala and parahippocampus as the neuroanatomical areas involved in the process of memory formation and storage (Squire and Wixted 2011). Finally, by the early 90s researchers identified the hippocampus as the anatomical area responsible for memory loss or impairment due to its high vulnerability to minimal circulatory changes after cardiac surgery (Levy, Kong et al. 1991; Matsuoka, Maeda et al. 1991).

Further advances in this field have continued into the new millennium. Different groups have tried to identify how the central nervous system reacts to non-cardiac surgery and how this response affects memory.

To enable the body of work described in this thesis to be more easily followed, there will be clear definition of words frequently used. This will be followed by a technical description of what is understood by learning and memory and then the deficits that are thought to be related to surgery and anaesthesia.

2.1 Definitions

Acquisition: it is the act of acquiring or gaining position. In this thesis it refers at the act of learning or acquiring knowledge.

Brain connectivity: it is the ability to make and maintain a connection between two or more points in a system. It is also refers to functional connectivity when and impulse reaches its final endpoint and an action is elicited.

Cognition: From the Latin *cognoscere* “to know”. It is the mental process of knowing, including aspects such as awareness, perception, reasoning and judgment.

Delirium: It is an acute condition characterized by fluctuating level of consciousness and temporary abnormalities in cognition, attention and memory. It also refers to early postoperative cognitive dysfunction or postoperative delirium (ePOCD or POD).

Episodic memory: It refers to memory associated to place, person and time.

Executive function: It has been operationally defined as higher order cognitive processes involving coordination, maintenance and control of information processing.

Explicit memory: It is an intentional form of conscious memory that encompasses recollection of previous experiences. It is also known as declarative memory.

Implicit memory: It is a non-intentional form of unconscious memory that included motor skills, habits and in general requires priming. It is also known as procedural memory.

Learning: It is defined as the act and process of experience of gaining knowledge or skills.

Learning by association (conditioning): It is the process by which an association between two stimulus or behaviour-stimulus is acquired.

Memory: it is the mental capacity to retained and recalled information or past experiences.

Memory processing: it is the ability to recognise, and establish short-term also known as working memory and long-term memory also known as processing memory.

Neurodegenerative diseases: It involves the progressive loss of structure or function of neurones and it also includes death of neurones.

Neuronal plasticity: It describes as the ability of the brain to undergo continuous molding and change as a result of one's experience. It is also known as cortical remapping.

Postoperative cognitive dysfunction/decline (POCD): It refers to impairment in concentration, memory, language, comprehension and social integration.

Postoperative memory decline (PMD): It refers specifically to implicit memory deficits in rodents.

Semantic memory: It refers to memory associated to knowledge of facts and it involves executive functions.

2.2 Understanding memory – physiology and anatomy

2.2.1 Cognition, learning and memory formation in humans

2.2.1.a Cognition

Cognitive processing is a unique and vital human experience. Cognitive function has been described in term of 4 different functions. These are input, processing, storage and output (Loring and Meador 2004). These domains of cognition are encompassed into two main areas; learning and memory (Figure 2.1).

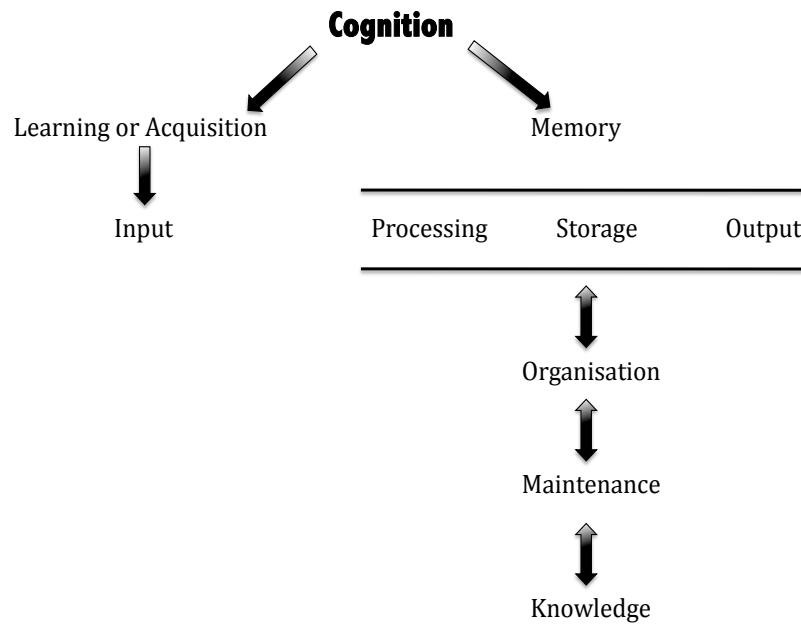


Figure 2.1: Schematic representation of the structure of cognition.

From a physiologic perspective, the process of cognition is dependent in part upon the neurone, which is the functional unit of the central nervous system (CNS). Neurologic function is vital to information processing and adaptation. It does so in different ways that may be important in cognition.

The neuronal network is through synapses. There are two main types of synapses; the classic synapse and the neuromodulatory synapse. The classical synapse is responsible for transmitting information from a presynaptic neurone to its target cell. In contrast, the function of a neuromodulatory synapse is to transmit information that will have long-term effects on the postsynaptic neurone, most importantly its response to succeeding input. It has been termed plasticity. The neuronal synaptic plasticity is an activity-dependent process, which involves continuous use of synaptic pathways or any lasting upregulation

or downregulation or synaptic strength. Neuronal synaptic plasticity was originally studied to identify experience-dependent changes in sensorimotor motor brains such as dynamic changes in the motor cortex following the amputation of limbs (Donoghue and Sanes 1988). Several mechanisms were identified to be involved in changes in synaptic strength, acting at the level of both the presynaptic and postsynaptic neurones. Direct modulation of the process of release of excitatory/inhibitory neurotransmitter and the subsequent changes in the amount of neurotransmitter released as well as modifications in the function and/or the numbers of neurotransmitter receptors are some examples of the pre and postsynaptic alterations by which the strength of synaptic connections may be altered (Mozzachiodi and Byrne 2010). Subsequent reactivation of the neurones comprising the altered connections constitutes the experience of memory for the external events with which the connection modification was initially associated (Martin and Morris 2002). This process is widely believed to be elemental to learning and memory, as well as providing an important role in the development of new neural pathways (Carew and Sahley 1986; Singer, Lindenberger et al. 2003). Neuromodulatory synapses could be modified throughout individual's life to promote learning and memory, and alter behavioural response (Culley and Crosby 2003; Thompson, Mata et al. 2005; Olson, Rao et al. 2006). Several models have subsequently identified that the variety of plastic changes gives rise to associative and non-associative learning and to short, intermediate and long term memory storage associated to these type of learning.

In summary cognition requires both the acquisition of information or learning and the establishment of memory. Brain adaptability or plasticity is paramount for the storage of information.

2.2.1.b Learning

Learning is the ability of every living organism to acquire information through its sensory input from the environment to adapt and survive. Learning requires perception, attention, concentration, comprehension and integration. In other words learning is the process of acquisition and encoding information. Cognitive skills are learned behaviours that are acquired through demonstration, replication, and practice (Carter, Howard et al. 1983; Blundon and Smits 2000). Learning is a basic mechanism in adaption and evolution, influencing the CNS at multiple levels (morphology, biochemistry and function).

Neuroimages has been used to hypothesise the process of learning comprises adaptations in motor functions, visuospatial functions, and body perception or control. These functions correspond to regions of the brain that undergo neuroplasticity and adaptation to process and store new encoded information. An association between learning motor skills and increased in grey matter due to neuronal adaptation, has been demonstrated, which is more pronounced with high intensity motor training (Bezzola, Merrillat et al. 2011), training induced neural plasticity.

2.2.1.c Memory

Memory encompasses the process of organisation, storing and retrieval of information. Attention, concentration, and memory are aspects of thinking that

are essential in everyday life. This is easily seen in terms of activities of daily living (ADL), where individuals need to be competent in managing finances, shopping, taking medications, transportation, and household management, all of which requires these and more components.

There are basically three stages of general memory processing: 1) registration, 2) short-term memory and 3) long-term memory (figure 2.2).

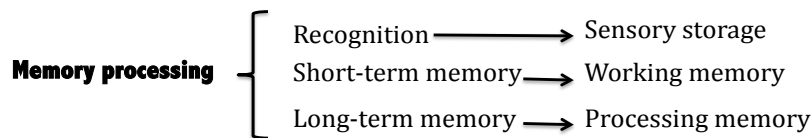


Figure 2.2: This flowchart illustrates the stages of memory processing.

2.2.1.c.1 *Recognition* involves briefly holding incoming information for one or two seconds in sensory storage (Lezak 1995). In this stage, the brain determines which memories to store as short-term memory by further processing the registered information, or alternatively, allowing the information to be forgotten (Lezak 1995).

2.2.1.c.2 *Short-term memory* also refer to as working memory, allows information to be held in mind for a brief period (Kolb 2003). The neuroanatomical substrates most closely related to short-term memory include the frontal and temporal lobes (Kolb 2003). The immediate memory substage of short-term memory lasts from approximately 30 seconds up to several minutes (Lezak 1995). Immediate memory typically allows for recollection of seven pieces of

information, plus or minus two pieces (Miller 1956). Rehearsal, defined as repetition of information that strengthens the memory trace, increases the likelihood that the information would be permanently stored (Lezak 1995). The formation of short-term memory requires executive functions. Executive functions are defined as higher-order cognitive processes involving coordination, maintenance, and control of information processing. Short-term or working memory comprises volition, planning, purposive action, and effective performance (Lezak 1995). It has been shown that executive functioning was significantly correlated with functional status in normal ageing, but memory decline was associated with functional status only through executive function impairment (Royall, Palmer et al. 2005).

2.2.1.c.3 *Long-term memory* represents the third stage of general memory processing. The organisation of long-term memory storage is based on the meaning of information (Lezak 1995).

Long-term and short-term memory systems work in concert with one another such that information is processed simultaneously; however, there is evidence of different neural substrates allowing for separate processing of information for long-term and short-term memories (Kolb 2003).

Once memory is processed it can be further divided into two major categories of memory: implicit (procedural) and explicit (declarative) memory (Lezak 1995) (Squire 1987)(Figure 2.3).

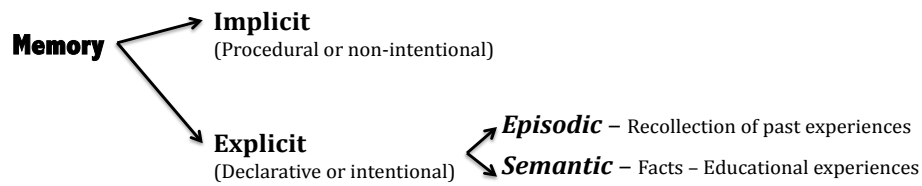


Figure 2.3: Schematic flowchart representing different type of memories.

2.2.1.c.3.a Implicit memory (procedural memory)

Implicit memory is a non-intentional form of unconscious memory that includes motor learning of skills and habits, priming (unconscious memory prompted by a cue) and classical conditioning (Kolb 2003). Lezak described implicit memory as knowledge that is outside of one's conscious awareness during task performance (Lezak 1995). It has been suggested that the neuroanatomical basis of implicit memory is associated with the neocortex, basal ganglia (caudate nucleus and putamen), and substantia nigra (Kolb 2003). The neurotransmitter dopamine appears to support implicit memory formation through projections from the substantia nigra to the basal ganglia (Hay, Moscovitch et al. 2002).

2.2.1.c.3.b Explicit memory (declarative memory)

Explicit memory is an intentional form of conscious memory that encompasses recollections of previous experiences (Kolb 2003). Lezak described explicit memory as an intentional process that involves knowledge within one's conscious awareness (Lezak 1995). Declarative memory answers the question “*what*” and included knowledge of facts such as places, people and things. The neuroanatomical basis for explicit memory is associated with the temporal lobe

(amygdala, hippocampus, and rhinal cortex), prefrontal cortex, and thalamus (Kolb 2003).

Explicit memory is further divided into two forms: a) episodic and b) semantic memory (Kolb 2003) (Squire 1987). The episodic memory refers to the recollection of past personal experiences (Kolb 2003). The episodic memory encompasses time and place, it relates the individual to his environment. Furthermore, episodic memory includes episodic verbal memory. Episodic verbal memory has been specifically conceptualized within a three-stage model involving encoding, consolidation, and retrieval (Ellis & Hunt 1983). Encoding entails information being taken in and transformed. Consolidation involves storing the transformed information for later use and retrieval refers to extraction of the stored information (Ellis & Hunt, 1983). Whilst neuropsychological and neuroanatomical studies have shown that encoding, consolidation, and retrieval processes demonstrate overlap (Nyberg, McIntosh et al. 1996), the terminology remains useful in the discussion of verbal learning and memory impairment. Episodic memory is closely associated with the hippocampus (Kolb 2003). Cermak suggested that over time, episodic memories lose their connection to a specific time or place and may become more generalized as semantic memories (Cermak, Stiasny et al. 1984).

The semantic memory refers to knowledge about the world and facts learned through educational experiences (Kolb 2003). This general store of information is shared in common with others and is not tied to personal experience, time, or place. Semantic memory is associated with the rhinal cortex of the temporal lobe

and left inferior prefrontal cortex (Kolb 2003). It would appear that the neurotransmitters acetylcholine, serotonin, and noradrenaline help support the neural circuit of explicit memory through input from the neocortex and brainstem (Kolb 2003).

In summary there are two main types of memory: implicit or procedural memory (memory for skills) and explicit or declarative memory (memory for facts). Memory is stored in specific areas of the brain with the hippocampus the key or central area for memory formation.

2.3 Assessment of memory in humans

Lezak asserted that verbal learning and memory functioning are essential areas to investigate, as they are fundamental to all cognitive tasks (Lezak 1995). To do so requires assessment tools. Learning and memory are assessed by using assays to quantify individual's abilities to recall and recognise objects, faces and familiar environment. There is now an increasing interest in using verbal episodic memory tasks to examine the components of learning and memory processes, such as the executive ability of applying a learning strategy.

Evaluation of learning strategies can help to elucidate the underlying cognitive mechanism of learning and memory impairment, which may inform treatment planning and potentially facilitate differential diagnosis (Poreh 2000). There is also growing interest in studying executive learning and memory strategies because impairment in e

executive abilities leads to memory decline and functioning deficits (Royall, Palmer et al. 2005). One type of executive learning strategy employed within verbal word list-learning tasks is semantic clustering. Semantic clustering refers to an organisational strategy involving consecutive recall of words from the same semantic category (Bousfield 1953). Spontaneous use of semantic clustering to organize verbal material is considered an effective learning strategy, as it allows more efficient encoding and enhances recall (Delis, Freeland et al. 1988).

An extensive body of research has examined verbal learning and memory ability on neuropsychological measures. An example of this is the California Verbal Learning Test-Second Edition (Wecker, Kramer et al. 2000) and variations of this word list learning task. Verbal learning and memory profiles, including degree of semantic clustering, have been assessed in various clinical populations. In general, deficits in the use of an active organisational strategy, such as semantic clustering, relates to impaired recall. Supporting this concept is the observation that utilisation of semantic clustering appears to facilitate overall memory performance in a variety of clinical populations, including dementia (Herlitz and Viitanen 1991), mild cognitive impairment (Ribeiro, Guerreiro et al. 2007), frontal lobe lesions (Gershberg and Shimamura 1995), traumatic brain injury (Crosson, Novack et al. 1988), human immunodeficiency virus (Peavy, Jacobs et al. 1994), schizophrenia (Brebion, Amador et al. 1997), obsessive-compulsive disorder (Deckersbach, Otto et al. 2000), bipolar disorder (Deckersbach, McMurrich et al. 2004), attention deficit/hyperactivity disorder (Hervey, Epstein et al. 2004), and Parkinson's disease (Buytenhuijs, Berger et al. 1994).

There is also an emerging interest in the use of the Hopkins Verbal Learning Test-Revised (Woods, Scott et al. 2005). The HVLT-R has been used to examine verbal learning and memory profiles related to recall and recognition, as an addition to component process variables such as semantic clustering. Utilizing the HVLT-R rather than the CVLT-II can be advantageous because it is a brief verbal learning and memory test with a shorter word list and fewer learning trials. Additionally, the HVLT-R is highly correlated with the more extensive CVLT-II (Lacritz, Cullum et al. 2001) and alternate forms with moderate to high test-retest reliability allow for repeated measures testing (Shapiro, Benedict et al. 1999). The test-retest reliability of the alternate forms is particularly important to evaluate postoperative neurocognitive decline over several time points.

The component process variable of semantic clustering has been examined within the HVLT-R, and research findings support the effectiveness of semantic clustering in discriminating between controls and various patient groups, including those with dementia (Gaines, Shapiro et al. 2006), mild traumatic brain injury (Bruce and Echemendia 2003), human immunodeficiency virus infection (Woods, Scott et al. 2005), and methamphetamine dependence (Woods, Rippeth et al. 2005). Semantic clustering was endorsed as a component process variable with sensitivity to more subtle verbal learning and memory impairment (Woods, Rippeth et al. 2005).

So this is what has been done in humans but in order to gain a better understanding of the molecular mechanisms regulating the process of memory

formation animal work is needed. Initially, we will gain an insight on memory formation in small rodents and its equivalent to human memory following by understanding of molecular mechanisms involved with the process of establishing and retrieving information from the brain.

2.4 Understanding of memory formation in animals

The rationale for using animal models to study memory is to have a better control of variables that may influence the mechanisms of memory formation such as variation on memory content and avoidance of linguistic complications. Ideally it would allow the creation of a model where the process of learning, specially the one associated with repetition and associative mechanisms can be controlled. It would be possible to concentrate on variables such as retention and forgetfulness. An animal model allows control of the stimulus and make it constant throughout experimental work.

The learning process in rodents is similar to humans when spatial memory is formed as there is an associative system that incorporates different sensorial pathways. The memory is formed in the hippocampus and storage is in other areas of the brain, the anatomical structures involved with memory formation in animals seem to be similar to those in humans. Unfortunately, executive functions such as verbal and mathematical memory are not easily reproducible in rodents.

Animal learning is mainly by association. This concept was initially introduced by Ivan Pavlov in 1927. Pavlov was interested in understanding the neuronal

mechanisms of glandular secretion during digestion. He studied salivation in dogs by administering food powder into the animal's mouth and measuring the amount of secreted saliva. He found that food increases saliva production in dogs. He also noticed that one of the dogs appeared to become experienced after several trials and what was more striking it was that the animal started salivating immediately after it saw the laboratory assistant coming in to feed them. This observation induced by Pavlov to design a more controlled experiment in which before offering the food to animals a bell was rung. After training with the bell the animal started salivating when the bell sounded in the absence of food. Pavlov concluded that an original reflex (salivation) could be generated if a neutral stimulus (bell) predicts the occurrence of a significant incentive (food) (Pavlov 2003). This association acquires a positive value and can act as a predictor of a pleasant event, thus establishing memory. These findings introduced the concept of associative learning memory which is the basis for the development of classical conditioning theory.

Animal memory or expression of learned behaviour was originally focused on basic conditioning or reflex activities. As this concept has evolved, memory in animals now refers to representations of events and relationships. Rescorla in 1988 explained Pavlov's findings on conditioning reflexes as a "process of learning that results from exposure to relationships between events in the environment" and as such provides a "primary means by which the organisms represents the structure of its world (Rescorla 1982).

Animals are usually conditioned to their environments so that 're' or new conditioning may happen immediately after an animal is taken from its own environment. Associative memory does not necessarily involve just one single stimulus and the association may be hierarchical rather than linear as multiple stimuli may elicit a response. Conditioning is normally predictable and slow so that contiguity seems to be a powerful force that sometimes suffices to generate an associative learning process.

In summary, classical conditioning is based on the association of two stimuli. The cause-effect relationship occurs only when the rewarding or warning stimulus is carefully sequenced and timed in order for an initially neutral stimulus to elicit a conditional or associative response. Repetition of stimuli in the same sequence generates memory.

2.4.1 Fear conditioning

The principle of classical conditioning is the basis for several behavioural paradigms used for assessment of learning and memory in animals.

Fear learned response requires rapid associations and reliable actions during learning and memory processing. It is primarily correlated with survival. Learning of fearful events can be rapid and long lasting, making fear suitable tool for experimental studies. Hence fear conditioning is one of the tools used for assessment of memory in this thesis.

In the model presented in this thesis the animals learnt a conditioned fear response in which a neutral stimulus is temporally associated to a noxious stimulus. The timing of the neutral stimulus and the intensity of the noxious stimulus affect the speed and rate of learning and repetition of the pair of them establishes memory. After pairing the neutral stimulus with the noxious stimulus, the neutral cue itself elicits an aversive response. Furthermore, the association of the neutral stimulus to the environment allows the formation of memory of the context or surroundings of the animal and an associative memory to the noxious stimulus. The innate reaction for fear in rodents is immobility, commonly referred to as freezing, which is scored as an index of memory (Quinn, Loya et al. 2005). Freezing is defined as the complete absence of mobility with the exception of respiratory movements (Kim and Fanselow 1992).

In the fear conditioning training, hippocampal functioning is key during the acquisition phase when the stimuli become associated but also, once the association is made, the hippocampus is required to create a uniform reconstruction of the environment or context. The contextual fear memory aims to access the hippocampus hence it is called the hippocampus-dependent memory. It has a clinical correlate in that hippocampal damage detected by altered fear conditioning is said to resemble clinical cases of amnesia following hippocampal damage (Kim and Fanselow 1992). At the same time, the conditional response allows the assessment of non-hippocampal dependent memory, which is in particular associated with the prefrontal cortex and the amygdala (Runyan, Moore et al. 2004; Misane, Tovote et al. 2005).

2.4.2 Morris water maze (MWM)

The Morris water maze has been used to assess hippocampal dependent memory. The MWM was originally described in 1982 for assessment of spatial memory deficit in rats after hippocampal lesion. In brief, the MWM consists of a round pool filled with water made opaque with milk and a hidden platform. The animals learn to identify the hidden platform under the water from different starting points. They also learn to identify the hidden platform by using different strategies. They can learn the sequence of movements required to identify the platform (praxic strategy), they learn proximal cues that guide them to identify the platform (taxic strategy). They also associate the location of the platform to distal cues (spatial strategy). There are several parameters used to identify the animals capacity to learn and retain the acquired information such as, time taken to reach the hidden platform, distant navigated to identify the platform, time spent on the area of the platform when this is removed. The MWM is a well characterised and highly sensitive tool for assessment of hippocampal dependent memory after therapeutic interventions in rodents. Morris et al showed in a very elegant experiment the importance of the hippocampus on spatial memory while he also confidently ruled out the role of the cortex on this type of memory by using the water maze technique (Figure 2.4).

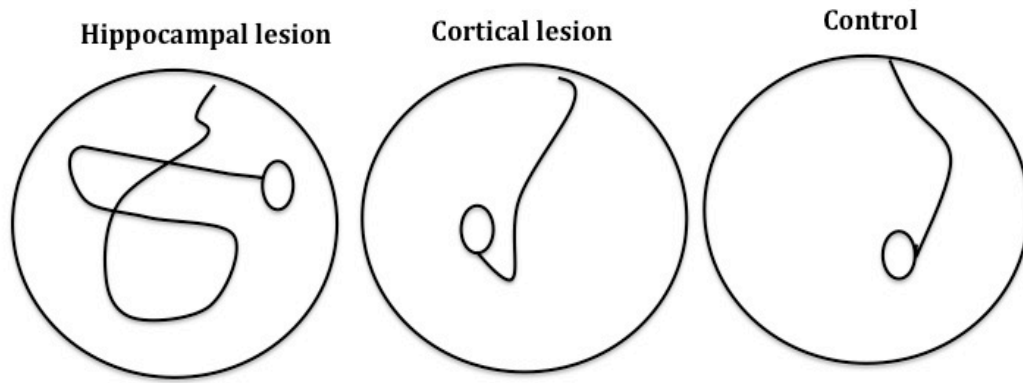


Figure 2.4: Representative image of rats' navigation path after a) hippocampal lesion, b) cortical lesion and c) control or no lesion. Modified from Morris et al. *Nature* 1982; 297: 281-283.

The rationale for using a second behavioural test in the studies in this thesis was to be able to corroborate and demonstrate changes in memory by different methods to categorically state that changes in animals behaviour was due to disruption of hippocampal dependent memory. Therapeutic interventions could then been translated into clinical practice promptly.

In summary, the MWM permits accurate and reproducible studies on spatial working memory and learning and it complements results obtained by fear conditioning training.

MWM behavioural training is used in this thesis as a second tool to validate the findings when assessing the effect of treatment interventions on hippocampal dependent memory changes after surgery.

2.5 The effect of neuroinflammation on memory

Memory and learning rely on the hippocampus. The hippocampus cells have Interleukin 1-beta (IL-1 β) receptor binding sites and are therefore highly prone to inflammatory insults (Kim and Fanselow 1992; Gemma, Fister et al. 2005). IL-1 β is paramount for the process of learning and memory.

The presence of IL-1 β in the synaptic cleft contributes to the modification of the postsynaptic neurone allowing conformational changes and in consequence enhancing the synaptic plasticity mechanisms. It would follow that high level of this pro-inflammatory cytokine is detrimental for learning and cognition (Gibertini 1998; Barrientos, Frank et al. 2009).

High level of IL-1 β in the neuronal milieu interferes with neurotransmitters involved in memory formation such as glutamate which is necessary for fast synaptic transmission as demonstrated in a murine model of pain (Lai, Shaftel et al. 2006). It has been shown that tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) interfere with the balance between excitatory and inhibitory neurotransmitters resulting in further disruption of learning and memory (Lai, Shaftel et al. 2006).

Deregulated connection between the peripheral immune system and the brain (Lee, Weindruch et al. 2000) results in elevated levels of inflammatory cytokines (IL-1 β , IL-6, and TNF- α) in the hippocampus and the forebrain (Ye and Johnson 1999; Richwine, Godbout et al. 2005). These two brain areas are associated with learning and memory, the former as a centre of consolidation of information from short term to long-term memory and the latter as the main centre of

acetylcholine production, a neurotransmitter that affects the ability of brain cells to transmit information to one another and encourages plasticity or learning.

Hence one might expect sickness in general to increase the risk of developing learning and memory changes due to production and release of pro-inflammatory cytokines within the brain and primary in areas with a considerable amount of IL-1 β production.

2.6 Sickness behaviour

The body response to infection and/or systemic inflammation is a primitive survival mechanism to protect the animal. Sickness behaviour is classically associated with a decline in cognitive function due to fever or disease. The animal remains immobile, lethargic, somnolent and reduces the intake of food. These symptoms are associated with high levels of certain types of cytokines systemic and in the CNS.

It is thought that cytokines act on a variety of brain structures to stimulate the behavioural and cognitive changes associated with sickness behaviour. IL-1 β levels change throughout the inflammatory reaction and at supra-physiological levels has been previously described as a cause of cognitive changes, reduced food intake and weight loss. Its role in cognitive changes and memory in particular is complicated by its importance in the process of long-term memory formation at physiological levels (Schneider H, Pitossi F, 1998). Supra-physiological and sub-physiological levels of IL-1 β load were demonstrated to impaired performance in cognitive tests (Goshen I, Kreisel T, 2007). Although

this has been shown to affect only long term memory (Oprica M, Zhu S, 2005, Spulber S, Mateos L, 2009).

IL-6 has also been identified as an important cytokine in memory formation as an inhibitor of long-term memory formation (Balschun D, Wetzel W, 2004). It has been postulated that IL-6 may form equilibrium with IL-1 β with regard to long term memory formation due to their roles in inducing production of the other (Reyes TM, Coe CL. 1998, Sparkman NL, Buchanan JB 2006).

These two pro-inflammatory cytokines and TNF- α have also been associated with sleep regulation (Opp MR 2005). This hypothesis arose due to variation in IL-1 β and TNF- α throughout the sleep-wake cycle which was believed to be important in the physiological control of sleep (Krueger JM, Fang J, 1998). Variation of these levels would therefore lead to changes in sleep as noted in 'sickness behaviour', which may consequently lead to lethargy. IL-6's role, however, is believed not to be directly regulating sleep (Hogan D, Morrow JD, 2003) but through inducing increased expression of both IL1- β and TNF- α as suggested previously. As can be seen there is a body of evidence suggesting that cytokines are involved in these behavioural events.

2.6.1 Mechanism of spread inflammation from a peripheral source to the central nervous system (CNS)

The CNS is surrounded by the blood brain barrier (BBB), which consists of endothelial cells connected by tight junctions. These capillaries are surrounded

by astrocytes, pericytes and neurones which come together forming the neurovascular unit (Reese TS 1966). The BBB acts as an effective barrier between the periphery and the CNS (Erol A 2008). Therefore inflammation must either pass through or bypass the BBB to enter the CNS. These potential routes are divided into 3 possible mechanisms into the CNS: 1) cytokine interaction with the BBB leading to inflammation spread; 2) cytokine interaction leading to leucocyte migration; 3) stimulation of neural afferents at the site of local inflammation to induce an inflammatory reaction in the CNS (Figure 2.5 and table 2.1).

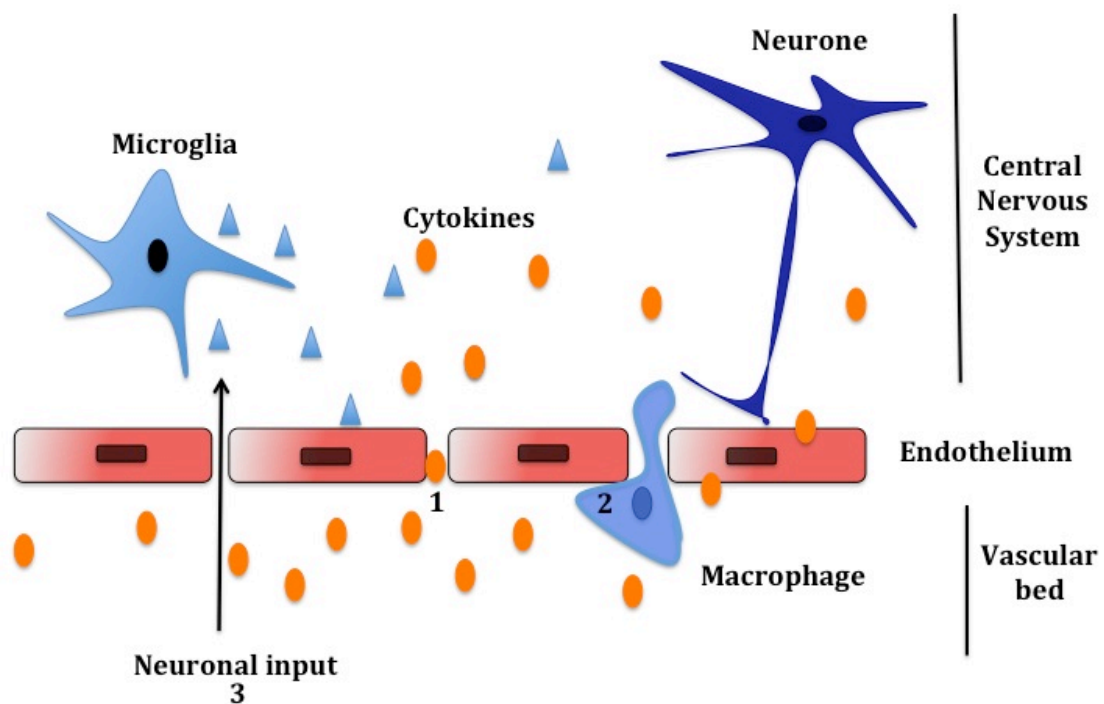


Figure 2.5: Schematic representation of the blood brain barrier (BBB) to illustrate peripheral to central nervous system communication: 1) cytokine interaction with the endothelium, 2) cellular migration and 3) Neuronal input bypassing the BBB.

Table 2.1: Summary of mechanisms associated with spread of inflammation from the periphery to the central nervous system.

Inflammation of the blood brain barrier	Mechanisms
Cytokines interaction with BBB	IL-1 β , TNF α
	Von Willebrand factor
	Toll-Like Receptors
	NF κ B pathways
BBB disruption and leukocyte migration	Activation of GTPases
	Phosphorylation of myosin light chain
	Decreased expression of ICAM-1
	Decreased expression of VCAM-1
	Decreased expression of E-Selectin
	Increased NO
	Increased MMP
	Increased calcium concentration
	Increased bradykinin activity
Stimulation of nerve afferent	Vagal stimulation
	Glossopharyngeal stimulation
	Cutaneous nerve stimulation
	Increased complement activity
	Increased PGE ₂ expression
	Increased cFOS expression

ICAM-1= Intracellular adhesion molecule-1. VCAM-1= Vascular adhesion molecule-1. NO= Nitric oxide. MMP= Matrix metalloproteinase. GTPases= Guanosine triphosphate hydrolyse enzymes. NF κ B= Nuclear factor kappa B. PGE₂= Prostaglandin E₂. cFOS= Cellular proto-oncogene.

2.6.1 Cytokine interactions with the BBB

Cytokines can cross the BBB through specific transport systems. This was initially demonstrated by an elegant study showing that uptake of radio-labelled

IL-1 α and IL-1 β cytokines was self-limiting and reduced by co-administration of non-labelled cytokines (Banks WA 1991). This evidence suggested that these two members of the IL-1 cytokine family may use the same transport mechanism to access the brain. However, this is unlikely to be a route of inflammatory spread into the CNS due to the small amount, only 0.07% of the administered IL-1 β being taken up (Banks WA 1991).

Receptor binding, however, may have a role in spread of inflammation through the BBB. Knockout of the both TNF receptors on the BBB has been demonstrated to abolish uptake of the TNF- α cytokine (Pan W 2002). An alternative role of the TNF- α receptors is in mediating activation of leukocyte entry into the CNS (D'Mello C 2009). The cytokine IL-1 β utilises the IL-1 receptor type 1 (IL1R1) which is co-localised with the general endothelial cell marker, von Willebrand Factor (Konsman JP 2004). Knockdown of these receptors prevented a CNS inflammatory reaction in response to both intravenous and intra-cerebroventricular administration of IL-1 β , highlighting its importance (Ching S 2007).

An alternative receptor that has been suggested is the toll-like receptor (TLR). These receptors can be triggered in areas of the CNS without the BBB. This stimulation can trigger expression of a plethora of pro-inflammatory cytokines by activation of the NF κ B pathway (Mukaida N 1996, Yang RB 1998). This pathway of inflammatory spread into the CNS was explored following lipopolysaccharide (LPS) challenge two peaks of IL-1 β mRNA levels were found within the brain at 2 and 12 hours after challenge (Quan N 1998).

The proposed mechanism is the binding of LPS to the Toll-like receptor-4 (TLR4) and stimulating IL-1 β production, this is then propagated by the IL-1 β binding to microglia further within the brain parenchyma brain (Spranger J 2006, Verma S 2006). The IL-1 β signal can also be passed through the stimulation of local neurones associating different areas of the brain including the amygdala (Silverman AJ 1981), hippocampus (Ciriello J 1981) and hypothalamus (Silverman AJ 1981, McKinley MJ 1994, Larsen P 1995). These neurones can also be stimulated by PGE₂ (Lazarus M 2007), which is produced following activation of the NF κ B pathway and cyclooxygenase-2 (COX-2) expression (Laflamme N 1999).

2.6.2 Mechanisms of increased BBB permeability and leukocyte migration across the BBB

Prostaglandins produced in response to cytokine stimulation also have a role in regulating the permeability of the BBB. They activate the Rho family of GTPases causing phosphorylation of myosin phosphatase which targets the subunit Rho-associated kinase (ROCK) (Schmeck B 2003). This leads to myosin light chain (MLC) phosphorylation and so actin-myosin contraction in the cells (Patil SB 2006), responsible for focal contraction of endothelial cells, which weakens the interaction between cytoskeleton and junctional proteins, thus increasing endothelial permeability. This has been demonstrated through the diminished rate of monocyte transmigration following endothelial Rho inhibition (Strey A 2002). An alternative mechanism of Rho inhibition is through the reduction in

expression of adhesion molecules: intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Wojciak-Stothard B 1999). It has also been postulated that reduced endothelial Rho may lead to reduced phosphorylation of tight junction proteins claudin-5 and occludin leading to increased barrier permeability (Yamamoto M 2008).

Nitric oxide (NO) can also increase the BBB permeability. It stimulates phosphorylation of MLC resulting in cellular contraction and tight junction changes (Haorah J 2005). That matrix metalloproteinases (MMP) are activated via the redox-linked signal transduction pathways has also been demonstrated (Haorah J 2007). These MMPs are able to degrade the vascular extracellular matrix and brain microvessels leading to leucocyte infiltration and microglial activation (Rosell A 2006, Rosenberg GA 2007).

Protein-kinase-C (PKC) is one of the most studied regulators of the endothelial tight junction and tight junction mediated permeability. It has been found to have a variety of mechanisms affecting different barrier involved structures. It can stimulate occludin phosphorylation (Harhaj NS 2006) leading to breakdown of the vascular endothelial cadherin junctions (Sandoval R 2001). PKC is also involved in the Rho family of GTPases and the NO pathway as previously discussed. It may also work by enhancing the action of Rho and NO by phosphorylating MLCK and so trigger increased actin-myosin motor function (Stasek JE,Jr 1992). The activation of the NO pathway is also possible through Akt/PI3K pathway. This causes phosphorylation of eNOS and an increase in the production of NO by several folds (Fulton D 1999, Dimmeler S 1999). The

Akt/PI3K pathway also reduces the expression of the claudin-5 protein, found in tight junctions, after treatment with the HIV-1 Tat protein. Furthermore, heat shock protein-72 (Hsp72) an intracellular chaperone prevents the extravasation of macromolecules from the systemic circulation into the CNS by preserving the protein occludin at the level of the endothelium tight junctions (Lu, Chen et al. 2004).

Contraction of endothelial cells leading to increased permeability may be due to increases in intracellular Ca^{2+} concentration (Ma TY 2000). This change in Ca^{2+} concentration is due to a Src-dependent mechanism and induction of TRPC1 channel expression (Tiruppathi C, Naqvi T 2001). An alternative mechanism of Ca^{2+} mediated permeability is through the induction of nitric oxide synthase (NOS) increasing NO production following bradykinin stimulation (Sarker MH 2002). Conversely, extremely low extracellular Ca^{2+} concentration is detrimental to BBB integrity due to its ability to cause loss of tight junction proteins (Ma TY 2000, Bowman PD 1983).

2.6.3 Neural pathway bypassing the BBB

The neural pathway has previously been described as a connecting pathway between different parts of the brain. It has also been postulated as a pathway for the inflammatory signal from the periphery to the CNS (Blalock JE 1985). Studies into the role of the vagus nerve have demonstrated cFOS expression throughout the brain after intraperitoneal LPS challenge (Wan W, Wetmore L 1994). This effect was suppressed following sub-diaphragmatic vagotomy

(Bluthe RM 1994, Watkins LR 1995). Other nerves involved in this reaction include both the glossopharyngeal and the cutaneous nerves linking the soft palate to the CNS (Romeo HE, Tio DL 2001, Turnbull AV 1997). This response by cutaneous nerves was suppressed by local anaesthetic administration highlighting the importance of sensory nerves (Ross G, Roth J 2000).

The exact role of nerves in inflammatory signal transmission remains unclear as other investigators have found vagotomy ineffective at low, medium and high doses of LPS (Hansen MK, 2001, Luheshi GN 2000). These studies, however, discount the role and effects of the BBB-dependent pathways linking the periphery to the CNS which is effective following intraperitoneal (i.p.) or intravenous (i.v.) administration of the LPS or IL-1 β (Quan N 2008). It is therefore important to direct the search for effects towards the nerve terminal fields within the brain separating the BBB-dependent and independent pathways (Quan N 2008). Strong evidence for the supporting a role for the neural pathway comes from a study where the administration of live bacteria led to fever induction (Campisi J 2003) and c-FOS expression within the brain (Goehler LE 2005) which occurred before the upregulation of cytokines either in the serum or the brain.

One possible mechanism for stimulation of local nerves involves the rapid release of PGE₂ and activation of complement proteins (Blatteis CM, Li S, 2004). This local release of PGE₂ has been demonstrated to stimulate local afferent vagal nerves leading to signalling in the preoptic area of the hypothalamus (Blatteis CM 2006). This reaction could also be inhibited by administration of a

cyclooxygenase inhibitor (Rummel C, Barth SW, 2005). PGE₂, however, is not the only mediator able to stimulate local nerves, IL-1 family of cytokines are also believed to be important (Miller AJ, Hopkins SJ, 1997).

2.7 Neuroinflammation and surgery

Surgery may cause remote injury including neuroinflammation. Surgical trauma activates the peripheral innate immune system and results in stress-related neuroendocrine system changes, such as increased levels of cortisol, decreased levels of thyroid hormone and release of cytokines (Rasmussen, O'Brien et al. 2005). The noted changes in hormone levels and the release of inflammatory mediators (such as cytokines, nitric oxide, arachidonic acid metabolites, endothelins and oxygen free radicals) (Lin, Calvano et al. 2000; Giannoudis, Dinopoulos et al. 2006; Karlidag, Unal et al. 2006) can result in further inflammatory changes. It is possible that reduction in neurotransmitter concentrations in the brain might induce postoperative memory dysfunction (Stoudemire, Anfinson et al. 1996; Bekker and Weeks 2003; Bekker and Weeks 2003; Wan, Xu et al. 2007).

The peripheral cytokines that are produced in the perioperative period of non-neurological surgery can exert their role in the CNS via both blood and neural pathways and induce further pro-inflammatory cytokine production by glial cells mainly in the hippocampus (Nguyen, Deak et al. 1998; Godbout, Chen et al. 2005). The hippocampal inflammation is demonstrated by local increase in expression of pro-inflammatory cytokines, such as IL-1 β , IL-6, TNF- α and reactive microgliosis that results in behavioural impairment. Elevated levels of

pro-inflammatory cytokines can impair both production of acetylcholine and synaptic plasticity in the dentate gyrus (DG) (Cunningham, Murray et al. 1996) and cornu ammonis (CA) regions (Bellinger, Madamba et al. 1993; Hellstrom, Danik et al. 2005) of the hippocampus, affecting hippocampal dependent memory and learning (Tancredi, D'Antuono et al. 2000; Tanaka, Ide et al. 2006).

TNF- α initiates peripheral inflammation that facilitates its entry to the brain through the BBB and promotes further neuroinflammation (Terrando, Monaco et al. 2010).

It is still questionable whether the observed glial reactivity is the cause or the effect of increased cytokine expression. Yet, the increased expression of IL-1 β and IL-6 in the hippocampus suggests a role for the local cellular population in the production and release of cytokines. The locally activated microglia secrete pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α), excitotoxins (glutamate), neurotoxins, amyloid precursor protein (APP) and reactive oxygen species (ROS), inducing the molecular changes responsible for cognitive impairment (Vereker, Campbell et al. 2000; van Rossum and Hanisch 2004; Griffin, Nally et al. 2006).

Amongst the pro-inflammatory cytokines, IL-1 β seems to play a pivotal role in the neuronal mechanisms associated with fear-related memory dysfunction and long-term neuronal plasticity through both direct and indirect (microglia activation) routes whereas IL-6 seems to facilitate IL-1 β effects (Ban, Haour et al.

1992; O'Connor, Johnson et al. 2003; Wieczorek, Swiergiel et al. 2005; Rosczyk, Sparkman et al. 2008; Cibelli, Fidalgo et al. 2010).

There is further evidence which supports surgery-mediated neuro-inflammation. The production of pro-inflammatory cytokines may exert a lasting effect on cognition after non-neurological surgery and it may be responsible for the pathogenesis of Alzheimer disease (AD) (Nguyen, Deak et al. 1998; Wan, Xu et al. 2007; Wan, Xu et al. 2010). The identification of brain damage and pre-existing neurodegeneration are also thought to be critical factors for delirium (Rudolph, Jones et al. 2007; Young and Inouye 2007).

In summary: Assimilating all this information provides a cogent argument that inflammation might be linked to cognitive decline. Taken together, current data suggests an association between non-neurological surgery and systemic inflammation with cognitive decline demonstrated by inflammation-induced impairment of hippocampal dependent memory (Giulian, Woodward et al. 1988; Wan, Xu et al. 2007; Rosczyk, Sparkman et al. 2008). This neuro-inflammatory process seems to be a promising target for future therapeutic interventions with great potential to ameliorate postoperative side-effects in the surgical population (Cibelli, Fidalgo et al. 2010).

2.8 Surgery and postoperative cognitive dysfunction (POCD)

POCD is a common complication following cardiac and non-neurosurgical procedures. It is associated with memory loss or other forms of cognitive dysfunction lasting for short (delirium) or long (dementia) periods of time.

2.8.1 Epidemiology

POCD is well defined, simple to diagnose and affects 5-62% of patients undergoing major surgery (Young and Inouye 2007; Deiner and Silverstein 2009); the percentage is significantly higher following cardiac or hip fracture surgery (Siegel, Agranoff et al. 1999).

In the immediate postoperative period the incidence of POCD has been reported to be as high as 80% (Arrowsmith, Grocott et al. 2000). Although reported as being transient, a review by Bekker and Weeks suggests that some patients may experience long-term dysfunction and this dysfunction may even become permanent (Bekker and Weeks 2003).

Newman and colleagues reevaluated patients 5 years after surgery and found that 42% showed decline in cognitive function from preoperative baseline assessment and decline in cognitive function in the immediate postoperative period was predictive of long-term decline (Newman, Grocott et al. 2001). The incidence of POCD is particularly prevalent in patients over 60 years of age (Mathew, Grocott et al. 2003). Studies suggest that patients who develop POCD may be at higher risk for cognitive decline or dementia later in life (Selnes, Royall et al. 2001).

Over the past 10 years, the reported incidence of POCD has varied widely ranging from 10% to 57% of adult patients 3 to 6 months after surgery (Moller, Cluitmans et al. 1998; Keith, Puente et al. 2002; McKhann, Grega et al. 2005). It is thought that this variation in reported incidence can be attributed to

methodological limitations, multifarious tests used to perform cognitive evaluation, disparate criteria to define cognitive impairment, variable follow-up intervals, and diverse patient populations (Murkin, Newman et al. 1995; Mahanna, Blumenthal et al. 1996; Symes, Maruff et al. 2000). Of note, the majority of studies to date have excluded patients with underlying cognitive disorders, psychiatric disorders or disorders of the central nervous system, and urgent or emergency surgical patients. These studies have also excluded patients who are taking psychotropic medications. While such stringent exclusion criterion avoids several confounding variables, they reduce the sample to a subpopulation of patients with limited generalisability (Ancelin, de Roquefeuil et al. 2001), hence underestimating the overall incidence of POCD.

Patients undergoing cardiac surgery are reported to have a higher incidence of cognitive dysfunction than any other major surgical procedures (Shaw, Bates et al. 1987; Gao, Taha et al. 2005).

POCD is traditionally associated with cardiac bypass surgery (found to affect 53% of patients at discharge and 24% at 6 months) (Newman, Kirchner et al. 2001) but it has also been reported following major non-cardiac surgery affecting 10 to 27% of patients in 1 week to three months after anaesthesia and surgery (Moller, Cluitmans et al. 1998)(Figure 2.6).

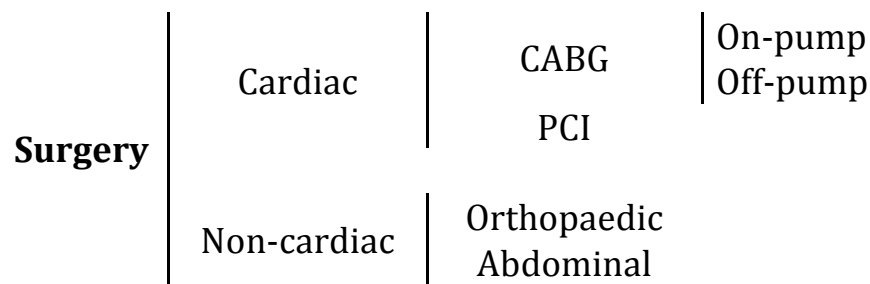


Figure 2.6: This flowchart attempts to simplify the commonest surgeries described in the literature associated with the development of POCD. CABG: Coronary Artery Bypass Graft. PCI: Percutaneous Coronary Intervention. On/Off-pump: Allude to cardiopulmonary extracorporeal circulation.

2.8.2 POCD and cardiac surgery

There are two common interventional procedures for cardiac patients: 1) non-surgical procedure or percutaneous coronary intervention (PCI) and 2) surgical intervention or coronary artery bypass grafting (CABG).

2.8.2.a PCI is performed under local anaesthesia and in some cases light sedation is provided. Anticoagulation may be used intravenously during the angioplasty to prevent blood clotting and thrombosis. PCI takes between 1 and 2h normally. Most patients stay overnight in the hospital for observation, and resume normal activities within a week. Transient cognitive changes have been reported up to one-year post intervention (Sweet, Finnin et al. 2008).

2.8.2.b CABG is performed under general anaesthesia. CABG takes between 1.5 to 4.0h normally. The typical hospital stay ranges from 4 to 7 days and a full recovery from the surgery may require 3 months. CABG procedure is performed

with or without an extracorporeal circulation also reported as on-pump or off-pump CABG to support the end organ function while the surgeons intervene the heart. POCD has been reported up to 5 years post-surgery (Newman, Kirchner et al. 2001; van Dijk, Dieleman et al. 2007).

Interventions on the heart have been modified over the years to reduce complications, length of stay in hospital and improve patient's outcome. However, it seems cognitive changes after cardiac interventions remain a problem in need of a solution to improve patient's quality of life after recovery from major organic insults.

2.8.3 PCI Versus CABG and POCD

A comparison study evaluating POCD and driving performance in post-CABG patients relative to a PCI group determined that significantly more CABG patients (n=27) experienced POCD, which negatively impacted traffic behaviour, but the PCI group (n=20) deteriorated in maneuvering the vehicle (Ahlgren, Lundqvist et al. 2003). Additionally, patients from both groups who experienced POCD also deteriorated in on the- road driving scores, relative to patients without evidence of cognitive decline indicating that the hippocampus spatial memory is affected.

2.8.4 Off-Pump CABG Versus On-Pump CABG and POCD

There is inconclusive evidence regarding the difference in POCD between off-pump CABG versus on-pump CABG. Some studies found that off-pump CABG was associated with decreased POCD at 5 days and 3 months post-operatively (n=35) (Murkin, Boyd et al. 1999), and at 6 months after surgery (n=35) (Stroobant, Van Nooten et al. 2002). One study assessing POCD following off-pump CABG (n=54),

claimed an improvement in the areas of language, memory, and attention when compared to on-pump CABG on day 3 after surgery (Sendelbach, Lindquist et al. 2005).

However, several large, randomized studies showed no difference in POCD between off-pump CABG and on-pump CABG (Taggart, Browne et al. 1999; McKhann, Grega et al. 2005; Jensen, Rasmussen et al. 2008).

2.8.5 Predictive risk factors

Several studies have looked for predictors of POCD following CABG surgery. In 448 CABG patients, normothermia, impaired left ventricular function, elevated preoperative creatinine, prolonged intensive care unit stay, and higher educational level predicted POCD occurrence (Boodhwani, Rubens et al. 2006). Patient age and heparin concentrations were associated with POCD immediately prior to discharge post-CABG (n=55) (Raymond, Radcliff et al. 2007). Additionally, preoperative depression, anxiety, and stress symptomatology predicted POCD (n=147) using measures of attention and memory (Andrew, Baker et al. 2000).

Nonetheless, it remains contentious whether patients with coronary artery disease experience deterioration in cognitive functioning after undergoing cardiac surgery. There are several large studies, where more than 50% of people demonstrated neurocognitive decline following cardiac surgery (Newman, Grocott et al. 2001; McKhann, Grega et al. 2005) with cognitive dysfunction persisting in nearly half of the individuals at 5 years post-surgery (Newman,

Grocott et al. 2001). Specific deficits in the area of verbal learning and memory have been demonstrated postoperatively, which persisted at three months (Knipp, Matatko et al. 2005) and six months (Chernov, Efimova et al. 2004) post-surgery.

In contrast, several studies determined cognitive improvement following cardiac surgery, with positive results specific to the same area of verbal memory (Sendelbach, Lindquist et al. 2005; Dupuis, Kennedy et al. 2006).

Alternative explanations for deterioration in cognitive functioning following cardiac surgery include neurocognitive decline as a natural progression for individuals with advanced coronary artery disease, or cognitive decline as generally associated with an elderly population. A lack of appropriate control groups such as patients with coronary artery disease not undergoing cardiac surgery and healthy elderly controls have made it difficult to establish baseline impairment, which may have contributed to inconsistencies in the literature.

In summary, discrepancies exist within the current literature as to whether POCD is an inherent complication of CABG. Alternative explanations relate to cognitive decline as a natural progression within the population of individuals with advanced atherosclerotic disease, or cognitive decline as generally associated with an elderly population. A lack of appropriate control groups, such as coronary artery disease (CAD) patients not undergoing CABG surgery and healthy controls, has made it difficult to establish the incidence and degree of

baseline, pre-surgical impairment and may have contributed to inconsistencies within the literature.

2.9 POCD following non-cardiac surgery

POCD is also well described following non-cardiac surgery and again multiple factors have been linked to memory changes in the postoperative period. There are three important studies looking at POCD in patients having non-cardiac surgery.

In the International Study of Postoperative Cognitive Dysfunction-1 (ISPOCD1), Moller and Colleagues investigated the neurological outcomes of patients (n=1,218) undergoing major non-cardiac surgery (Moller, Cluitmans et al. 1998). As noted previously neuropsychological surveillance was conducted preoperatively and on postoperative week 1 and week 12. They also found a significant relationship between both postoperative infectious complications ($P=0.04$) and postoperative respiratory complications ($P<0.05$) and POCD.

Rosenberg and Kehlet conducted a randomized study comparing patients undergoing major abdominal surgery (n=30) matched with patients undergoing minor ear surgery (n=10) to investigate the role of postoperative hypoxia on POCD. Oxygen saturation (SpO₂) was monitored in the preoperative and postoperative periods by pulse oximetry (Rosenberg and Kehlet 1993). Neuropsychological evaluation was conducted preoperatively and on day 3 and day 7 in the postoperative period. The patients in the control group did not experience any hypoxic episodes whereas the patients in the major surgery

group experienced several hypoxic episodes on postoperative day 2 ($P<0.05$). Cognitive decline was significant on postoperative day 3 in the major surgery group ($P<0.05$) and multiple regressions revealed a significant relationship ($P<0.05$) between postoperative SpO₂ and cognitive decline.

Wang *et al* conducted a prospective cohort study (n=225) of patients aged 65 and greater that underwent non-cardiac surgery investigating the association of pain and postoperative analgesia with POCD (Wang, Sands et al. 2007). They reported that only postoperative analgesia administered via patient controlled anaesthesia (PCA) was associated with POCD ($P=0.02$). It is evident from the substantive published studies that the predictors, risk factors, and etiology of POCD are complex and multifarious. While studies focused on decreasing the incidence of POCD are of great importance, intervention studies designed to enhance cognitive function in the postoperative period after non-cardiac surgery are also necessary to promote improvement in patient outcomes.

2.10 Risk Factors associated with the development of POCD

There are several risk factors associated with the development of early and long lasting memory decline after surgery. A common denominator seems to be the stress response to surgery, but anaesthesia and pain will also precipitate a cascade of events resulting in memory decline (Figure 2.7).

Risk Factors for POCD

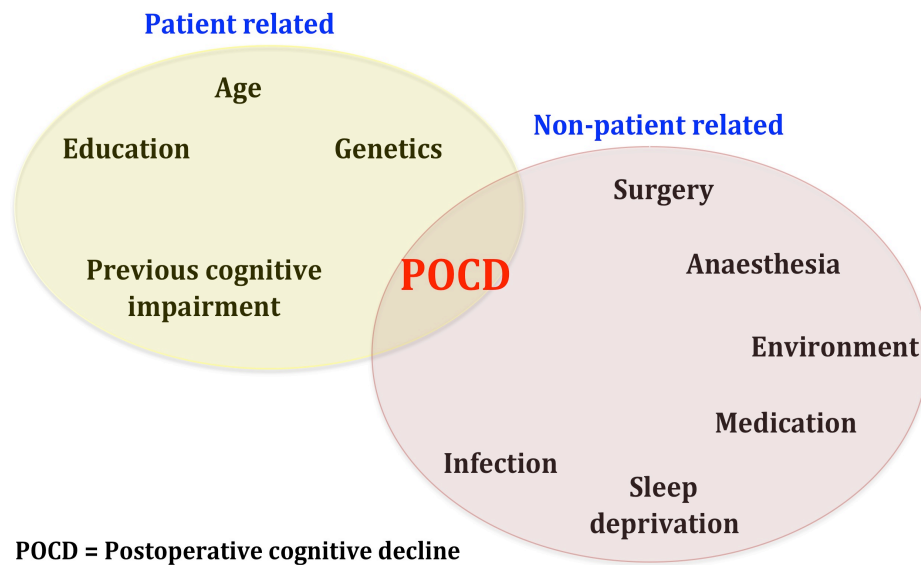


Figure 2.7: Schematic illustration of risk factors associated with the development of POCD.

2.10.1 Patient's related risk factors for the development of POCD

2.10.1.a Age

Age is the only pronominal factor for late POCD, highlighting the role of age in cognitive decline. Both the very young and the very old are vulnerable for POCD development, presumably since brain reserve capacity is reduced. The threshold theory would explain why elderly patients are more vulnerable to cognitive deterioration postoperatively (Bekker and Weeks 2003). According to the threshold theory the reaction to a stressful event depends on an individual's brain reserve capacity. Thus if an individual has necessary neuronal networks and therefore greater reserve capacity a stressful event will remain subthreshold whereas an individual with diminished reserve capacity will be more vulnerable

and probably unable to function normally after the insult (Satz, Morgenstern et al. 1993).

The very nature of the elderly patient supports the threshold theory as they generally show pre-existing neurodegeneration, lower body metabolism, decreased ability to preserve normal homeostasis and increased risk of myocardial ischaemia and chronic diseases; that is they exhibit a number of risk factors that added to environmental precipitants make them more prone to cognitive decline (Moller, Cluitmans et al. 1998; Young and Inouye 2007).

As people age, many individuals develop health problems that require surgery. Additionally, because of ageing, pharmacological agents administered during surgery can have an altered effect (Monk, Weldon et al. 2008). For instance, a pharmacokinetic consequence of age related changes in renal function is prolongation of the elimination half time of anaesthetic drugs and metabolites requiring renal clearance (Peduto, Peli et al. 1998; Bleeker, Vree et al. 2008).

The ageing process has been shown to be an independent risk factor for the development of POCD. When we look at the ageing person, we may see wrinkles and greying hair. What we do not see are the organs changes inside the body. The "oxidative stress theory" states that free radicals cause damage to deoxyribonucleic acid (DNA), which eventually leads to the production of abnormal proteins (Golden, Hinerfeld et al. 2002). The oxidative stress theory has become increasingly accepted as playing a role in the ageing process, based primarily on a substantial accumulation of circumstantial evidence, albeit a

substantial quantity. In recent years, the hypothesis that mitochondrially-generated ROS play a role in ageing has been tested in both invertebrate and mammalian model systems. In mammalian model systems, the effect of oxidative stress on lifespan is less clear, but there is some evidence that antioxidant treatment protects against age-related dysfunction, including cognitive decline (Golden, Hinerfeld et al. 2002).

2.10.1.b Genetics

Genome-wide association studies (GWAS) have demonstrated a relation between certain genotypes and propensity for cognitive decline and dementia. Variant forms of the apolipoprotein E (APOE) gene that encodes the APOE protein responsible for repair of neuronal injury make patients more susceptible to develop POCD (Lelis, Krieger et al. 2006). APOE is a protein essential for the normal metabolisms of triglyceride-rich protein constituents. The APOE was primarily studied for its role in lipoprotein metabolism and in cardiovascular diseases but more recently research is focused on its role in the nervous system where APOE is shown to have a role in myelin and neuronal membrane maintenance during development or after surgery (Boyles, Zoellner et al. 1989; LeBlanc and Poduslo 1990). Grupe *et al* and Coon *et al* were the first to confirm a role of the APOE gene as a major susceptibility gene for Late Onset Alzheimer's Disease (LOAD) (Coon, Myers et al. 2007; Grupe, Abraham et al. 2007). Alzheimer's disease is characterised by the aggregate formation of amyloid beta (A β) peptide. APOE enhances proteolytic cleavage of this peptide within and between cells. Some APOE isoforms (such as APOE- ϵ 4) are not so effective and

the individuals that carry them exhibit increased vulnerability to AD and other forms of cognitive decline (Dik, Jonker et al. 2000; Jiang, Lee et al. 2008).

As a consequence several studies have tried to demonstrate a relationship between APOE ϵ 4 allele and the occurrence of cognitive decline. The results so far are conflicting for POCD (Steed, Kong et al. 2001; Abildstrom, Christiansen et al. 2004; Askar, Cetin et al. 2005; Lelis, Krieger et al. 2006) but a relationship between ApoE ϵ 4 allele and the occurrence of delirium has been established (Leung, Sands et al. 2007). If delirium and POCD are similar phenomena this could suggest that postoperative cognitive decline has a genetic predisposition (Leung, Sands et al. 2007). This is supported by findings suggesting that isoflurane anaesthesia may be safe for normal animals but potentially harmful for mice with genetic risk factors for AD (Perucho, Rubio et al. 2010).

2.10.1.c Cognitive state of individual before surgery

There is a large variability on individual memory throughout life which is accentuated in the ageing individual. There are individual differences in how people process tasks which may be a factor that allows some of them to cope better with brain pathology than others. This concept led to the development of the brain reserve theory and the concept of a passive threshold model. It seems that preservation of memory in old age is determined by the levels of performance prior to the onset of senescent decline rather than the magnitude of the decline itself so, that when the brain resources fall below a given, or predetermined threshold the brain abnormalities may manifest as behaviour or cognitive disorders. The higher the level initially the greater the decline needed

to reach the threshold where disorders begin to be seen. It has been found that a high level of education seems to protect the individual from POCD so that education appears to pre condition the brain and make it more resilient to withstand injury (Langa, Larson et al. 2008). This could be comparative to the way that physical exercise protects the body against injury (Monk, Weldon et al. 2008). It has also been hypothesised that people who have more education may have developed different brain circuitry that facilitates them continuing to function at a high level (Langa, Larson et al. 2008).

The psychological well being of an elderly person prior to surgery is another variable that may contribute to the development of POCD. If an individual is depressed, the immune system is functioning at a slower level, the neuroconnectivity in the hippocampus is affected leading to a reduction in hippocampal volume having a direct impact on memory (Sachdev, Lipnicki et al. 2012). Depression in older adults has been associated with significant cognitive impairment particularly in domains of attention and cognitive control (Schneider and Lichtenberg 2011). It has also been shown that persistent depressive symptoms in patients requiring cardiac surgery significantly affected their global cognition, executive function and verbal fluency at 30 months after the intervention (Freiheit, Hogan et al. 2012). The lack of energy, which accompanies depression, contributes to a lengthier healing period, prolonged stay in hospital as shown in burns patients with history of depression and psychological distress (Wisely, Wilson et al. 2010). Pre-existing psychiatric diagnosis and in hospital psychological distress may not only impact on psychosocial, but also physical

aspects of recovery of an individual as well as contributing to the long term cognitive decline (Laxton and Perrin 2003; Wisely, Wilson et al. 2010).

2.10.1.d Alzheimer's disease (AD)

An association between AD and neuro-inflammation was established in early post-mortem brain studies of Alzheimer's sufferers (Uchihara T 1997), using magnetic resonance imaging (MRI) and positron emitting tomography (PET) imaging (Cagnin A 2001). These studies all demonstrated microglial infiltration into the brain (Uchihara T, 1997 Cagnin A). Using the in vivo marker of activated microglia [^{11}C](R)-PK11195, activated microglia were found in the inferior and middle temporal gyrus, fusiform gyrus, left parahippocampal gyrus, left amygdala, left posterior cingulate and inferior parietal lobules (Cagnin A 2001). It has also been shown that the inflammatory reaction within the brain can be exacerbated by systemic inflammatory events (SIE) such as infection (Perry VH 2003). This is supported by the association of the increased decline in cognitive function seen in AD sufferers following surgery and is also associated with an increased baseline level of TNF- α (Holmes C 2009). It has been suggested that the relevance of TNF- α is its ability to up-regulate matrix metalloproteinases (MMPs) as well as the increase in the expression of cellular adhesion molecules such as ICAM1, VCAM1 (Zeni P 2007) and COX-2. These changes facilitate an increased migration of monocytes from the periphery which in turn leads to β -amyloid protein deposition within the brain (Lee JW 2008). Systemic inflammation may also have a role in the pathogenesis of AD. This was demonstrated in a 'twins' study. A twin who had experienced a serious systemic

infection tended to develop AD at an earlier age than their counterpart who had not (Nee LE 1999).

2.10.2 Non-patient related risk factors for development of POCD

2.10.2.a Surgery

Surgery, as opposed to the combination of surgery and anaesthesia, is considered a major risk factor for development of POCD. Specific types of operation, such as cardiac surgery, are well documented as associated with, and probably contributing to, POCD. In particular the development of cognitive changes after surgery. These neurocognitive changes were initially attributed to the effects of the cardiopulmonary bypass on the brain and were assessed through neurological and neuropsychological tests (Savageau, Stanton et al. 1982b; Shaw, Bates et al. 1987; Townes, Bashein et al. 1989; Newman, Kirchner et al. 2001; Newman, Kirchner et al. 2001). More recent studies could demonstrate no differences in neurological outcomes after on- or off-pump surgeries. Furthermore in the late 90s non-cardiac surgeries were also implicated in the development of POCD. Amongst these the commonest operations associated with a high incidence of POCD were orthopaedic surgery especially hip and knee replacement. Orthopaedic surgeries were reported to have an incidence of 75% at discharge from hospital and 45% after 3 months of the surgical procedure (Koch, Forteza et al. 2007). Major abdominal surgeries, gynecological procedures (Chiang, Gerten et al. 2009) and renal surgery including renal transplant (Harciaiek, Biedunkiewicz et al. 2009) have all been associated with the development of POCD.

2.10.2.b Length of surgery

The length of surgery is another risk factor for development of memory changes in the postoperative period. Surgeries lasting more than 90 minutes carry a very high risk of POCD (Monk, Weldon et al. 2008). In this, surgery and anaesthesia cannot be dissociated but the duration of surgery is definitely implicated and it seems independent of age. Young, middle-aged and elderly patients undergoing abdominal, thoracic and orthopaedic surgery all developed detectable POCD after being under general anaesthesia for more than 100 minutes. The same authors showed that a proportion of patients from each of the 3 groups had POCD at 3 months post discharge from hospital (5.7, 5.6 and 12.7% respectively). An obvious confounder is that longer or prolonged surgical times are in general due to more extensive procedures which, may be associated with a higher incidence of stress and pain, which in turn may trigger a cascade of events described as a stress response. This then contributes to an inflammatory response which could lead to neuroplasticity disruptions and poor outcome (Feldt and Oh 2000; Ganai, Lee et al. 2007).

2.10.2.c Anaesthesia

Anaesthesia is probably a contributor to POCD but it is important to look at specifics of the anaesthesia. The types of anaesthetic, the pharmacology and the duration of time the individual is under anaesthesia might all be associated with POCD. The lingering effects of anaesthesia, sequelae, may contribute to the development of POCD.

Anaesthesia has been implicated in the neurocognitive changes in the postoperative period. The effects of anaesthesia on mental decline were based

on observations of prolonged memory deficits after surgical interventions which were considered to be the long-lasting effect of anaesthetic agents on the brain (McMahon, Bolshakov et al. 1996). These effects were longer than the half life of the drug used. This was more marked in the aged brain (McMahon, Bolshakov et al. 1996). These effects were thought to be responsible for the observed deterioration of pre-existing neurodegenerative processes and hence POCD (Rees and Gaines 1985; Moller, Cluitmans et al. 1998; Bone and Rosen 2000).

There are a wide range of anaesthetic techniques and agents and many of them have been implicated in memory disruption. Anaesthetic techniques can be divided into two main groups: general and regional anaesthetic techniques. There are two main type of anaesthetic agents; inhalational and intravenous anaesthetic agents.

Alteration of cognitive dysfunction due to general anaesthesia has been identified and it has been suggested that the cholinergic signalling might be the cause of altered memory processing from dose-dependent anterograde amnesia to changes in neuroplasticity resulting in neuro-disruption and long lasting cognitive impairments.

2.10.2.c.1 General anaesthesia and POCD

2.10.2.c.1.a Inhalational anaesthetics, predominately nitrous oxide and halothane but also isoflurane, have been studied either in isolation or combined, they have been evaluated using neuropsychological and memory tests. This has

corroborated the hypothesis of anaesthesia-induced mental changes (Galindo 1969; Nicoll 1972; Richards and Smaje 1976; Pocock and Richards 1993). They identified several alterations in memory processing. There are dose-dependent effects on anterograde memory. The main effect of inhalational agents seem to be at the postsynaptic membrane with cholinergic neurotransmission and hence signalling which is probably responsible for changes in neuroplasticity (Richards 1983; McMahon, Bolshakov et al. 1996; Anagnostaras, Maren et al. 1999; Dutton, Maurer et al. 2002). This results in neuro-disruption and cognitive impairment.

A secondary negative effect of inhalational anaesthetics is on the circadian rhythm which again it is a contributor on itself for development of POCD (Rasmussen, O'Brien et al. 2005).

More recent studies (Mawhinney, de Rivero Vaccari et al. 2012; Wang, Xu et al. 2012) suggest that anaesthetics contribute to the POCD pathology through both direct and indirect pathways. They can exert direct toxic effects, such as alterations in calcium homeostasis in the neuronal endoplasmic reticulum (ER) (Wei, Kang et al. 2005; Yang, Liang et al. 2008), blockage of *N*-Methyl-D-aspartic acid (NMDA) receptors, activation of gamma-aminobutyric acid (GABA) receptors (Siegel, Agranoff et al. 1999; Young and Inouye 2007). They also suppress neuronal stem cell activation which affects the release of CNS neurotransmitters (acetylcholine, dopamine, norepinephrine). This disrupts normal memory regulation controlled by the cholinergic neurones of the basal forebrain (Blundell 1967; Rudolph, Jones et al. 2007). This may significantly contribute to POCD as well contributing to accelerating pre-existing endogenous

ageing and neurodegenerative processes. They also have indirect actions through systemic inflammatory effects presumed secondary to the surgical trauma (Deiner and Silverstein 2009).

2.10.2.c.1.b Intravenous anaesthetics also exert potential effects. Propofol has been shown to contribute to the development of POCD especially in elderly patients with a similar incidence to sevoflurane at 1 day after surgery (Rohan, Buggy et al. 2005). Interestingly, when propofol is compared to isoflurane it seems that the cholinergic function is better preserved in animals anaesthetised with propofol as less memory impairment has been reported (Wang, Xu et al. 2012). Propofol also preserves the cerebral oxygenation state as assessed by jugular venous saturation but this does not modify the manifestations of POCD (Kadoi, Saito et al. 2003).

2.10.2.c.1.c In summary, inhalational anaesthetic agents have been identified as risk factors for development of POCD and to a lesser extent the intravenous anaesthetic agents. However the inflammatory changes as assessed by levels of IL-6, TNF- α and phosphorylation of tau protein are similar after both types of anaesthesia (Ribot 1887) which suggests an underlying and still unknown mechanism responsible for the development of POCD.

2.10.2.c.2 Regional anaesthesia and POCD

Data on regional anaesthesia and POCD are more controversial (Savage 1887). Perrier et al suggested that preoperative fascia iliaca plexus block for hip

surgery in 65 year old patients or older before general anaesthesia with propofol prevents early POCD when compared to general anaesthesia with propofol following by bolus of morphine for pain relief (Perrier, Julliac et al. 2010). There are no human data showing a benefit from regional anaesthesia for prevention of POCD and so there is a degree of urgency for randomized clinical trials to assess whether there is a difference between regional and general anaesthesia in terms of subsequent long-term memory decline after surgery.

2.10.2.c.3 Co-adjuvant pharmacological agents during anaesthesia and POCD

Co-adjuvant drugs, may also have a role in the development of POCD.

2.10.2.c.3.a Opiates: Intraoperative morphine is an associated risk factor for POCD after hip surgery when compared with fascial iliac compartment block (Perrier, Julliac et al. 2010). Fentanyl has been implicated with early POCD even at low dose although there is no difference between low and high dose of fentanyl in the incidence of POCD at 3 and 12 months after cardiac surgery (Silbert, Scott et al. 2006). There is some evidence suggesting an effect of opiates on the immune system and the pro-inflammatory response after cardiac anaesthesia is more pronounced when fentanyl analgesia is used (Silbert, Scott et al. 2006) and so is the incidence of POCD when compared to ketamine (Hudetz, Patterson et al. 2009). However, the cellular mechanism by which opiates in the acute setting contributes with the development of POCD has not been fully elucidated, yet.

The chronic use of opiates may increase the risk of developing POCD, although memory impairment may not be permanent (Pratico, Quattrone et al. 2005). One issue is that because of the symptoms it may be very difficult or indeed impossible to differentiate from acute withdrawal symptoms from delirium in the early postoperative period.

2.10.2.c.3.b Benzodiazepines: These drugs have a long acting effect when given as a co-adjuvant drug during anaesthesia (Abildstrom, Rasmussen et al. 2000). Rasmussen et al tried and failed using multiregression analysis of patients undergoing non cardiac surgery to demonstrate that benzodiazepines or their metabolites were responsible for POCD in elderly patients (Rasmussen, Christiansen et al. 1999).

2.10.2.c.3.c Psychotropic drugs: Psychotropic medications taken concurrently, seem to result in a more difficult recovery from general anaesthesia (Stockton, Cohen-Mansfield et al. 2000; Laxton and Perrin 2003). This may be due to drug interaction (Laxton and Perrin 2003). Interaction with the pharmacodynamics or kinetic profile may result in minor to moderate psychological e.g. drowsiness and impairment of executive skills including ability to concentrate. The interaction may be harmful or hazardous to the patient as it may counter balance the side effects of psychotropic drugs. Haloperidol and thioridazine for instance has been shown to affect brain activities well after the drug level in plasma is undetectable after six weeks of administration when compare to lorazepam while levels of prolactin remained higher in the haloperidol and thioridazine groups (Cohen-Mansfield, Taylor et al. 2000) and this may be another factor for

accelerating the manifestations of cognitive dysfunctions after surgery in elderly patients.

A mechanism that has been suggested is illustrated by the selective serotonin reuptake inhibitors (antidepressants) which interact with anaesthetic drugs by inhibiting the cytochrome P450 (Stevens and Wrighton 1993). This interaction may result in an increase in plasma concentration of drugs metabolized by this enzyme which includes benzodiazepines, thiopentone and neuromuscular agents. An alternative mechanism is the interaction with opioids which may result in serotonergic syndrome (Mills 1997), which can be mistaken with acute onset of delirium or early POCD.

Tricyclic antidepressants like amitriptyline have a multimodal mechanism of action that goes from inhibition of cholinergic pathways to enhancement of noradrenergic and serotonergic pathways (Butler and Meegan 2008). These modes of action increase the risk of prolonged sedation after anaesthesia and hence early POCD.

Monoamine oxidase inhibitors (MOIs) act by inhibition of the metabolic breakdown of norepinephrine and serotonin by the MAO enzyme thereby, increasing the level of serotonin and norepinephrine at the receptor side. The effect of MAOIs may be exacerbated by the concomitant use of isoflurane anaesthesia which has been associated with an increase in DA at presynaptic level and hence enhance the effect of MAOIs (Adachi, Yamada et al. 2005). MAOs trigger an excitatory CNS reaction attributable to a central serotonergic over

activity or serotonergic syndrome (Mills 1997) therefore pethidine and dextromethorphan need to be avoided as they may contribute to a development of a serotonergic syndrome which could easily be mistaken with a hyperactive delirious status. A second type of reaction caused by MAOs is a depression of the CNS due to inhibition of the hepatic microsomal enzymes leading to an accumulation of opiates and their metabolites (Stack, Rogers et al. 1988) and also interfering with the normal brain neuroconnectivity resulting in attention deficits and decrease in level of alertness that can be mistaken for early signs of POCD.

As most of the co-adjuvant drugs in anaesthesia carry an intrinsic risk for development POCD it is often advised that surgical patients should refrain from taking these types of drugs at least 24 hours prior to surgery (Pratico, Quattrone et al. 2005). This strategy may carry a risk of withdrawal and psychiatric recurrence or relapse so that the differential diagnosis of memory impairment is a relapse or exacerbation of the neuro-psychiatric disease.

2.10.2.d Sleep deprivation

Disrupted sleep has been considered a predisposing factor for developing neurocognitive dysfunction and psychiatric disorders such as depression (Roman, Hagewoud et al. 2006). It is associated with stress. Sleep disorders are recognised risk factors for both POCD and exacerbation of confusional status in patients suffering from dementia (Cohen-Mansfield, Garfinkel et al. 2000). Disruption in sleep-wakefulness following gastrointestinal surgery under general anaesthesia has been shown to be a risk factor for developing delirium,

and hence POCD, in elderly patients (Kaneko, Takahashi et al. 1997). Circumstantial evidence can be derived from the observation that when melatonin decreases daytime sleeping there is an improvement in the level of alertness and psychological test performance. It also decreases agitation and confusion in the evenings in elderly people with cognitive deficits living in nursing homes (Cohen-Mansfield, Garfinkel et al. 2000).

2.10.2.e Environment

In the elderly a change in environment such as hospital admission or just disruption of routine increase the risk for developing POCD. In the postsurgical setting these factors added to by the noise on the ward, disturbance from the regular monitoring of vital signs by different personnel throughout the day and night may also contribute to disruption of temporo-spatial memory, diurnal rhythm and induce sleep imbalance (Husted, Holm et al. 2008). Gross examples of this such as a prolonged stay in intensive care or long periods of hospitalization are also risk factors (Radtke, Franck et al. 2012).

2.10.2.f Infection

The International study for POCD (ISPOCD-1) has identified infection in the postoperative period as a contributing factor for developing long term cognitive dysfunction (Moller, Cluitmans et al. 1998; Abildstrom, Rasmussen et al. 2000).

2.11 POCD pathogenesis

Memory impairment has been associated to neuro-inflammatory changes.

Neuro-inflammatory changes, in turn have also being linked to the physiological process of ageing and chronic neurodegenerative processes such as Alzheimer's disease. Furthermore, non-neurological surgeries have been linked to neuro-inflammatory changes constituting the substrate of the POCD pathophysiology.

Neuroinflammation has been described as the pathogenic substrate for the development of sickness behaviour after systemic inflammation secondary to infection. The systemic inflammatory mediators trigger a cascade of events resulting in endothelial cell activation, increase in the release of adhesion molecules, high expression of cyclooxygenase-2, activation of central nervous system cells and de novo synthesis of pro-inflammatory cytokines in areas of the brain with high synaptic activities responsible for memory formation. The glial cell membrane up-regulates danger receptors, the IL-1 receptors, and toll-like receptors (TLRs) within others leading to activation of different transcription factors responsible for the synthesis and release of pro-inflammatory cytokines.

Amongst the pro-inflammatory cytokines responsible for neurocognitive changes after different type of insults, the IL-1 β seems to play a pivotal role in the neuronal mechanisms associated with fear-related memory dysfunction and long-term neuronal plasticity through both direct and indirect (microglia activation) routes whereas IL-6 seems to facilitate IL-1 β effects (Ban, Haour et al. 1992; O'Connor, Johnson et al. 2003; Wiczorek, Swiergiel et al. 2005; Rosczyk, Sparkman et al. 2008; Cibelli, Fidalgo et al. 2010). The previously described neuroinflammatory changes result in an imbalance of neurotransmitters such as

acetylcholine and glutamate which result in disruption of neuronal connectivity and in consequence memory impairment.

All the above evidence suggests that the pathogenesis of POCD may be initiated by an inflammatory insult that results in neuroinflammatory changes.

In this section I will elaborate further the pathophysiology of POCD and expand on the pathogenic mechanisms I will attempt to elucidate in my thesis.

The role of anaesthesia on the pathogenesis of POCD- potential mechanisms anaesthetic drugs upset certain types of brain functions such as consciousness and appreciation of pain while preserving others such as respiration within the CNS. Thus, it is logical to hypothesise and it is established by many studies (Galindo 1969; Nicoll 1972; Richards and Smaje 1976) that, irrespective of the molecular pathway that they follow or cellular modulations that they exert, anaesthetics produce their effects by disrupting the neuronal communication. The synapse is the main route of neuronal communication and it is considered to be the main site at which anaesthetics exert their actions. The activities at the synapse can be divided into four main processes:

1. Impulse conduction in the afferent neurone.
2. Release of neurotransmitter in the synaptic cleft.
3. Recognition of postsynaptic receptors and binding of the released transmitter to the postsynaptic membrane modulating the threshold depolarisation required for the generation of action an potential and the

function of neurotransmitter receptors GABA-A, glycine, glutamate, nicotinic, acetylcholine) (Franks and Lieb 1998; Mennerick, Jevtovic-Todorovic et al. 1998; Narahashi, Aistrup et al. 1998).

4. Change in the conductance of the postsynaptic membrane (Richards 1983).

Synaptic transmission is regulated by families of synaptic vesicle proteins, such as synapsin (Greengard, Valtorta et al. 1993) and synaptophysin (McMahon, Bolshakov et al. 1996).

Anaesthetics act upon neuronal elements and modify membrane conductance and transmission (Antognini, Carstens et al. 2003). Previous work in the field pointed that there is no general mechanism of anaesthetics' action on synaptic transmission and that different types of anaesthetics exert different actions. Despite this it would appear that compromised synaptic transmission seems to be the main route of anaesthesia-mediated mental decline (Xie and Tanzi 2006a; Tang, Eckenhoff et al. 2010).

An alternative action might be through neuroinflammation. It has been shown that commonly used inhalation anaesthetics (isoflurane, sevoflurane) at clinically relevant concentrations can generate neuro-inflammation (Tang, Eckenhoff et al. 2011). Anaesthesia-induced neuroinflammation is driven either directly by enhancing the levels of proinflammatory cytokines (Tang, Eckenhoff et al. 2011) or indirectly by modulating the permeability of the BBB (Tétrault, Chever et al. 2008). Inhalation anaesthetics have been shown to induce such insults in glial

cells, which are traditionally associated with the generation of neuroinflammatory processes after a transient insult (such as ischaemia) (van Groen, Puurunen et al. 2005). Anaesthesia may induce neuroinflammation, A β accumulation and apoptosis, which could contribute to cognitive deterioration and AD (Eckenhoff, Johansson et al. 2004; Xie, Culley et al. 2008; Dong, Zhang et al. 2009).

Neurones also contribute to the production of proinflammatory cytokines, especially TNF- α , and play a direct role in neuroinflammation (Murphy, Borthwick et al. 1999; Heneka and O'Banion 2007; Wu, Lu et al. 2011) although the molecular mechanisms of these processes are currently unknown.

Isoflurane is speculated to exert its unique effects on both neuronal and glial cells through differential regulation of the nuclear factor kappa light-chain-enhancer of activated B cells (NF κ B) and the receptor for advanced glycation end products (RAGE) dependent pathways (Wu, Lu et al. 2011).

This could tie in with the severe neuroinflammation described in the elderly due to age-related, deregulated connection between the peripheral immune system and the brain (Lee, Weindruch et al. 2000) that results in elevated levels of inflammatory cytokines (IL-1 β , IL-6, and TNF- α) in the hippocampus and the forebrain (Ye and Johnson 1999; Richwine, Godbout et al. 2005). As previously discussed the hippocampus is the centre for consolidation and transfer of information from short term to long-term memory while the forebrain is the main centre of acetylcholine production, a neurotransmitter heavily involved in

the brain plasticity during the learning process. The hippocampus and forebrain communicate through the septo-hippocampal pathway.

In either situation, elevated levels of inflammatory cytokines might impair production of acetylcholine and hence alter synaptic plasticity in the dentate gyrus (DG) (Cunningham, Murray et al. 1996) and cornu ammonis (CA) regions (Bellinger, Madamba et al. 1993; Hellstrom, Danik et al. 2005) of the hippocampus. If so this would affect hippocampal-dependent memory and learning and would provide a potential mechanism for anaesthesia to cause POCD (Tancredi, D'Antuono et al. 2000; Tanaka, Ide et al. 2006).

A series of recent studies associate anaesthetics with neuroinflammation and the neuropathogenesis of AD (Xie, Dong et al. 2006b; Xie, Dong et al. 2007; Xie, Culley et al. 2008). These findings, combined with current data suggest that neuroinflammation *per se* can contribute to AD pathogenesis (Games, Adams et al. 1995; Guo, Yu et al. 2002; Sastre, Dewachter et al. 2003) and by association suggests it could be exacerbated after surgery.

2.12 Neuroinflammatory changes and POCD

There is therefore considerable circumstantial evidence to implicate neuroinflammation and specific cytokines in POCD (Cibelli, Fidalgo et al. 2010). Various cytokines act on a different brain structures to 'influence' the behavioural and cognitive changes associated with central inflammation. IL-1 β levels change throughout the inflammatory reaction. It has been previously

described as a cause of cognitive changes (Cibelli, Fidalgo et al. 2010), reduced food intake and weight loss when at supraphysiological levels (Morley and Farr 2008). Interpretation of its role in cognitive changes and memory in particular is complicated by its importance in the process of long-term potentiation (LTP) at physiological levels (Schneider H, Pitossi F, 1998). In animal work it has been shown that both supra-physiological and sub-physiological levels of IL-1 β were associated with impaired performance in cognitive testing although only long term memory (Goshen I, Kreisel T, 2007)(Oprica M, Zhu S, 2005, Spulber S, Mateos L, 2009).

IL-6 has also been identified as an important cytokine in memory formation. It is an inhibitor of long-term memory formation and it has been postulated that it may form an equilibrium with IL-1 β with roles for each in inducing production of the other (Balschun D, Wetzell W, 2004)(Reyes TM, Coe CL. 1998, Sparkman NL, Buchanan JB 2006).

These two pro-inflammatory cytokines and TNF- α have also been associated with sleep regulation (Opp MR 2005). This hypothesis arose due to variation in IL-1 β and TNF- α throughout the sleep-wake cycle suggesting an important role in the physiological control of sleep (Krueger JM, Fang J, 1998). Variation of these pro-inflammatory cytokines levels would lead to changes in sleepiness as occurs in 'sickness behaviour'. This may consequently lead to overt lethargy. IL-6's role, however, is not believed to directly regulate sleep (Hogan D, Morrow JD, 2003) but rather acts via inducing expression of both IL-1 β and TNF- α as suggested previously.

Danger signalling through the purinergic receptors particularly P2X7 promotes further synthesis and release of IL-1 β to the extracellular space accentuating and propagating the inflammatory process within the brain (Bernardino, Balosso et al. 2008). High levels of normal cell waste products such as reactive oxygen species (ROS) perpetuate the inflammatory signalling cascade by stimulating the toll-like receptors and hence further synthesis of IL-1 β , and TNF- α (Dasu and Jialal 2011).

It has been suggested that the expression and released of IL-1 β is highly regulated by the activation of the P2X7 receptor under stressful conditions. Furthermore, the continuous production of ROS triggers further synthesis of proinflammatory cytokines and also induces chaperone heat shock protein 72 (Hsp72) to protect the cells from further injury. It appears from all the above that IL-1 β is a key player in unlocking the inflammatory mechanisms involved in POCD. There are many things that affect IL-1 β but amongst these things are the P2X7 receptor and ROS. These links will be elucidated below but these are potentially fruitful areas for study for the reasons explained later.

It is clear that the inflammatory response to stress is widely orchestrated however for the purpose of my thesis I will focus on the role of danger receptor, P2X7 receptor, ROS and the role of Hsp72 on the pro-inflammatory cascade and how these proteins are associated to cognitive changes in the postoperative period (Figure 2.8).

Signalling pathways for synthesis and release of IL 1- β

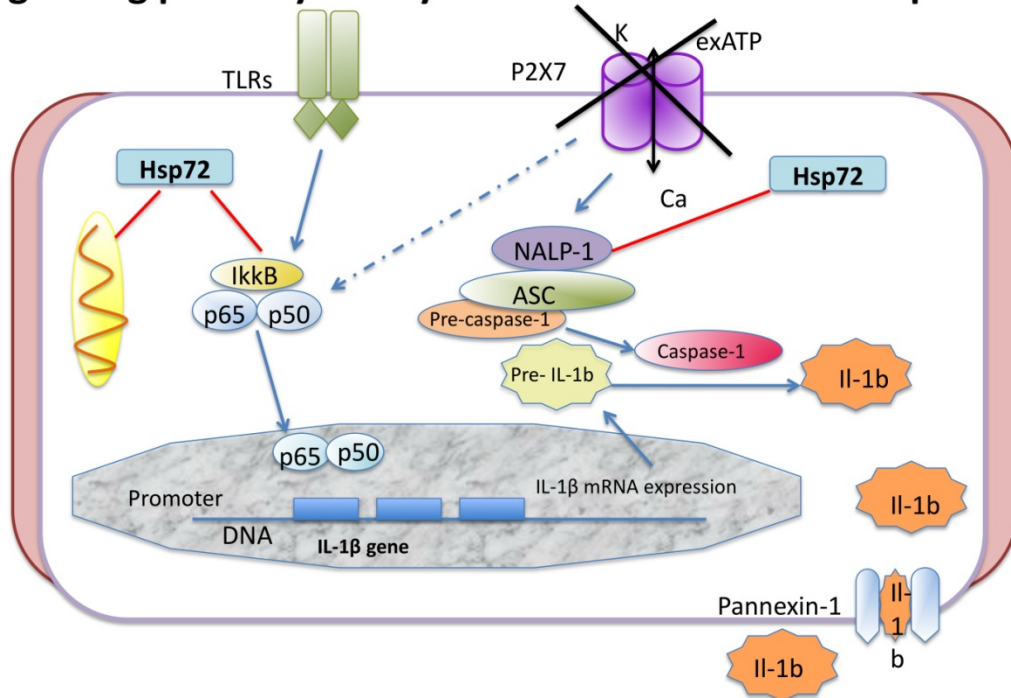


Figure 2.8: Schematic illustration on the production of IL-1 β after cell activation via P2X7 and TLR-4 receptors and the role of Hsp72 on suppressing the synthesis of IL-1 β . The P2X7 receptor is located in the cell membrane and it is activated by extracellular ATP (exATP). The activation of the P2X7 receptor leads to an efflux of potassium (K) and an influx of calcium (Ca). The high level of intracellular Ca leads to the assembly of the inflammasome (NOD-like receptor-1 (NALP-1), adaptor protein (ASC), pro-caspase-1, Pro- IL-1 β) resulting in cleavage of pro-caspase-1 into caspase-1. The caspase-1 converts the Pro- IL-1 β into active IL-1 β that is contained in microvesicles and released to the extracellular space through the hemichannel Pannexin-1. The activation of the P2X7 receptors also leads to activation of the transcription factor NF κ B which translocates into the cell nucleus to increase the synthesis of pro-inflammatory cytokines. The NF κ B is also directly activated through the TLRs receptors. These intracellular mechanisms are

negatively regulated by the heat shock protein 72 (Hsp72) at the level of the mitochondria, NFκB and inflammasome. The suppression of the P2X7 activity may also abort the process of synthesis and release of pro-inflammatory cytokines, mainly IL-1β.

2.12.1 Production of proinflammatory cytokines through activation of the P2X7 receptor

The P2X7 receptor has multiple physiological functions and it is a key player in the activation of microglia cells (Soto, Garcia-Guzman et al. 1996; Solle, Labasi et al. 2001; Mariathasan, Weiss et al. 2006; Pelegrin and Surprenant 2006; Skaper, Facci et al. 2006). It has also been linked to regulation of cell proliferation, cell death *via* apoptosis and/or necrosis (Chen and Brosnan 2006), and release of pro-inflammatory cytokines particularly, IL-1β (Ferrari, Chiozzi et al. 1997; Qu, Franchi et al. 2007).

The P2X7 receptor is a ATP-gated non-selective ion channel receptor permeable to sodium, potassium and calcium (Baraldi, Di Virgilio et al. 2004). It has a selective cellular distribution. The P2X7 receptor is expressed in macrophages (Di Virgilio, Ferrari et al. 1996), monocytes, microglia (Bianco, Ceruti et al. 2006) and dendritic cells (Baroni, Pizzirani et al. 2007) and it seems to be involved in host-defense reaction. The P2X7 receptor has a very high selectivity for ATP and ATP is the only known physiological activator of this receptor. Under normal physiological conditions, extracellular ATP is present in very low concentrations in the extracellular space but the cytoplasmic concentration of ATP is much

higher, in the millimolar range, and activated immune cells such as macrophages and microglia may release significant concentrations of ATP under stress or inflammatory conditions.

Activation of the P2X7 receptor by extracellular ATP leads to a reversible formation of a plasma membrane pore permeable to hydrophilic solutes up to 900Da (North 2002). After the membrane pore formation the cells release microvesicles (MV) enriched in IL-1 β (MacKenzie, Wilson et al. 2001; Ferrari, Pizzirani et al. 2006; Di Virgilio 2007; Pizzirani, Ferrari et al. 2007).

The IL-1 β is part of the IL-1 superfamily that consists in IL-1 α , IL-18 and IL-1 receptor antagonist (IL-1RA). IL-1 β synthesis is promoted by the activation of P2X7 receptor. The IL-1 β is synthesised in the form of a biologically inactive cytokine that remains dispersed in the cell cytoplasm until a second stimulus triggers its cleavage by caspase-1. The caspase-1 is an IL-1 β converting enzyme and it is the key intracellular protease responsible for processing IL-1 β . In resting cells caspase-1 is presented as an inactive enzyme known as a pro-caspase-1. The activation of caspase-1 is initially primed via activation of toll like receptors (Kahlenberg, Lundberg et al. 2005). The activation of pro-caspase-1 occurs in a multimeric specialized structure named IL-1 β inflammasome protein complex (Martinon, Burns et al. 2002). The inflammasome comprises different proteins; caspase-1, caspase-5, apoptosis-associated speck-like protein containing a CARD (ASC), and NACHT-, LRR- and PYD-containing protein that transiently oligomerise upon cell activation (Martinon and Tschopp 2004). The IL-1 β is accumulated into endocytic vesicles also known as secretory lysosomes

together with caspase-1. When the P2X7 receptor is stimulated there is an efflux of potassium from the cell and influx of calcium that activates calcium-dependent phospholipase-A2 that releases IL-1 β (Andrei, Margiocco et al. 2004) into the extracellular space through the pannexin-1 channels (Pelegriin and Surprenant 2006; Orellana, Froger et al. 2011).

Once the IL-1 β is released it binds to the IL-1 receptor (IL-1R1). Once bound to its receptor it recruits the accessory protein (IL-1R-AcP) a member of the immunoglobulin superfamily and initiates a stimulatory signalling cascade. There is on the cell membrane a decoy receptor that does not trigger a cascade of events, as it does not couple to IL-1R-AcP (Colotta, Re et al. 1993). There is also an IL-1R2 receptor that competes with IL-1R1 to control and balance the IL-1 β activity (Colotta, Re et al. 1993). To complete this fine regulatory mechanism, when IL-1 β is released the IL-1bRa is also secreted and it competes with the IL-1 β binding sites on the IL-1R1 and IL-1R2. The IL-1bRa lacks the interacting domain with IL-1R-AcP therefore its binding to the IL-1R1 and IL-1R2 does not result in biological signaling therefore the synthesis of pro-inflammatory cytokines is not activated (Wang, Zhang et al. 2010).

The IL-1 β is a key mediator in the host response to infection and inflammation while the IL-1Ra binds to the 1 IL-1R and blocks the IL-1 dependent signal transduction, thus functioning as an endogenous, IL-1 selective inhibitor of inflammation (Dinarello 2000).

The release of IL-1 β leads to upregulation of gene products that contribute to the inflammatory state, including matrix metalloproteases, COX-2 and cell adhesion molecules (Guan, Buckman et al. 1998). IL-1 β causes accumulation of arachidonic acid metabolites, upregulation of inducible NO synthase and sustains NO production. The IL-1 β initiates a cascade of events that leads to further stimulation of the inflammatory response through further production of IL-6, COX-2, interferon gamma (IFN γ), monocyte chemotactic protein-1 (MCP-1) and superoxides. This promotes extravasation of leucocytes in areas where there is endothelium disruption and an increase in cells permeability (Dinarello 2002). The feedback from the proinflammatory cytokines results in a further increase in sensitivity and expression of P2X7 receptor (Guan, Buckman et al. 1998; Parvathenani, Tertyshnikova et al. 2003; Narcisse, Scemes et al. 2005).

2.12.3 Signalling through P2X7 receptors

The signalling through the P2X7 receptor allows cells to sense and respond to events occurring in the extracellular environment, modulate the transcription of genes involved in cellular inflammatory processes to thus regulate the cytokine responses. It has been shown that the lack of P2X7 receptors leads to the loss of ATP-dependent leukocyte functions including release of IL-1 β and L-selectin shedding (Labasi, Petrushova et al. 2002). The level of IL-1 β in plasma increased after LPS + ATP challenge in wild type mice while plasma level of IL-1 β is minimally modified after the same challenge in P2X7 receptor deficiency mice (Labasi, Petrushova et al. 2002).

The P2X7 receptor links to the immune system and to the CNS. Evidence suggests that an increase in the number of P2X7 receptors lead to microglia activation. This phenomenon may be implicated in the process of neurodegeneration (Le Feuvre, Brough et al. 2002) due to its ability to modify the microglia reactivity within the CNS (Skaper, Debetto et al. 2010). There are several neurodegenerative conditions that exhibit high level of expression of P2X7 receptor in the neuroinflammatory area where microglia activation is a coexisting feature. Increase in pro-inflammatory cytokines coincides with an up-regulation of P2X7 receptors and cognitive decline that occurs with advancing age, ischaemia and neurodegenerative disease including Alzheimer's and multiple sclerosis (Parvathenani, Tertysnikova et al. 2003; Franke, Gunther et al. 2004; Yiangou, Facer et al. 2006; Crain, Nikodemova et al. 2009).

There is an association between memory decline and an increase in neuroinflammation in rat hippocampi (Wan, Xu et al. 2007), which is in line with previous findings where increased microglia activation was observed following trauma (Wan, Xu et al. 2007; Bernardino, Balosso et al. 2008; Rosczyk, Sparkman et al. 2008; Monif, Reid et al. 2009). It has also been shown that the P2X7 receptor is regulated by mechanical injury at post-transcriptional level (Franke, Grosche et al. 2001). The P2X7 receptor can also act as an 'emergency' receptor to control excessive cell proliferation (astrogliosis) after brain injuries (Afework and Burnstock 2000).

It is therefore of interest to know if modification of receptor function will result in functional changes. The use of P2X7 receptor antagonists in mice does

improve functional recovery and neuronal survival after spinal cord injury (Wang, Arcuino et al. 2004). The recovery from spinal cord injuries and neuropathic pain also improved significantly with the antagonist of the P2X7 receptor, Brilliant Blue G (BB-G). The BB-G is a competitive antagonist of extracellular ATP and selective inhibitor of P2X7 receptor (Nieber, Eschke et al. 1999; Chessell, Hatcher et al. 2005; Peng, Cotrina et al. 2009). Nancy Rothwell showed a reduction in brain lesions induced by middle cerebral occlusion in rats after administration of systemic IL-1 β RA which results in a reduction of the activation of the synthesis and release of IL-1 β (Clark, McMahon et al. 2008).

The microglia cells are the immunological system within the central nervous system and they react against different noxious stimuli by activation, synthesis and released of cytokines and or activation of apoptotic pathways. There is an established role for P2X7 on both signalling and the release of cytokines from the glial cells and hence its contribution in perpetuating the inflammatory response within the CNS and remote organs.

Extracellular ATP does not only stimulate the P2X7 receptor but it has been linked to the increase in Hsp72 in plasma during and after aerobic exercise (Ogawa, Seta et al. 2011) which suggest that this chaperone may be implicated in the regulation of the inflammatory response under stressful condition.

In summary, P2X7 receptor are pivotal in triggering the synthesis and release of pro-inflammatory cytokines, mainly IL-1 β which is found in abundance in areas of the brain where memory is formed.

2.12.4 Heat shock protein 72 (Hsp72) and POCD

Hsp72 is a highly stress inducible cytosolic heat shock protein has many cell protective functions including acting as a protein chaperone, regulating inflammation, and regulating cell death and survival pathways (Giffard, Han et al. 2008). It is also is an assistant protein that interacts with other proteins or protein subunits to stabilise unstable structures and at the same time prevent the formation of non-functional structures or protein aggregates that could induce inflammatory and apoptotic processes (Hartl 1996).

Within individual cells, Hsp72 is induced by hypoxia, NO, fever, endotoxaemia (Kume, Yamamoto et al. 1996; Meng, Banerjee et al. 1999). It is also activated by denaturalised proteins and ROS and the expression of Hsp72 is associated with down regulation of subsequent cell response to stress. Hsp72 requires high concentration of intracellular calcium (Kiang, Carr et al. 1994) to be activated and this activation is known as the Hsp72 response. The Hsp72 response involves the ATP-dependent dissociation of the Hsp72 from its heat shock factor (HSF) in the cytosol. When the HSF is phosphorylated it then translocates into the nucleus where it promotes further synthesis of Hsp72 (Wang, Chen et al. 2007). The evidence shows that Hsp72 is responsible for protein translocation across membranes of organelles. Hsp72 unfolds proteins during transit across the membrane then reconfigures them after the internalization process (Kiang and Tsokos 1998). This process of unfolding proteins is again an ATP dependent process but also requires interaction with other proteins to complete its action (Schatz and Dobberstein 1996). Cells that over express Hsp72 under stress may become desensitized in the presence of persistently high intracellular

concentration of calcium or its desensitization may be due to alteration of the balance of Na/Ca exchange cell membrane pump system (Kiang, Ding et al. 1996) resulting in a leak of Hsp72 to the extracellular space with a relative reduction of the intracellular Hsp72 and subsequently an increase in the synthesis of pro-inflammatory cytokines.

Hsp72 is also responsible for sphingomyelin degradation but also maintaining proper protein folders preserving lysosome membrane integrity (Yamashima 2012). A dysfunctional Hsp72 or lack of inducible cytoplasmatic Hsp72 promotes activation of NF κ B (Yamashima 2012). That in turn increases pro-inflammatory cytokines. This then activates microglia cells which interferes with normal neuronal connectivity. Therefore a high level of inducible Hsp72 may obtund damage associated molecular pattern molecules (DAMP) by affecting the continuous production of ROS hence pro-inflammatory cytokines.

Over expression of mitochondrial heat shock protein 70 decreases ROS production and preserves mitochondria membrane potential during oxygen glucose deprivation in astrocytes in an *in vitro* models of ischaemic injury (Voloboueva, Duan et al. 2008).

Hsp72 suppresses the synthesis of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-12) in monocytes and macrophages stimulated with LPS (Ding, Fernandez-Prada et al. 2001). Intracellular Hsp72 also prevents the cell response to TNF- α and IL-1 β as nicely demonstrated in animals over expressing Hsp72 after exposure to exogenous TNF- α (Van Molle, Wielockx et al. 2002). The mechanism by which

the intracellular Hsp72 seems to control the inflammatory response is through blocking the activation of the transcription factor NF κ B. This is the result of inhibition of I κ k, inhibition of ubiquitination of tumour necrosis factor associated factor 6 and stabilisation of the complex with I κ B (Giffard, Han et al. 2008). The suppression of the inflammatory cascade by Hsp72 may be also associated with a reduction in the production of reactive oxygen species. This suppresses the expression of inducible form of nitric oxide synthase (iNOS) and NADPH oxidase in glial cells (Beckman, Beckman et al. 1990). High level of Hsp72 prevents the formation of superoxide by controlling the activity of NADPH oxidase induced by inflammatory process in circulating phagocytic leucocytes in the periphery and also in the CNS (Robinson and Badwey 1995). The neurones and in particular hippocampal neurones and endothelial cells with high activity of the NADPH oxidase has been linked to the process of ageing (Park, Anrather et al. 2007). These findings have suggested the role of the DAMP on neuroplasticity and how Hsp72 interferes with astroglia activation and neuroinflammation hence this suggest that high level of inducible Hsp72 may prevent the development of POCD.

Physiologically, Hsp72 activity seems to reduce with age. Ageing has being associated with low level of Hsp72. A reduction in inducible heat shock protein has been reported in aged rodents (Tandara, Kloeters et al. 2006) and this has also been found in humans. Rea et al found a reduction in plasma levels of Hsp72 in elderly patients (> 60 year old) when compared to young patients (< 40 year old)(Rea, McNerlan et al. 2001). It less clear if this low expression of Hsp72 is related to chronic neurodegenerative processes including AD. In neuronal cells

the Hsp72 prevents microtubules associated protein such as tau protein hyperphosphorylation a microtubular protein responsible for architectural cell stability therefore interferes with the normal cellular architecture and as a consequence modifies the cells connectivity by triggering local inflammatory process (Kirby, Merrill et al. 1994).

There is growing evidence supporting the idea that Hsp72 is released into the extracellular space. Hsp72 is released into the extracellular space from lysosomes. This was demonstrated by an increase in the expression of the intralysosomal protein on cell surfaces (Mambula and Calderwood 2006). Broquet et al has suggested that the sphingolipid-cholesterol-rich micro-domains in cell membrane also play a role in the release of Hsp72 into the extracellular space (Broquet, Thomas et al. 2003).

It therefore seems that under stress conditions the glial cells surrounding the neuronal axon produce Hsp72 which is secreted into the extracellular space and then internalized into the neurones as part of the physiological neuroplasticity mechanisms (Morimoto, Kline et al. 1997; Calderwood, Mambula et al. 2007). It has been postulated that the extracellular Hsp72 released from necrotic cells take part of the danger signalling mechanisms and promotes the release of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6 through activation of TLRs (Asea, Rehli et al. 2002). LPS signalling through TLRs receptors is mediated by a large complex which includes Hsp72 and depending on the composition of the complex the LPS signalling results in induction and or inhibition of an immune response (Triantafilou and Triantafilou 2004). It is evident that the role of

Hsp72 in the extracellular space is not yet fully elucidated and there could be positive or negative effects on the immune system depending on the overall physiological response to different type of stressors.

An example is that statin treatment is associated with low level of Hsp72 in plasma monocytes in healthy individuals but also in patients suffering from peripheral vascular disease(Madden, Coward et al. 2010). The Hsp72 is suppressed by simvastatin when the endothelium cells are exposed to oxidised low-density lipoproteins (oxLDL) (Pirillo, Jacoviello et al. 1997). This provides linkage with pharmacological agents that might, potentially have an interventional or modifying role.

Hsp72 is probably protective. The induction of Hsp72 before the onset of an insult such as severe haemorrhage protects the individual from further organ damage (De Maio 1999). A high level of Hsp72 in plasma on admission after trauma correlates with hospital survival (Pittet, Lee et al. 2002). It has been shown in animal and cell models of stroke that injury is reduced in animals or brain cells over-expressing Hsp72 (Sun, Ouyang et al. 2006) and that this is associated with reduced activation of the pro-inflammatory transcription factor NFκB (Zheng, Kim et al. 2008).

In other circumstances it seems that after very major insults even the induced levels of Hsp72 may be insufficient to prevent the injurious effect of stress (Okaya, Blanchard et al. 2005; Tandara, Kloeters et al. 2006; Rumora, Lovric et al. 2007).

Nevertheless it has been demonstrated that Hsp72 confers neuroprotection in the setting of acute neurological injury (Hoehn, Ringer et al. 2001). In addition to its roles in stress, survival and inflammation, Hsp72 has also been implicated in learning and plasticity, with up-regulation of Hsc70 reported in a learning paradigm (Pizarro, Haro et al. 2003) and changes in Hsp72 noted in the hippocampus of animals trained in a radial maze task (Ambrosini, Mariucci et al. 2005). It therefore has potential as a focus for seeing if its manipulation can have an effect on POCD. Hsp72 seems to be affected by statins and so not only the role of hsp72 on memory should be explored but also drugs such as statins that may impact on it. Statins may be suitable targets for investigation.

2.12.5 Reactive oxygen species and POCD

Reactive Oxygen Species (ROS) are products of normal cell metabolism. ROS is found in microglia, the resident macrophages of the brain. These play an important role in the immune response to injury in the CNS and activated microglia produces ROS and cytokines (Min, Jou et al. 2003). An enhanced production of O_2^- is seen when microglia cells are incubated with TNF- α (Lull and Block 2010). ROS also promotes recruitment of leucocyte but in excess ROS activates TLRs receptors resulting in an increase in the synthesis of pro-inflammatory cytokines (Kaneko, Ota et al. 2012). Hence there is a potential link between ROS and neuroinflammation.

ROS may be influenced by the activity of superoxide dismutase (SOD) and catalase enzymes. SOD is found in 2 locations within the cell; in the

mitochondria as manganese (MnSod) and in the cytoplasm as copper and zinc (CuZnSod). Superoxide radical (O_2^-) is the result of the reduction of electrons passing through the respiratory chain. Accumulation of O_2^- has significant effects on the cell. The accumulation of O_2^- leads to cell membrane peroxidation, release of cytochrome-C and cell apoptosis by formation of apoptosomes and activation of the caspase cascade (Huang, Hsu et al. 2005).

Oxidative stress affects the immune response to stress and it has been associated with the danger signalling mechanisms by activating the P2X7 receptors (Helfand and Rogina 2003) and affecting the induction of heat shock proteins during the stress response acting as part of the DAMP (Chen, Chan et al. 2006).

Experiments using *Drosophila* which showed lifespan could be increased by increasing the cell antioxidant activities (Helfand and Rogina 2003). It would also appear that oxidative stress is involved in many diseases, such as Alzheimer's disease and atherosclerosis (Sopher, Fukuchi et al. 1996).

Therefore, it transpires that high level of inducible cytosolic Hsp72 obtunds the neuroinflammatory cascade by suppressing the production of ROS, synthesis and release of pro-inflammatory cytokines and hence reduction on neuronal disruption responsible for the development of POCD. It seems reasonable to propose that targeting Hsp72 or ROS or both is a sensible approach for looking at POCD.

2.13 Proposed preventative mechanisms for development of POCD

A schema is shown below and includes two possible means of intervening in the neuro-inflammatory cascade using existent agents (Figure 2.9).

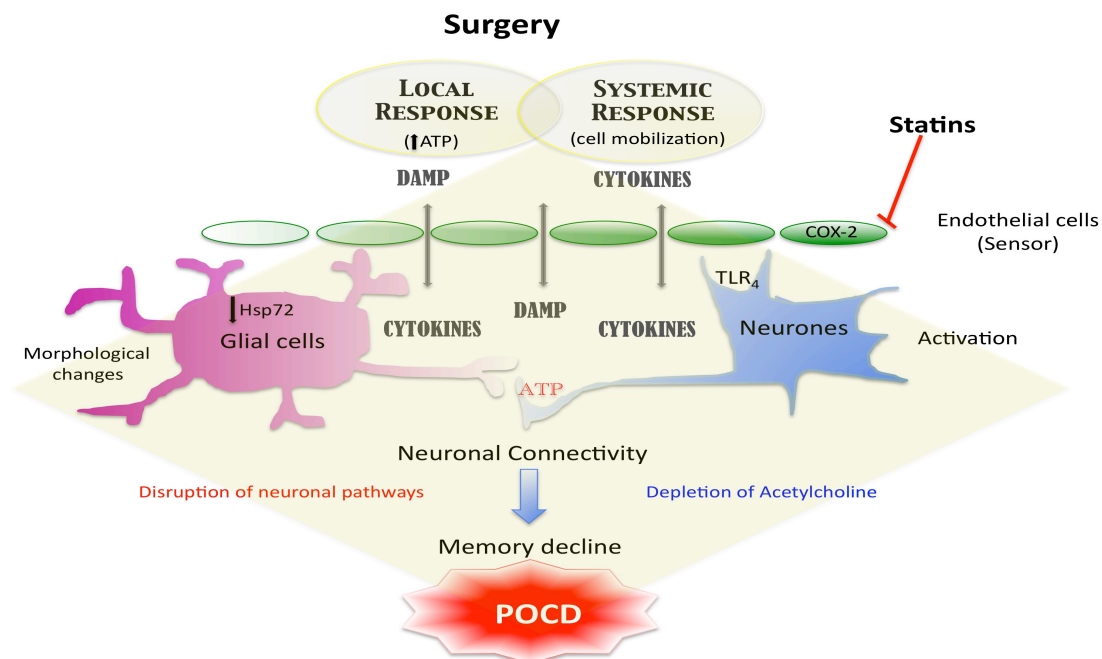


Figure 2.9: Schematic representation of the inflammatory response after surgery and anaesthesia highlighting the key mechanisms by which memory is impaired. Surgery and anaesthesia trigger a local and a systemic inflammatory response resulting in the release of damage associated molecules patterns (DAMPs) and cytokines. The inflammatory response leads to activation of endothelial cells with expression of cyclooxygenase COX-2). The astroglia and neuronal cells react to the stimulation of toll-like receptors (TLRs), purinergic receptor (P2X7) and cytokines. The activation of these receptors promotes the activation of the danger signalling mechanisms with further synthesis of cytokines by glial cells and neurones. The cells try to control this inflammatory response by inducing the Hsp72 which inhibits the activation of NFκB and subsequently further synthesis of pro-

inflammatory cytokines. The de novo synthesis of cytokines in combination with the reactive and active cells disrupt the normal neuronal connectivity and acetylcholine neuronal neurotransmission. The disruption of neuronal connectivity may leads to memory decline and consequently POCD. Reducing the expression of COX-2 in the endothelium cells with statins and inducing Hsp72 with xenon and statins may prevent the development of POCD.

POCD is a devastating complication. From the introduction it can be seen that there may be ways of modifying some pathways that might be implicated in POCD. These include ameliorating the inflammatory response by suppressing the synthesis of cytokines and upregulating the Hsp72.

This can be taken a step further. If major surgery and the stress response are implicated then as major surgeries are performed generally under general anaesthesia, it may be possible to ameliorate they effects by modifying the anaesthetic or the agents. Xenon is a noble gas with demonstrated neuroprotective properties (Homi, Yokoo et al. 2003; Limatola, Ward et al. 2010). If this is correct then using this as pre-treatment at induction this may be an option to prevent memory decline.

The prevention of memory decline by xenon is through the antagonism of the NMDA receptor(Preckel, Weber et al. 2006), inhibition of the synthesis of pro-inflammatory cytokines (Fahlenkamp, Coburn et al. 2010)and hence the disruption of neuronal connectivity.

A different approach might be to look for a different agent that might influence POCD because xenon even though potentially beneficial it is a very expensive and cumbersome anaesthetic agent. Statins become an option as they are cholesterol lowering drugs with anti-inflammatory effects, widely used and with a good safety profile. Statin suppresses the signalling through inhibition of the pro-inflammatory cytokines, including cyclooxygenase at the endothelium level and inducing the expression of cytosolic Hsp72. Therefore, statin interferes with the activation of immune cells systemically and cells confined to the CNS. Prevention of immune cell activation and induction of Hsp72 may lead to prevention of development of POCD.

A great number of patients requiring major surgical interventions are elderly, suffer from multiple co-morbidities and a vast majority of them take statin normally.

To this end, I will explore these two drugs to identify their role in POCD (Figure 2.9).

2.13.1 Xenon and POCD

Xenon is one possibility by which POCD could be prevented.

Xenon is a noble gas, used experimentally in clinical practice since 1948 (CULLEN and GROSS 1951). It possesses many properties of an ideal inhalation anaesthetic, such as low blood-gas partition co-efficient, rapid penetration of the BBB and absence of significant side-effects (Dingley, Ivanova-Stoilova et al. 1999; Wilhelm, Ma et al. 2002). It can be an effective anaesthetic but also has anti-inflammatory activity and is considered to be an excellent neuroprotective agent.

Its anti-inflammatory activity relates to the suppression of caspase-3 mediated apoptosis (de Sousa, Dickinson et al. 2000). Xenon, unlike other antagonists of N-methyl D-aspartic acid (NMDA) receptors (including common anaesthetic agents such as isoflurane and nitrous oxide), binds to the glycine site of the NMDA receptors. Once the NMDA receptor is antagonised by xenon it induces phosphorylation of cyclic adenosine monophosphate (cAMP), which activates the responsive element-binding protein (pCREB) leading to the transcription of the anti-apoptotic B-cell lymphoma-2 protein (Bcl-2) and by this conferring neuroprotection (Wilson, Mochon et al. 1996; Ma, Hossain et al. 2006). The neuroprotection is thought to be due to prevention of activation of apoptotic pathways (Cattano, Williamson et al. 2008) and some evidence though more controversial through amelioration of the synthesis of pro-inflammatory cytokines (Clark, Ma et al. 2005).

In vivo, xenon has been shown to have a neuroprotective effect in a model of hypoxia/ischaemia in rats (Dingley, Tooley et al. 2006) and to prevent acute neuronal injury caused by middle cerebral artery occlusion (ischaemia) in mice (Homi, Yokoo et al. 2003) and ameliorates the effects of cardiopulmonary bypass in rats (Ma, Yang et al. 2003) through inhibition of the NMDA receptor in the brain and also by reducing the level of IL-6 and TNF- α in plasma (Hein, Roehl et al. 2008).

Recent *in vivo* and *in vitro* studies support a substantial neuroprotective role of xenon not only when used as the main anaesthetic agent but also as a preconditioning agent (Wilson, Mochon et al. 1996; Homi, Yokoo et al. 2003; Ma,

Yang et al. 2003; Ma, Williamson et al. 2007; Cattano, Williamson et al. 2008).

Although the actual mechanisms of xenon-mediated neuroprotection are not yet identified it is though that xenon exerts its role through two main routes:

1. Antagonising the NMDA receptor by binding to the glycine site (Dickinson, Peterson et al. 2007).
2. Suppressing the systemic inflammatory response reducing the levels of pro-inflammatory cytokines (Hein, Roehl et al. 2008; Saravanan, Exley et al. 2009).

In view of the current level of evidence and the intricacy of the neuro-inflammatoritory mechanism, it may be possible that xenon induces cytoplasmic Hsp72 and prosurvival proteins such as phospho-protein kinase B (pAkt), Bcl-2 and hypoxia-inducible factor-1 α (HIF-1 α) (Ma, Hossain et al. 2005; Limatola, Ward et al. 2010). Furthermore, xenon may reduce the cell damage by ameliorating the systemic inflammatory response and DAMPs both in the periphery and in the CNS and therefore prevent neuronal disruption and hence POCD.

This then provides a rationale for exploring this agent as a means of intervention in the development of POCD.

2.13.2 Statin and POCD

There is some evidence supporting the beneficial effect of statin in POCD. Statins exert their cholesterol lowering effects via competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting step of the

mevalonate pathway, which is the cellular mechanism for the production of cholesterol (Corsini, Bellosta et al. 1999).

The pivotal role of inflammation in atherogenesis lead to the suggestion that statin exerted an anti-inflammatory effect (Weitz-Schmidt 2002). It has been hypothesised that statin as a potent inhibitor of cholesterol biosynthesis affects the production of pro-inflammatory cytokines by inhibiting the activation of the transcription factor NFκB via the TLR4 signalling pathways. This is the same pro inflammatory pathway that was mentioned previously in association with ROS and hence a common pathway for POCD. It has also been shown that TLR4 in macrophages are upregulated by oxidised low density lipoprotein (oxLDL) (Xu, Shah et al. 2001) which leads to activation of the transcription factor NFκB, ultimately leading to the synthesis of inflammatory cytokines.

Its effects on POCD are uncertain but statin improves functional neurological deficit and reduces level of microglia activation in the hippocampus after traumatic brain injury in an animal model (Wang, Lynch et al. 2007).

Gang Chen et al explored the effect of statin on traumatic brain injury in a rat model of cortical contusion and demonstrated a dramatic reduction in the expression of TLR4 after administration of statin when compared to placebo and the NFκB expression and proinflammatory cytokines mirrored the effect of statin on the TLR4 (Aberg 1995). This study also showed a preservation of the BBB integrity by reduction of Evans blue extravasation in the group of animals treated with atorvastatin. Following this Haichen Wang et al looked at the effect

of statin on cerebral blood flow and behaviour after traumatic brain injury in a mouse model. The animals treated with statin prior to the trauma showed better motor activity when compared to non-treated animals. There was less neuronal injury demonstrated by reduction in hyperaemia and microglia activation in the hippocampus of the animals treated with statin (Wang, Lynch et al. 2007).

This is encouraging and suggests there may be an antiinflammatory mechanism. More recently, there has been some evidence supporting the effect of statin on delirium in intensive care (Katznelson, Djaiani et al. 2009). Although there is limited information there is a basic rationale for assessing the efficacy of statin in preventing POCD.

2.14 Statement of the problem

The patient population is ageing and more high-risk patients are undergoing surgical procedures (Alexander, Newby et al. 2007). Even if the surgical procedure itself is uneventful, it may be followed by a decline in cognitive functional status. Cognitive function is of critical importance in relation to independent living, need for care, personal and economic cost, and quality of life. Risk reduction should be part of the preoperative assessment and planning but at present there are no obvious interventions that can be employed. Nevertheless when optimising patients for surgery, perhaps we should consider the potential for development of POCD. In doing so, perioperative management should include a long-term perspective with consideration of cognitive assessment as well as patient education regarding the potential impact of POCD.

Despite progressive changes in anaesthetic management with the development of new drugs and improved monitoring techniques, as well as improvements in surgical techniques, POCD continues to be reported in the literature as one of the major morbidities following surgery and anaesthesia and awareness of the problem grows (Bekker and Weeks 2003).

Even though there are several tests to assess cognition in the postoperative period in humans we do not fully understand the mechanisms by which memory is impaired. Work is needed to elucidate the mechanisms by which memory is impaired to be able to develop techniques to prevent or at least ameliorate the development of POCD. To this end, several animal models have been developed to understand the pathogenesis of POCD.

2.15 Animal models used for assessment of the pathogenesis of POCD

2.15.1 Thoracic model of POCD

2.15.1.a Cardio-pulmonary bypass

Ma *et al* has previously used a rat model of cardio-pulmonary bypass (CPB) to ascertain the role of xenon on the neuro-cognitive changes induced by cardiac surgery (Ma, Yang et al. 2003). In brief, adult male Sprague-Dawley rats were anaesthetized with 5% isoflurane in oxygen enriched air in a plastic box.

Following orotracheal intubation animals were ventilated to maintain normal oxygenation and PaCO₂ concentration in plasma throughout the surgical intervention. Anaesthesia was maintained with 1.5 – 2.0% isoflurane throughout the surgical intervention, analgesia was achieved by using buprenorphine intraperitoneally, and physiological variables were maintained within normal range. The CPB is a cumbersome technique with multiple variables that could influence the final result, therefore it is mainly used when there are specific research questions related to cardiac surgery. Memory tests are used immediately after surgery and then at 3 and 12 days post intervention to assess the animals' performance.

2.15.2 Orthopaedic model of POCD

2.15.2.a Tibial fracture

Professor Nanchahal, from the Kennedy institute, London introduced initially the tibial fracture model to ascertain the local inflammation after fracture and the process of healing (Harry, Sandison et al. 2008). In collaboration with Professor Maze at Imperial College the model was used for assessment of POCD (Cibelli, Fidalgo et al. 2010). This model is a completely aseptic model of surgery. Animals are anaesthetised with 5% isoflurane in oxygen-enriched air in a plastic box and anaesthesia maintained with 1.5±0.3% isoflurane throughout the surgical procedure delivered via a nose cone. Cardio-respiratory monitoring is performed throughout the surgical intervention and central body temperature is kept within normal parameters. Once the surgery is terminated the animals are allowed to recover from anaesthesia in a warm environment enriched with oxygen until they were able to eat and drink. The fracture is generated under

anaesthesia which takes away the additional effect of the 2-hit model. It is highly reproducible and it has been used widely for the assessment of POCD in animals (Fidalgo, Cibelli et al. 2011; Terrando, Eriksson et al. 2011). The assessment of POCD was performed by fear conditional training, water maze and social behaviour.

2.15.3 Abdominal surgical models of POCD

2.15.3.a Splenectomy

For a non-cardiac surgery model the rodent splenectomy was the first model developed for assessment of POCD after abdominal surgery (Wan, Xu et al. 2007). Once again for this type of surgery the rodents were induced with 20µg/Kg fentanyl and 500µg/Kg droperidol intraperitoneal. Oxygen-enriched air was delivered through a face mask throughout the maintenance of the neuroleptic anaesthetic. Cardio-respiratory monitoring was performed throughout the surgical intervention and central body temperature was kept within normal parameters. Once the surgery was terminated the animals were allowed to recover from anaesthesia in a warm environment enriched with oxygen until they were able to eat and drink. Approach to the retroperitoneal space was achieved via the abdomen. The spleen was identified and all vessels ligated proximally and distally then the spleen was removed, the skin infiltrated with 0.25% bupivacaine and the abdominal cavity was closed. The assessment of memory after the surgical intervention was performed by using the T-maze and it was performed on day 1, 3 and 7 post surgery. This model is highly reproducible and it can be easily performed in small rodents. The downside of this model is that the immunological system may be affected by the removal of

the spleen and therefore the findings may not fully reflect the intrinsic mechanisms involved in the pathogenesis of POCD.

2.15.3.b Hepatectomy

Partial hepatectomy was performed for assessment of POCD and development of Alzheimer's disease in aged mice (Wan, Xu et al. 2010). Once again for this type of surgery the rodents were induced with 0.03ml/10g 10% chloral intraperitoneal. Oxygen-enriched air was administered throughout maintenance of anaesthesia. Cardio-respiratory monitoring was performed throughout the surgical intervention and central body temperature was kept within normal parameters. Once the surgery was completed the animals were allowed to recover from anaesthesia in a warm environment enriched with oxygen until they were able to eat and drink. Approach to the liver was achieved via a medial abdominal incision. The intra-abdominal organs were exposed and then covered with a warm and wet gauze to reduce the loss of temperature. The liver segments were identified and hepatic vessels and corresponding branches of the biliary tree ligated both proximally and distally, then the segment of the liver was removed and the abdominal cavity was closed. The assessment of memory after the surgical intervention was performed by using the Morris water maze and it was performed on day 1, 3 and 7 post surgery. This model even though reproducible carries the risk of hypotension and hypothermia. These are variables that may modify the inflammatory response, therefore this model is not popular with researchers for studying the pathogenesis of POCD.

2.15.3.c Nephrectomy

Nephrectomy was originally performed in rats for assessment of the uraemic syndrome in the brain and its effect on the cholinergic system and memory (Ballesta, Del Pozo et al. 2012). Nephrectomy is a simple retroperitoneal procedure performed also under general anaesthesia. It is highly reproducible and does not carry the risk of hypotension and body temperature which are preserved in a much more controlled fashion than during hepatectomy.

In summary, there are a number of surgical procedures described in the literature attempting to reproduce a clinical setting allowing the investigators to study the mechanisms by which memory is impaired after surgery.

The animal models allow control of most of the potential variables including co-morbidities, age, sex and genetic susceptibilities that could influence the behavioural response to the injury and hence the development of POCD.

The type of anaesthesia and analgesia was the same in all the above described surgical procedures hence one could argue that the cause of the neuroinflammatory changes and development of POCD could be the anaesthesia. The effect of anaesthesia on the neurocognitive changes is difficult to unveil, as it has not been assessed consistently as a solo technique in those studies.

2.16 Hypothesis

The hypothesis is that the mechanism underlying POCD is inflammation mediated in particular by low levels of inducible Hsp72 and activation of P2X7 receptors.

I have suggested that by using atorvastatin, an anti-lipid drug with ample anti-inflammatory effect or pre-treatment with xenon, a noble gas I would be able to increase the expression of Hsp72 and reduce the activity of the receptor P2X7 and in consequence obtund the neuroinflammatory response accountable for the development of POCD.

2.17 Aims

My aims are to use existing animal models for assessment of memory changes and inflammatory response after surgery. I will also attempt to identify techniques to study the systemic and central nervous system inflammatory response to surgery and anaesthesia and develop a model to predict the development of POCD based on the inflammatory response. Then I will elucidate the role of P2X7 receptors and Hsp72 in POCD and finally I will identify preventative mechanisms through up regulation of Hsp72 to complete my thesis by testing the effect of xenon and statin on POCD.

Chapter 3:

Material and methods

This chapter provides the pillars of the thesis. The structure of this chapter encompasses the description of the background experimental work, techniques and methods that were used to analyse the results. The means by which techniques and methodology were validated are also described.

The background work and techniques used are presented in the same order as the aims were proposed to prove the hypothesis of the PhD thesis. This is followed by the description of the statistical methods used.

3.1 General animal handling

3.1.1 Animals

The development of an animal model for the assessment of POCD is paramount for the investigation of the pathogenesis of POCD. It also allows testing of interventions or preventative strategies that may influence the development of POCD.

The United Kingdom animals' scientific procedures and the Stanford animal care and use committee shared common grounds for the care of animal welfare. All the animal experiments carried out at Chelsea and Westminster campus, Imperial College London were approved by the UK Home Office and conformed to the United Kingdom animals (Scientific Procedures) Act of 1986 and were performed under licence 70/6966. All the experiments performed at the University of Stanford (USA) were performed in accordance with a protocol approved by the Stanford Animal Care and Use Committee, Stanford University,

USA, and following the NIH guidelines under NIH grant R01 GM49831 to RGG and ICS travelling fellowship award to MPV. All efforts were made to minimise any animal suffering and the minimum number of animals necessary to achieve a statistical significance was used.

Small rodents were used for developing memory paradigms to test memory changes after surgery and anaesthesia and also characterisation of the pathogenesis of POCD. Wild type animals were chosen for most of the experimental work as they have not been manipulated genetically. These animals were virgin to treatments and were kept in a normal environment making them an ideal model for assessment of their “clinical” response to major insults such as surgery and anaesthesia. Wild type animals serve as control in experiments where a particular pathway is modified genetically to ascertain the underlying mechanism responsible for the development of POCD (Table 3.2) for instance; knock out animals in which a particular protein is deleted from their genetic material.

Table 3.2: This table summarises the type of animals and rationale for their used in my thesis. The rationale of using a C57BL/6 background in genetically modified animals is to keep consistency with the expression of the rest of proteins.

Animal	Type	Protein Modified	Indication	Rationale
C57BL/6	Wild type	None	Characterisation Of the POCD model	Control and good resemblance to humans
P2X7-KO with C57BL/6 background	Knockout mouse (KO)	P2X7 receptor deleted from myeloid cell line	Identification of the role of IL-1 β in glial activation and development of POCD	Expect an on / off effect if it is the responsible protein for the development of POCD
Hsp72-Tg with C57BL/6 background	Over expressor mouse	Hsp72 cytosolic protein is transgenically over expressed	Cytoprotective mechanisms to prevent neuro-inflammation	Prevention of POCD after surgery and anaesthesia

3.1.1.a Wild type mouse

Wild type animals with no genetic manipulation, were used for developing models for memory assessment, surgical interventions and characterisation of the inflammatory response after surgery under general anaesthesia and after anaesthesia alone.

Wild type mice were C57BL/6-Wt male mice purchased from Harlan, Bicester, Oxon, UK. These animals were 14 weeks old (12-16) weighing 25-30g. C57BL/6-Wt male mice were also purchased from Charles River Laboratories, Wilmington, Massachusetts, USA for the experiments completed at Stanford University. The C57BL/6 wild type mice used at Stanford were older than the one used in UK. They were 24 weeks old (12-50) weighing 23-51g. The only

rationale for using different providers was the fact that these are the official providers for the laboratories where I carried out my experiments.

3.1.1.b P2X7 knock-out mouse

Genetically modified mice were used to characterise the danger signalling mechanisms and particularly the role of the P2X7 receptor on the neuroinflammatory changes seen in areas of the brain where memory is formed.

The P2X7 deficient mice were obtained from Glaxo-Smith-Kline (Harlow, Essex), these animals were backcrossed onto C57BL/6 wild type (Chessell, Hatcher *et al.* 2005). The P2X7-KO mice were 14 weeks old (12-16) weighing 21-40g. To maintain consistence through out the experiments and to avoid variability associated with age all genetically modified animals were young and C57BL/6 backcrossed.

The P2X7-KO mouse is a genetically modified mouse in which the gene responsible for encoding the proteins for this receptor was deleted by bioengineering. The lack of the P2X7 receptor on the myeloid cell line results in prevention of synthesis and release of pro-inflammatory cytokines when the cells were primed with LPS for 75m and then incubated for further 30m with P2X7 agonists and then compared with control cells from wild type mice (Solle, Labasi *et al.* 2001) therefore it would be expected that there will be an amelioration of glial activation and neuro-inflammation, on stimulation and hence a less chance of the development of POCD.

The P2X7-KO mouse (P2X7-KO) was generated according to published protocols (Conquet 1995) by GlaxoSmithKline laboratories (GSK). In brief, the P2X7 gene was isolated from a genomic library obtained from 129/Sv mice. Partial sequencing of the 5' exons allows ligation of 2 fragments into the neomycin resistant 'knock-out vector' pGN, a plasmid described previously (Le Mouellic, Lallemand et al. 1990). This resulting plasmid DNA which is an homologue recombination was injected into an embryonic stem cell which then produced cells with disrupted P2X7 gene hence the P2X7 receptor was not synthesised. These cells, also called "germline chimaeras", were injected into C57BL/6 wild type females to generate mice that were heterozygous for the P2X7 receptor mutation. These heterozygous mice were then crossed to produce homozygous P2X7-KO, homozygous wild type and heterozygous P2X7 offspring in the expected Mendelian ratios of 1:1:2. The homozygous P2X7-KO and wild type mice were then bred to produce pure breeding colonies of P2X7-KO mice and wild type mice. These animals were backcrossed 6 times to guarantee deletion of the P2X7 gene on the offspring before we received them for use at Chelsea and Westminster campus. Confirmation of absence of the P2X7 receptor was made by polymerase chain reaction (PCR) by GSK.

3.1.1.c Hsp72 Transgenic over expressing mouse

In order to gain a better understanding of the pathogenesis of POCD, a second and different type of genetically modified animal was used, the Hsp72 transgenic over expressing mouse.

The inducible cytosolic Hsp72 is cytoprotective and it has been implicated in reduction of neuroinflammatory changes and hence improving neurological outcome after stroke in animal models (Giffard, Han et al. 2008). Over expression of the cytosolic Hsp72 may well also prevent the neuroinflammatory changes associated with POCD.

The Hsp72 over expressing mice were bred by Dr Lijun Xu at Stanford University. The creation of Hsp72 transgenic mice (Hsp72-Tg) started with the use of adult wild type male mice expressing a chimeric transgene which consisted of a rat-inducible Hsp72 gene inserted into a vector called pCAGGS under control of the chicken-actin promoter and human cytomegalovirus enhancer which were originally produced by Dillmann and coworkers (Marber, Mestril *et al.* 1995). These mice were useful because the inducible Hsp72 amino acid sequence is 95% identical to that of the human inducible Hsp72 and 97% identical to that of the mouse inducible Hsp72. These mice over expressing Hsp72 will help to ascertain the intracellular mechanisms responsible for the development of POCD.

The founder mice were bred with mice of the same strain (C57BL/6 wild type). The DNA extracted from the tail of the resulting litters were then analysed by polymerase chain reaction using protocols described previously (Zheng, Kim *et al.* 2008). The brain levels of Hsp72 were previously shown to be elevated previously to about 10 fold in litters used to bred animals for the experiments carried out for this PhD thesis (Zheng, Kim *et al.* 2008).

3.1.2 General management

All animals were very carefully managed through out the experiments. They were allowed acclimatisation to the animal house for a week before allocating them to take part in experimental work.

Animals were also handled on a daily basis facilitating their adaptation to their new environment and to familiarise them to handling. This was to reduce the level of stress that comes with continuous handling, manipulation and change of environment. Every effort was made to limit the level of stress and only one or two operators handled them through out the experiments.

Animals were housed in groups of five in a 12-h-12-h light dark cycle environment in which temperature and humidity were controlled. The animals were allowed free access to food and water. The animal holding and experimental room was maintained at a temperature of 20-22°C and humidity at 35-55 %. Recognition and assessment of pain and distress was performed after each experiment. A table for assessment of animal's well being was created based on the recommendation made the central biomedical service at Imperial College London and the National Research Council, " guide for the care and use of laboratory animals" (http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals_Prepub.pdf). Animals' physiological scoring was documented in the perioperative period to ascertain their well-being (freedom from discomfort, distress and pain). The scoring system consisted in documentation of animals weight, locomotor activity, general appearance and behaviour. Score of 0 indicates no evidence of symptoms of suffering while a

score of 16 indicate severe illness. When animals score 7 or above they were terminated by schedule 1 method to the animal (scientific procedures) act 1986 (issued under section 21). Refer to appendix 1 for details of the physiological scoring used in the perioperative period.

Allocation of animals to experiments was performed in a random manner. Single animals from groups of five housed together, were individually allocated to a treatment group as they were collected from their cage on the day the experiments were initiated. Each cage of five animals contained all the corresponding treatment groups to avoid environment variability. Animals allocated to treatment groups were identified by their mark on their tails e.g. no mark for control/naïve group, 1 mark for anaesthesia group, 2 mark for surgery, etc.

3.1.3 Anaesthetic procedures

3.1.3.a Anaesthesia

Animals in all experiments received the same type of anaesthesia and analgesia. General anaesthesia consisted of the use of isoflurane anaesthesia delivered in air enriched with 30% oxygen.

Each animal received general anaesthesia, which lasted for 20 ± 5 min. General anaesthesia was induced with 5% isoflurane in a 30% oxygen-air mix at a flow rate of one L/min. Anaesthesia was maintained with $1.8 \pm 0.6\%$ isoflurane (approximately 1.4 MAC for mice) in 30% oxygen in air at a flow rate of one L/min.

The administration of anaesthesia was provided via a semi-closed circuit attached to a facemask. The vaporiser used in all experiments was an Ohio, Model 100F, Louisville, KY. All the residual gases were scavenged via a passive scavenging system (f/Air, Omnicon, A.M. Bickford inc. New York, Lot: 20050) (Figure 3.1).

The concentrations of isoflurane and oxygen were continuously monitored by using a gas analyser from VetEquip. Inc., Pleasanton, CA through out the surgical procedure. The depth of anaesthesia was monitored clinically by assessing the respiratory rate and heart rate.

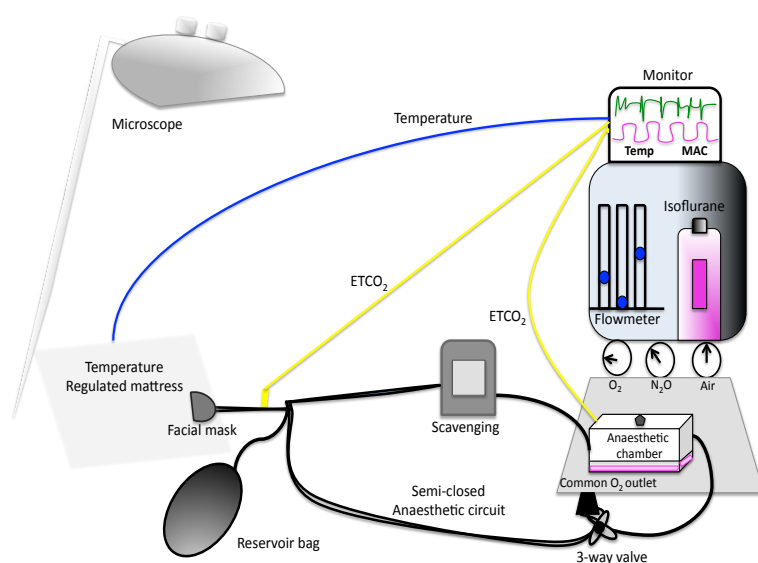


Figure 3.1: Schematic representation of the anaesthetic equipment utilised to deliver anaesthesia to rodents.

Five minutes after induction of anaesthesia, supplemental analgesia was provided by buprenorphine (Buprenex), 0.1mg/kg in saline, injected intraperitoneally (i.p.) in <1ml given over 5 min. Buprenorphine was chosen for all the experiments as it does not alter the immune response, at different concentrations, for up to 7 days after nociceptive stimuli while morphine does. (Martucci, Panerai *et al.* 2004).

Once animals were anaesthetised they were monitored closely to ensure they did not become either too deep and hence be injured or die, or too light and experience pain from the surgical procedure. Adequacy of analgesia and anaesthesia was assessed prior to surgical intervention by applying a firm pressure to the rear paw with a Gilles forceps (Fine Science Tools, GmbH, Fahrtgasse, Germany). If muscle contraction or purposeful movement were observed the animals' anaesthesia was deepened by increasing the concentration of isoflurane (increased MAC) and the stimulus was repeated prior to knife to skin. Furthermore, the depth of anaesthesia was monitored using the physiological variables seen as reflex responses to stress such as those described in (Table 3.3).

Table 3.3: Physiological variables utilised for assessment of depth of anaesthesia during surgical procedures.

1. Reflexes and simple tests

-
- a) Response to pain
 - b) Alteration in eye reflexes
 - c) Muscle tone

2. Physiological parameters

-
- a) Heart rate
 - b) Respiratory rate
 - c) Temperature

For postoperative analgesia, an eutectic mixture of local anaesthesia (EMLA) was applied to the surgical site on completion of the surgical procedure, followed by daily application until day 3 postoperatively. The assessment of mice normal activity, including signs of pain and hydration in the postoperative period was performed by using a physiological scoring system described in table 3.1, Appendix 1.

3.1.3.b Xenon anaesthesia

One study (chapter 8) used xenon anaesthesia as pre-treatment to try to prevent the development of POCD. The rationale for administering xenon in the acute setting was to try to identify a novel mechanism by which this gas could exert its function through modulation of proteins released after surgery.

The technique for anaesthesia was modified as follows (Figure 3.2).

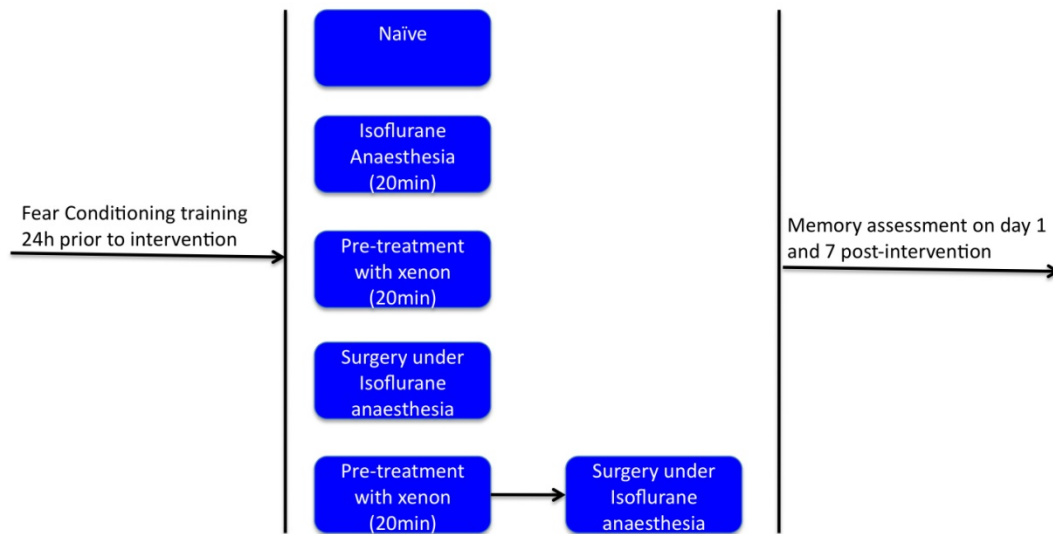


Figure 3.2: This is a schematic representation of the experiments where xenon was used as pre-treatment to prevent the development of POCD. There were two cohorts of animals. Cohort 1 was terminated at day 1 after surgery or allocated treatment and cohort 2 was terminated at day 7 after corresponding interventions.

The animal was subjected to breathing 70% xenon anaesthesia balanced with 30% oxygen for 20 minutes. It was then either terminated at different time points and the group served as a pre-treatment with xenon alone or it was immediately followed by introduction of isoflurane and surgery.

As the administration of xenon was performed just before induction of anaesthesia with isoflurane it was called 'pre-treatment' to differentiate this type of administration from xenon preconditioning. Xenon was administered in a closed system designed by Dr Ma (Figure 3.3).

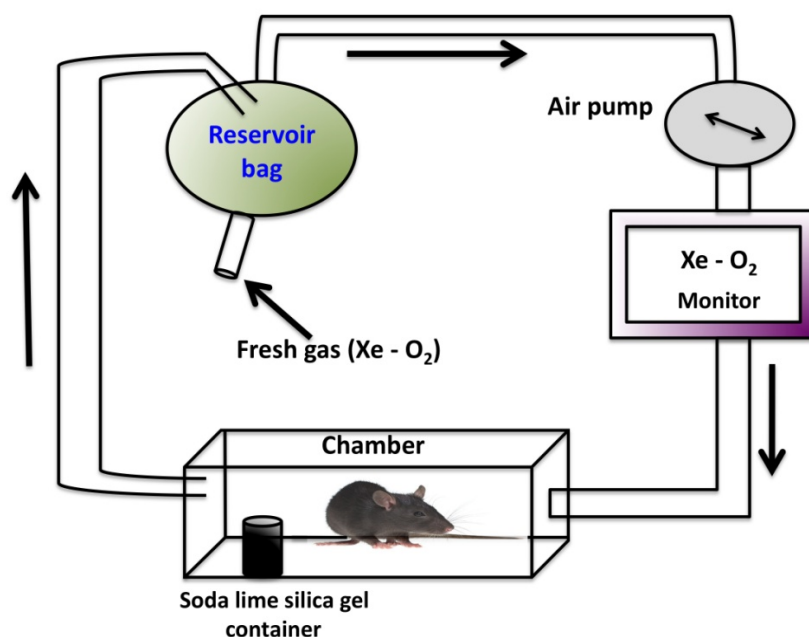


Figure 3.3: Schematic representation of the closed circuit used for the delivery of xenon in experiments performed in chapter 8.

Temperature was monitored throughout the surgical procedure using a rectal probe (CSi8D, Series, Omega) and temperature controlled regulated mattress (Harvard, apparatus).

3.1.3.c Regional anaesthesia technique

The spinal injection technique was a modification of an intrathecal technique described by Janice Hylden 1980. Animals were sedated with 3% Isoflurane for 5min before performing spinal anaesthesia. The site of injection chosen was based on previous illustrations published by Sidman 1971. Details of the mouse spine anatomy were obtained from the anatomy of the laboratory mouse website (Janice L.K. Hylden and George L. Wilcox. Intrathecal morphine in mice: a new technique. Eur J of Pharmacology 1980;67:313-316, R.L Sidman, J.B Angevine Jr, E.T Pierce. Atlas of the mouse brain and spinal cord. Harvard University press, Imperial College London

Cambridge, Mass 1971, <http://www.informatics.jax.org/cookbook/figures>, S.B.A Akerman. A methodological study of spinal (subarachnoid) anaesthesia in the rat and the mouse, BJA 1985;57:329-332).

A spinal technique was used when mouse restraining was achieved by using a homemade mice restrainer. The mice restrainer consisted of a vertical metal frame with grooves for a cushioned metal leaf that adjusted from the high onto the mouse body just below the xiphisternum angle to allow free movement of the upper part of the body. The mouse was held by the pelvic girdle with one hand, while the syringe was held on the other hand at an angle of about 20° above the vertebral column. The needle was inserted into the tissue on the middle line of the L4-L5 spine processes (Figure 3.4). The needle was then moved carefully forward to the inter-vertebral space as the angle of the syringe was decreased to about 10°. The tip of the needle was inserted approximately 0.5cm within the vertebral column. Spinal anaesthesia consisted of an injection of 10µl of 0.5% heavy bupivacaine. All intrathecal injections were performed with 30 Gauge ½ inch needle and 50µl luer tip glass syringe.



Figure 3.4: This figure illustrates the homemade physical restrainer used to perform spinal anaesthesia in mice.

To examine and confirm the staining of the spinal cord, three mice were injected with methylene blue dye then terminated by cervical dislocation (schedule 1 termination) five minutes after the injection. The distribution of dye was intradural in 2 mice. The distribution of the methylene blue was extended cephalic up to middle thoracic spine. The methylene blue was in soft tissues in 1 mouse.

A different cohort of mice (5 wild type C57BL/6) was anaesthetised with isoflurane before spinal anaesthesia was performed. There were 3 fatalities due to total spinal anaesthesia and in 2 mice the physiological scoring was 10 at 12h after their spinal anaesthesia and therefore they were terminated by schedule 1.

The dose of 0.5% heavy bupivacaine was reduced to 4 μ l and injected to 5 mice. A motor block was confirmed by observing the ability of mice to mobilise with their rear paw. This cohort developed adequate motor block allowing performance of tibial fracture surgery under light anaesthesia ($0.8 \pm 0.2\%$ isoflurane). There was 1 death within 24h of the surgical procedure and 2 mice were terminated the following day by schedule 1 as their physiological scores were 8 and 10. In view of all the difficulties of the procedure, number of complications and deaths, regional anaesthesia was discontinued.

3.2 Surgical techniques

The assessment of memory was performed after several different types of surgeries. This was to first confirm the robustness of the tests used and second with the intent of assessing the reproducibility of the findings after different type of surgeries.

The main surgical procedures performed were tibial fracture under general anaesthesia, nephrectomy and middle cerebral artery occlusion to emulate ischaemic stroke in order to ascertain the degree of involvement of P2X7 receptors in microglial activity after primary neurological events.

Surgical techniques were practiced in dead animals initially. There were 5 animals allocated to each type of surgery. Once I was familiar with the techniques I performed surgery in further 10 animals under general anaesthesia. Animals were kept alive for 24h and 10 days for assessment of immediate and

late complications related to surgery. A total of 61 animals were used for assessment of surgical reproducibility through out the PhD project (Table 3.4).

*Table 3.4: This table shows the number of surgical procedures performed before setting out to assess the development of POCD and different strategies to prevent it. In the group of cadavers the animals were terminated by schedule 1 and then proceeded to surgery. The group 'terminal anaesthesia' indicates the number of animals that were alive while surgery was performed but then were immediately terminated after the procedure. The groups under general anaesthesia include animals which received general anaesthesia as described previously and then were kept alive until day 10. *2/7 animals in the middle cerebral artery occlusion group did not have lesions compatible with stroke at day 10.*

Practice – Developing the technique					
Type of surgery	Cadavers	Terminal anaesthesia	Standard general anaesthesia (survival)		Physiological scoring
			Day 1	Day 10	
Tibial fracture	5	5	9/10	9/9	1 - 4
Unilateral nephrectomy	5	5	8/10	8/8	1 - 4
Middle cerebral artery occlusion	8	3	7/10	7/7*	1 - 2

Surgery was performed under general anaesthesia with fully aseptic technique. Animals were prepared and positioned under a microscope which was used to identify structures not properly visible under the naked eye. All animals were tested for pain as described under the anaesthetic section prior starting the experiment (Figure 3.5).

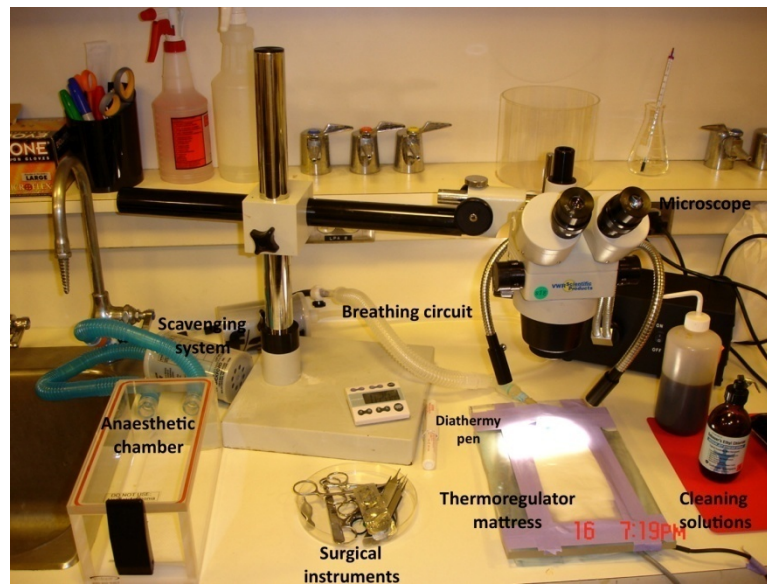


Figure 3.5: This photography illustrates the surgical environment where surgeries were performed.

3.2.1 Surgical experimental models

3.2.1.a Tibial fracture

This model was designed to provide reliable and reproducible results on memory deficits after surgery which may mimic POCD findings in humans. This is an aseptic orthopaedic model of surgery. This technique has been performed previously by Harry et al (Harry, Sandison *et al.* 2008) to assess the healing process of bone fracture. This model has also been used for assessment of POCD and to gain better understanding of the role of inflammation on the pathogenesis of memory dysfunction after surgery (Cibelli, Fidalgo et al. 2010)

In brief, the tibial fracture surgery was performed under general anaesthesia and it was based on Harry *et al* (Harry, Sandison *et al.* 2008). The surgery consisted

of skin preparation with chlorexidine gluconate 0.5% and isopropyl alcohol 70%. After the skin incision below the knee, soft tissue was reflected to expose bone through which a 0.3mm stainless steel rod was inserted into the medullary cavity (Figure 3.6).

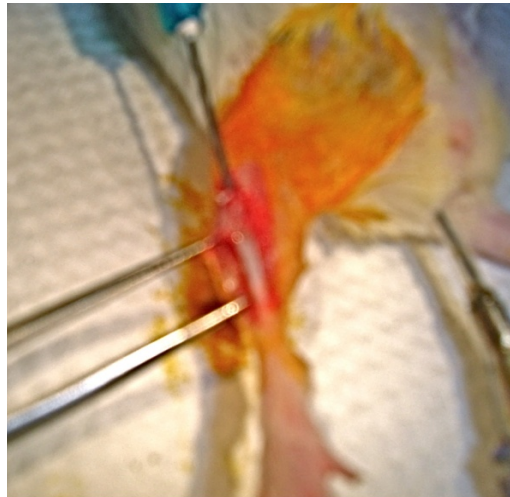


Figure 3.6: this is an illustration of the internal fixation of the tibia prior to the fracture.

Once the tibia was internally fixated, the bone was fractured in the mid-diaphysis (tibial, mid-shaft) using surgical pliers. The wound was closed using 2-0 Nylon suture (Ethicon Inc., Somerville, NJ). Post-intervention animals were left to recover on heated pads, closely monitored while recovering from anaesthesia and then transferred to their own cages, where food and water were available. Physiological scoring tests were done daily for the first 72h.

Tibial fracture surgery was performed in 300 mice for assessment of POCD. There were 7 deaths (2.3%) within first 24h of the operation, 5 termination by schedule 1 (1.6%) as the physiological scores were above 7 and 9 animals were

removed from the study (3%) as these were used to build up the cell response to surgery over time (in hours) for the flow cytometric analysis. There were further 48 tibial fracture surgeries performed for validation of the memory paradigm and 12 tibial fracture surgeries were performed for retrieval of tissues for assessment of peripheral and CNS inflammatory response.

3.2.1.b Unilateral nephrectomy

This model was designed to assess reproducibility of POCD from the tibial fracture surgery findings. This surgery was also used for assessment of other type of memory paradigm which requires swimming skills hence no trauma or limb surgery around the time of training and assessment of POCD was paramount.

In brief, the surgery consisted of skin preparation with chlorhexidine gluconate 0.5% and isopropyl alcohol 70% then a 5cm longitudinal abdominal skin incision was made in the midline. The intestine was gently displayed from the abdominal cavity and irrigated with warm 0.9% normal saline and covered with a warm and wet gauze. The retroperitoneal structures were identified under the microscope. The left kidney was mobilised the ureter identified and ligated and the vascular pedicle ligated proximally and distally and finally the left kidney was removed (Figure 3.7).

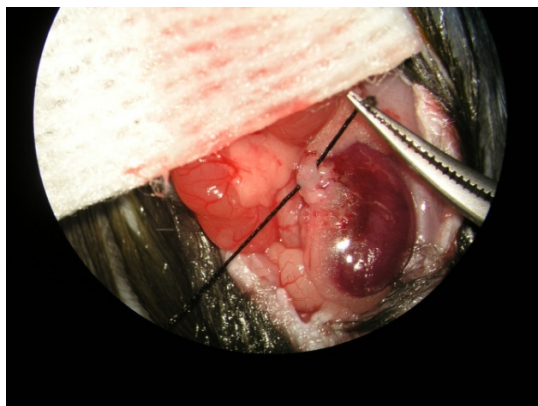


Figure 3.7: This figure shows the isolation of the left kidney and identification of the vascular pedicle.

The intestine was returned to the abdominal cavity and the peritoneum was closed with size 4-0 sutures and the skin was closed using metal surgical clips (Harvard Apparatus, Kent, UK). Buprenorphine 0.3mg/Kg was injected subcutaneously around the wound incision to provide analgesia in the postoperative period. A bolus of 800µl of 0.9% normal saline was administered in the peritoneal cavity to replace the insensible loss.

Post-intervention animals were left to recover on heated pads. They were closely monitored in the recovery box until they fully recovered from anaesthesia. Once animals were able to drink and move freely around the recovery box they were then transferred to their own cages, where food and water were available. A further bolus of 800µl of 0.9% normal saline was administered in the peritoneal cavity 8h and 24h after surgery. Physiological scoring tests were done daily for the first 72h.

Unilateral nephrectomy was performed in 50 mice. There were 6 deaths (12%) within the first 24h after surgery. Two mice (4%) were excluded at day 3 after surgery as their weight was less than 90% of the initial weight and they also showed signs of poor grooming. These 2 animals were terminated by schedule one and tissues discarded.

3.2.1.c Transient middle cerebral artery occlusion

Transient focal ischaemia was induced by transient occlusion of the left middle cerebral artery. This surgical model of ischaemic stroke was used for assessment of the role of P2X7 receptors on the reactive glial cells and also for the assessment of the infarct size after a primary neurological insult. The rationale was to modulate the neuroinflammatory changes with P2X7 receptor antagonists and if there was a clear reduction in infarct size then the second step could be testing P2X7 receptor antagonists for prevention of developing POCD after tibial fracture surgery.

In brief, the surgery consisted of skin preparation with chlorhexidine gluconate 0.5% and isopropyl alcohol 70%. The left common carotid artery was identified after a vertical middle line neck incision. Then the internal and external carotid arteries were identified under the operating microscope. The confluence of the common carotid artery and left carotid artery was mobilised very gently displaced and then ligated. A 6/0 silicon-coated nylon filament was introduced into the common carotid artery and advanced about 9 mm beyond the carotid bifurcation for temporal occlusion of the middle cerebral artery (Figure 3.8).

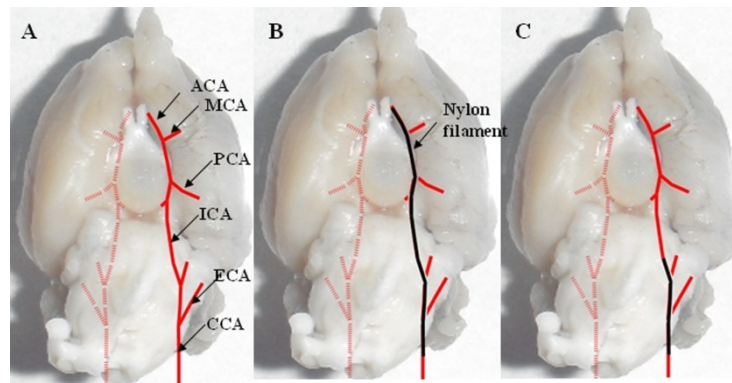


Figure 3.8: This is an schematic illustration of the cerebral arteries distribution and how the filament is advanced. A. Illustrates the main artery branches: Anterior cerebral artery (ACA), Middle cerebral artery (MCA), posterior cerebral artery (PCA), internal carotid artery (ICA), external carotid artery (ECA), common carotid artery (CCA). B. Illustrates the position of the nylon filament (black) and C. This illustration represents a failed operation, also used as a sham model. This is an illustration reproduced with permission of Al-min Ji from his paper published in the *Neuroscience letters*, Vol 519, Issue 1 on 12th June 2012.

The skin was sutured with silk 2/0. Animals were recovered from anaesthesia and then transferred to their home cage. The total procedure took around 12 ± 4 min. After 120 min animals were re-anaesthetised briefly for filament removal allowing reperfusion of the ischaemic brain and intraperitoneal injection of Placebo (S-P) or brilliant blue-G (S-BB-G) was given.

MCAO surgery was performed in 18 wild type C57BL/6 mice. A total of 3 mice were excluded from the study; one mouse with no neurological deficits (placebo group), one mouse with haemorrhagic stroke (treatment group) and one mouse died within 24 h (placebo group).

3.3 Other treatments

3.3.1 Lipopolysaccharide (LPS) challenge

Sickness behaviour due to a systemic infection is classically described in the literature and it has been explored extensively (Godbout, Chen et al. 2005; Cunningham, Campion et al. 2007; Swiergiel and Dunn 2007) therefore, it was decided to use LPS derived from *Escherichia coli* endotoxin (0111:B4, InvivoGen, USA, 1mg/Kg) dissolved in 1ml of normal saline and injected intraperitoneally to test the memory paradigm developed for assessment of POCD.

The dose of LPS used for experiments in this thesis was based on previous experiments carried out at Chelsea and Westminster campus by Fidalgo et al (Fidalgo, Cibelli et al. 2011). A small pilot study where mice (3 mice per group) were treated with 10mg/Kg, 3mg/Kg and 1mg/Kg of LPS intraperitoneally was performed before the hypothesis for this thesis was tested. Mice treated with 10mg/Kg died within the first 2h of treatment (100% mortality). Mice treated with 3mg/Kg developed severe sickness behaviour, 1 died within 24h of the treatment and 2 were terminated by schedule-1 as their physiological score was 12 for the 2 of them). Mice treated with 1mg/Kg behaved normally at 24h after treatment and their physiological score were 0 to 1. Mice challenged with LPS served as positive control for initial validation of memory tests conducted by fear conditioning training (Figure 3.9). See details of behavioural studies below.

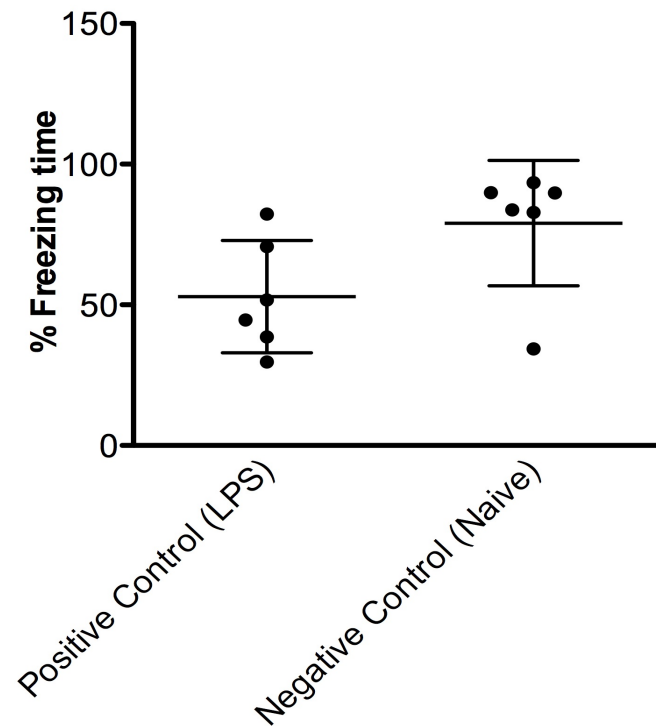


Figure 3.9: This scatter histogram illustrates the negative effect of LPS challenge on memory after 48h of fear conditioning training. There were 6 wild type C57BL/6 mice per group. All mice were trained by fear conditioning following a challenge of 1mg/Kg LPS intraperitoneally in the positive control group. The negative control group received 800 μ l of normal saline intraperitoneally in an attempt to maintain the same level of stress in both groups of animals. The % Freezing time alludes to the ability of the mouse to recall the environment where it learnt by association and therefore, developed its memory. The higher the % of freezing the better the memory preservation is. The % freezing time (median/IQR/range) was much lower in the positive control group (48.2/36.7-73.5/29.7-82.3) than in the negative control group (86.8/70.7-90.8/34.2-93.4) indicating the memory test was suitable for assessment of POCD and the dose of LPS safe enough not to cause any accidental death.

The intraperitoneal route was the preferred route for administration of LPS for experiments that involved assessment of memory as animal handling was similar to the one utilised for the memory experiments and therefore, animals were not distracted from their normal daily activities. LPS was injected in 20 mice. These animals were monitored closely for signs of sickness behaviour for 2, 6 and 24h post injection. None of the animals died or were terminated by schedule 1 before the stipulated period for completion of experiments to which they were allocated.

3.3.2 Retrieval of brain tissue from newborn mouse pups

The assessment of hippocampal cells were performed in tissues obtained from adult mice and newly born pups. The retrieval of the hippocampal tissue from newly born pups mice was performed after the pups were terminated by schedule 1. The brains were retrieved from the pups cranium and freed from meninges and the cortical hemispheres removed from the rest of the brain under microscopic guidance. The hippocampus was identified, retrieved then minced and treated with 0.09% trypsin for 20m at 37°C. After centrifugation at 4000rpm (40G) for 3m the pellet was re-suspended in 2ml normal media by gentle trituration through a flame-polished Pasteur pipette. The hippocampal cell suspension was then used for FACS experiments.

3.4 Behavioural studies for assessment of POCD

3.4.1 Pre-intervention assessment of physiological variables

In this thesis, memory was explored in animals after surgery and anaesthesia. It was therefore important that physiological variables were measured before

exploring differences in memory within groups as well as between wild type and genetically modified animals.

The fear conditioning paradigm requires an intact sensorial reflex, specifically the reflex to pain. It also requires an intact acoustic reflex, and normal motor activity. The MWM requires coordination hence normal motor activity too. The following techniques are described in more detail below. The reflex to pain was assessed by using the hot plate. The acoustic reflex was assessed by using the startle reflex chamber and the motor activity was assessed by using the rotarod and or a smart cage where animal's activity was recorded in real time into a computer software over a period of time. The techniques and equipment used for assessment of the above physiological variables are described below.

3.4.1.a Hot plate

Assessment of the pain threshold was part of the battery of tests used for assessment of physiological variables before establishing memory. Mice that were subjected to neither anaesthesia nor surgery were assessed for sensory and motor activity to detect possible behavioural differences between genotypes.

Pain threshold was assessed using a hot plate at 55°C (IITC, Life Science) to test acute thermal nociception. The latency to the nociceptive response was the time from the moment the mouse was put onto the hot surface which was surrounded by a transparent cylinder with a top cover until the mouse started licking the hind paw or jumping. A cut-off time of 30s was applied to protect animals from burn injury or distress (*Figure 3.10*).



Figure 3.10: This picture illustrates the hot plate. The hot plate is used for assessment of physiological variables before fear conditioning training. The pain pathway is of vital importance as the noxious stimulus (foot-shock) is one of the components of the training paradigm used for establishing memory.

3.4.1.b Acoustic reflex

The acoustic function was performed as part of the assessment battery for assessment of the physiological sensorial activity of the mice and it is carried out before performance of fear conditional training. It was of vital importance to identify if there was any acoustic deficiency as a cue (Tone) was used for establishment of memory before surgery and anaesthesia. Acoustic function was tested in a mouse startle reflex chamber made of plexiglass vented with holes that are attached to a spring-loaded platform hinged to a support stand (Figure 3.11).

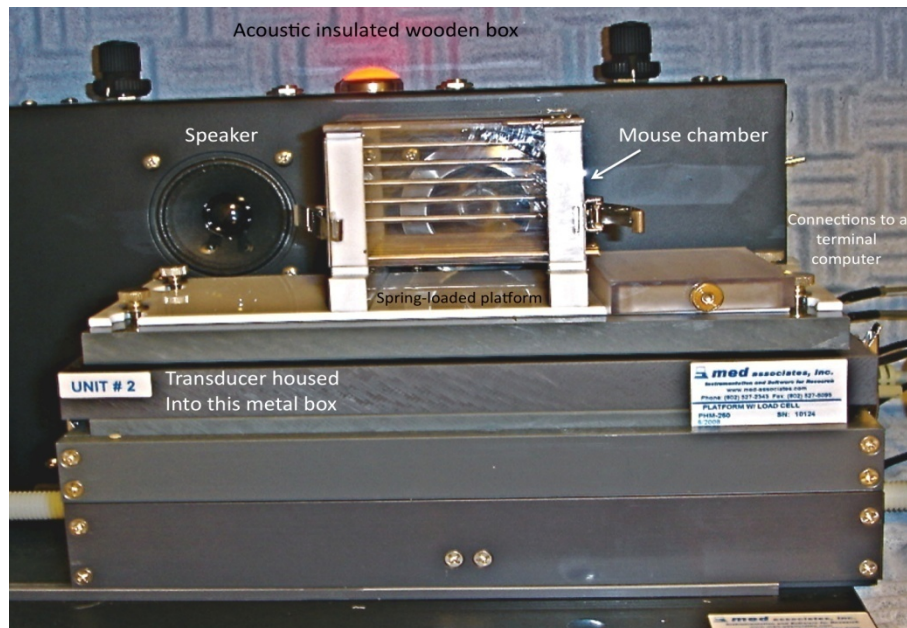


Figure 3.11: Mouse startle reflex chamber from Med Associate Enc. was used for testing mice acoustic reflex.

A transducer assessed the displacement force of the animal chamber. The entire apparatus is housed in an acoustically insulated wooden box containing speakers (Med Associates Inc.). The startle stimulus consisted of applying 110dB of white noise lasting 10s. Latency to startle response was used as a measure of sensorineural hearing (acoustic reflex).

3.4.1.c Rotarod

The motor function assessment was performed for experiments with genetically modified animals. The animals' motion and equilibrium was assessed by using the rotarod. The latency to fall from the rod running at 60rpm was used to identify any discrepancy between groups. A cut off of 250s to fall was used to stop the experiments (Figure 3.12).



Figure 3.12: This picture shows the boxes where motion and equilibrium was assessed in mice. Up to 4 mice were assessed simultaneously and directly recorded on the local computer terminal.

3.4.1.d Spontaneous motor activity

Spontaneous activity was another form of assessment of mice normal locomotion. Mice were transferred to cages that were similar to the ones in which they were housed normally. Spontaneous locomotion was assessed for a period of 60m, 72h prior to surgery using the Smart-Home Cage System (Afasce, Inc; loaned by Dr. Xingmin (Simon) Xie). Active count and travel distance was used as a measure of motor activity detected as breaks of the infrared sensors crossing the cage (Figure 3.13).

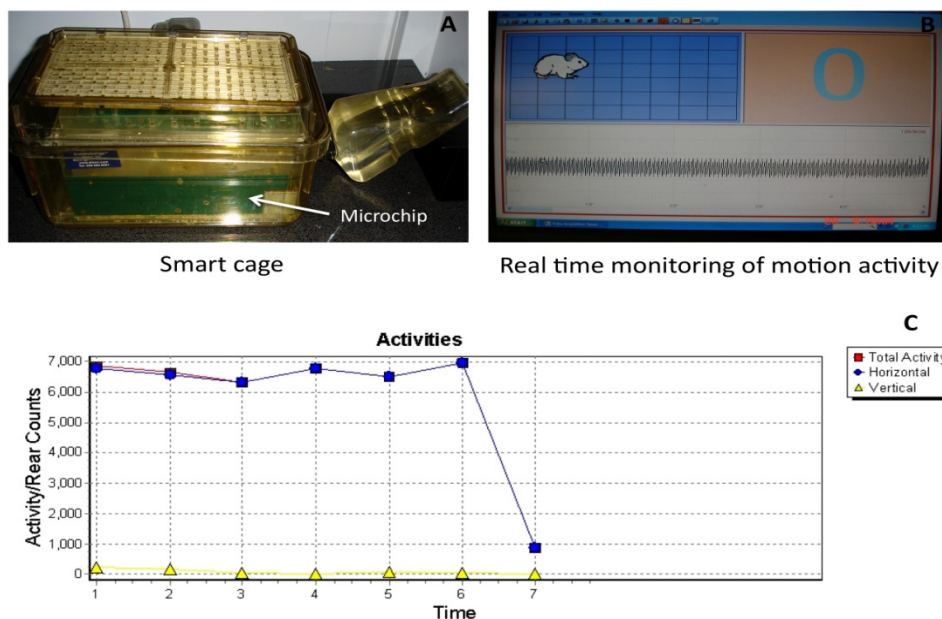


Figure 3.13: This figure illustrates how motion activity was assessed in the smart chamber. A) An ordinary mouse chamber is converted into a smart cage by adding microchips around the 4 walls of the cage. Food and water was provided throughout the assessment so the mice were not stressed and their behaviour and level of activity resembled that in their own normal and familiar habitat. B) This shows a snapshot of the computer screen recording the mouse activity in real time. C) An illustration of how the data for total activity is analysed for each individual mouse based on the mouse motion during the assessment period.

In summary the outcome measures used to assess the physiological variables prior to the experimental work were the acoustic reflex in the mouse startle reflex chamber (latency to startle in seconds, pain threshold in the hot plate (latency to pain in seconds) and motor activity in the smart cage or rotarod (travel distance in cm). Results of the cohorts of animals assessed are summarised in table 3.5, appendix 1.

3.4.2 Optimisation of behavioural tests for assessment of POCD

Behavioural studies were performed to assess memory in animals. To this end, animals were allowed to learn by 'association' and then memory was assessed at different time points after surgery and anaesthesia to ascertain the development of POCD.

3.4.2.a Fear-conditioning

Learning and memory was established by fear-conditioning training (FCT). The FCT paradigm consisted of a training phase prior to surgery and an evaluation or assessment phase after surgery when memory was assessed (Figure 3.14).

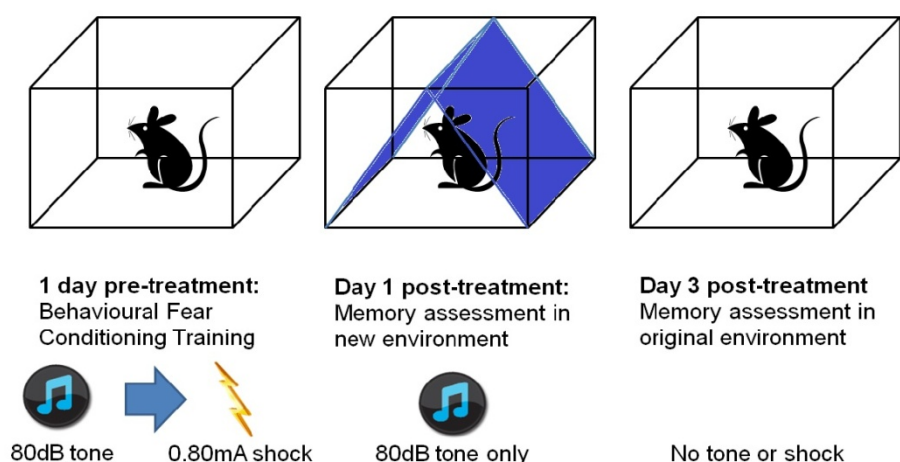


Figure 3.14: This is a representation of the different phases of the fear conditioning training (FCT). 1 day pre-treatment animals were trained to learn by associating a cue (tone of 80dB) with a painful stimulus which followed (shock of 0.80mA). 1 day post-treatment animals memory was assessed in a new environment and again at day 3 post-treatment (it could also be day 7 post-treatment) memory was once again assessed but at this time in a familiar environment. (This figure was published as part of a poster for the ARS in 2011).

A fear conditioning paradigm was developed in which mice learnt conditional reflexes by association to establish both hippocampal dependent and hippocampal independent long-term memory which was used as a surrogate of retrograde memory. In developing this paradigm the time for training, the number of conditional stimuli, and the intensity of stimuli for establishment of long-term memory were standardised. The time of exposure to a familiar environment, called context or contextual period, was investigated, prior to a subsequent conditional stimulus.

There was no difference in acquisition between training during light and dark times of the cycle when a cohort of 8 wild type C57BL/6 mice was trained during day time and another cohort at night time (a total of 8 mice per group); however, animals trained during daytime freeze more (median $\pm$ SD) than mice trained at night during the assessment phase (64.5 $\pm$ 24.4 and 51.7 $\pm$ 18.4 respectively) indicating that recollection was better during daytime. Therefore, animals were trained and assessed during the light cycle, consistent with prior results (Chaudhury and Colwell 2002).

A cohort of C57BL/6-Wt mice was assessed with a short paradigm (2-pair of conditional-unconditional stimuli), a medium length paradigm (6-pair of conditional-unconditional stimuli) and a long paradigm (8-pair of conditional-unconditional stimuli). These mice were trained to ascertain the numbers of conditional-unconditional pairs of stimuli required to develop and consolidate new information (acquisition). Two or 8 pairs were less effective for establishment of long-term memory as there was large intergroup variability

(median freezing time $\pm$ SD expressed as percentage of total freezing time was used as surrogate of memory; 59.4 ± 16.9 and 30.6 ± 9.1 respectively) than 6 (50.6 ± 5.1) (Figure 3.15). Therefore, 6 pairs were used for the subsequent studies. The pairs of conditional-unconditional stimuli were separated by random intervals between 45 and 60sec each to prevent associative conditioning in between trials.

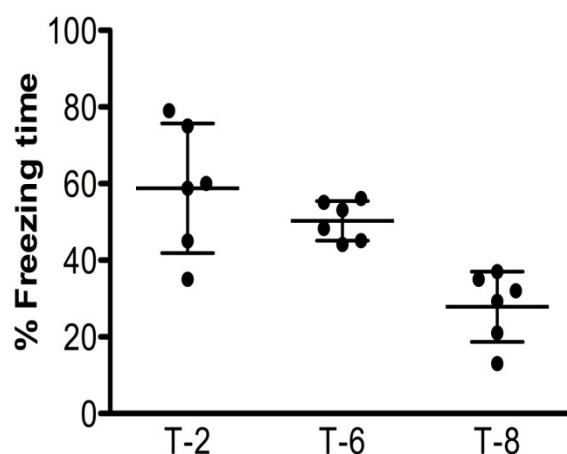


Figure 3.15: This scatter histogram illustrates the development of the fear conditioning training. Wild type C57BL/6 mice virgin from treatment were trained in cohorts of six each at each time point. Training -2 (T-2); represents the cohort of mice trained with 2 pairs of conditional-unconditional stimulus. Training -6 (T-6); represents the cohort of mice trained with 6 pairs of conditional-unconditional stimulus and Training -8 (T-8); represents the cohort of mice trained with 8 pairs of conditional-unconditional stimulus. Data is presented as median $\pm$ SD.

The time required to establish long-term memory was also assessed. Twenty four hours was identified as the minimum interval to establish long-term memory. This was assessed in a different group of C57BL/6-Wt mice prior to the experiment. Animals were trained with a set of conditional stimuli and then

assessed for memory formation in their training context and also in a novel context at different time points. Memory was established at 24h post training and then surgery was performed.

The final optimisation of the fear conditioning training paradigm was achieved by testing mouse ability to retain information by association after cleaning the training chamber with different cleaning solutions e.g. alcohol gel, alcohol spray and alcohol-free spray. The % freezing time (median $\pm$ SD) was higher in the alcohol gel group (89 $\pm$ 8.9) than in the alcohol spray (51 $\pm$ 8.9) or alcohol-free spray (38 $\pm$ 6.3) (Figure 3.16). In view of these findings the cleaning solution utilized for experiments performed in this PhD project was alcohol-free gel or alcohol-free spray.

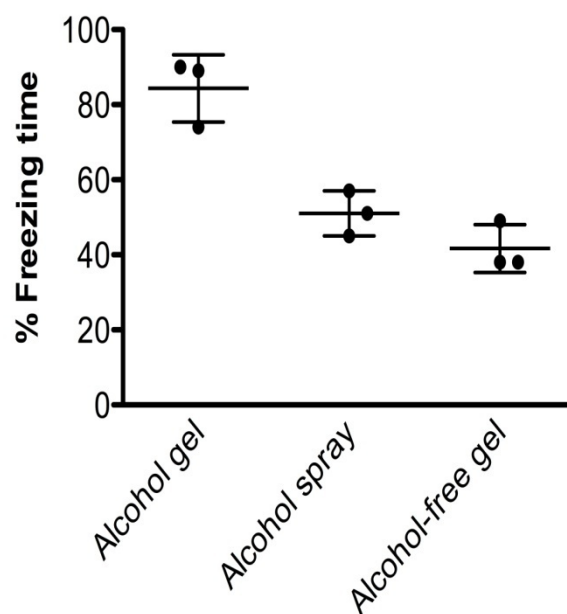


Figure 3.16: This histogram illustrates the effect of different cleaning solutions on memory acquired by association during fear conditioning training. There were 3 cleaning solutions tested in wild type C57BL/6 mice: 1) Alcohol gel, 2) Alcohol

spray and 3) Alcohol-free spray. A total of 3 mice were tested per group. Data is presented as median $\pm$ SD.

In brief, the fully developed and optimised fear conditioning training paradigm used to test the hypothesis is illustrated in Figure 3.17. This paradigm is divided into 3 phases: A. Pre-exposure phase also known as a training phase. During this phase the animals' acquired/learned by association of a cue to a noxious stimulus that is repeated in pairs up to a total of 6 pairs. B. Post-exposure phase or memory assessment phase which is performed in a familiar environment (context) or in a new environment where the cue was present. The post-exposure phase takes place at different time points after surgery and or anaesthesia. Normally these time points were on day 1, 3 and 7 after corresponding interventions.

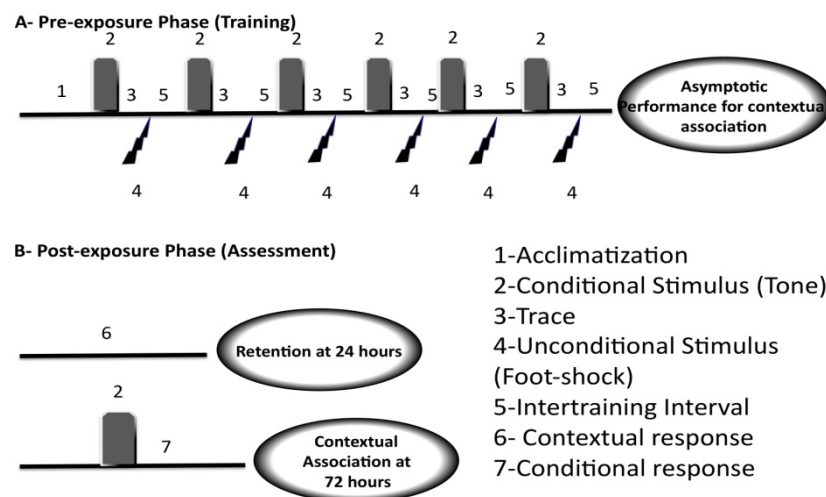


Figure 3.17: Schematic representation of the fear-conditioning training paradigm developed for assessment of memory. A- Pre-exposure phase or training phase takes place 24h before surgery. It consisted of an acclimatisation period following by pairs of cue (Tone) and noxious stimulus (shock). The mice learnt by association

after 6 continuous trial sessions of pairs of conditional (Tone) and unconditional stimulus (foot-shock) delivered in the fear conditioning training chamber. The mice achieved maximal performance by trial 6, maximal performance was also described as an asymptotic performance for contextual association. B- Post-exposure phase or assessment phase: This took place at different time points after surgery or planned interventions. In the assessment phase it was intended to differentiate animals that were able to retain their memory from those which developed POCD. The difference between groups was ascertained by the % of freezing which indicates the mouse ability to recall what it was learnt by association before the intervention.

The fear conditioning training consisted of an acclimatisation phase where the animals were allowed to explore the box; then this period was followed by a cue (Tone) or conditional stimulus for 20sec and then a contextual interval of 25sec (Trace) separated the cue from the noxious stimulus (Shock) also known as unconditional stimulus. The contextual interval (Trace) is basically the time between conditional and unconditional stimulus. The trace was used for assessment of contextual memory which is also known as hippocampal dependent memory. An interval of 25sec was chosen as it was previously shown that less than 18sec is not enough for establishment of hippocampal memory and above 30sec other regions of the brain may be involved (Misane, Tovote *et al.* 2005).

The cue or conditional stimulus consisted of a tone of 0.80dB. The rationale for choosing 0.80dB was that a weak cue (less than 50dB) may not trigger the associative mechanisms as if it is too weak it does not increase connectivity within the learning centres and if it is too strong, more than 85dB, it acts as an unconditional stimulus and the pain centre may be activated (Davis and Astrachan 1978; Ito, Pan et al. 2009).

For the unconditional stimulus a 0.80mA shock was chosen which triggers maximum freezing behaviour (Young and Fanselow 1992). The shock generates fear, so if it is too weak the stimulus may not trigger fear while if it is too strong it may activate pain pathways creating a confounding variable which may make difficult to interpret the effects on behaviour.

Training was accomplished 24h before surgery. Mice were allowed to familiarise themselves with the surroundings (Context) for 120sec. A 20sec 80dB tone (Conditional stimulus) was then again delivered after a delay of 25sec. This contextual interval was terminated by an unconditional stimulus, which was a 0.80mA for 2ms electrical foot-shock.

After 6 pairs of conditional-unconditional stimuli the mice learned the association and established long-term memory. The pairs of conditional-unconditional stimuli were separated by random intervals of between 45 to 60sec. This was the inter-training interval. The inter-training interval allowed the mice to disengage from the process of association before a new set of stimuli was introduced. After the fear was established, the mouse would freeze in

response to exposure to the environment where it has received a noxious stimulus or if in a new environment when the cue is applied. The percentage of time spent not moving (freezing time) was determined (% freezing time). Freezing time measured during exposure to the known context or after a conditional stimulus in the known context reflects hippocampal dependent memory while assessment during delivery of the conditional stimulus (tone) identifies hippocampal independent memory.

Following the training session animals were returned to their normal chambers and allowed to disengage for 24h from their fear conditioning training activity. Surgery, anaesthesia or planned intervention was performed 24h after their training and once animals fully recovered from anaesthesia they were returned to their cages.

The post-treatment phase consisted of assessment of memory retention. Animals' memory was assessed in a normal environment to them (Contextual memory) also known as hippocampal dependent memory. It was initially at, 6h, 12h, and on the post-experimental day 1, 2, 3, 5, 7 and 10 to gain full understanding of mice behaviour and memory retention after corresponding interventions (Table 3.6)

Table 3.6: This table illustrates the hippocampal dependent or contextual memory results after surgery and their counterpart naïve controls at different time points. This was a cohort of wild type C57BL/6 mice. There were 2 groups; 1) Naïve group (7 mice) and 2) Tibial fracture surgery group (6 mice). Data is presented as median±SD. There is a large intergroup variability and it could well be due to the small number of animals per group. Different cohorts of animals were used at each single time point.

Time of memory assessment after surgery	Naïve (Total 7) median±SD	Tibial fracture surgery (Total 6) median±SD
6h	70±22.2	39.5±12.4
12h	50±6.7	35±10.7
24h	51.3±6	33±9.5
48h	48±8.7	23.5±10.4
72h	46±6	23.6±10.6
5d	47±5.9	27.3±9.7
7d	48±3	33.5±5
10d	41.7.5	30.5±9.2

Assessment of hippocampal dependent and hippocampal independent memory was also performed in a novel environment.

To create a novel environment (Context) which was different from the normal or known environment, the shape of the chamber was changed from rectangular to triangular, the ceiling colour was black instead of white, the flooring rods were covered with a smooth white surface and background noise was removed (Figure 3.18).

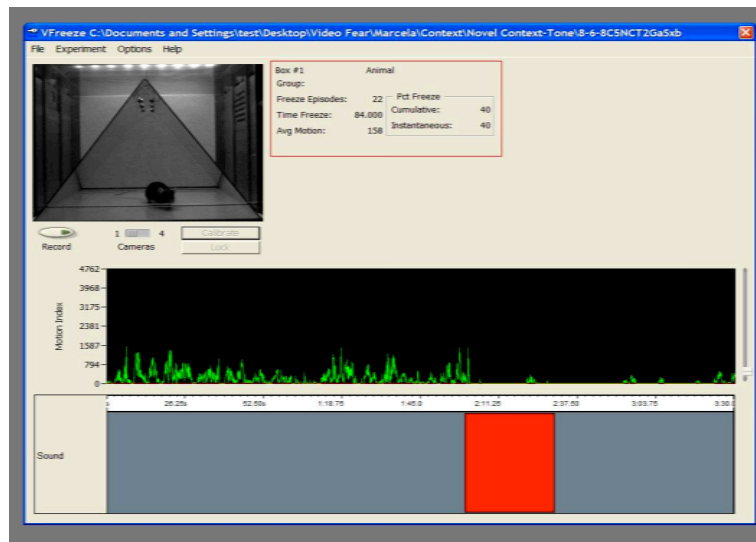


Figure 3.18: This snapshot illustrates the assessment of memory on day 3 after surgery. On the top right corner of the photography is the modified chamber used to create a novel environment for assessment of memory after a cue (tone) is introduced (red bar in the photography). The green oscillations indicate the mouse is mobile or wandering about in the chamber. The first part shows how mobile the mouse was (no freezing) oscillations of high amplitude and frequency (green waveforms) as the environment was new to it but soon after the introduction of the tone, the mouse became less active (oscillations are reduced in amplitude and frequency suggesting the presence of an associative memory hence there is an increase in the freezing time illustrated by the flattening or practically disappearance of the green line on the photography).

Following surgery or anaesthesia hippocampal dependent memory was assessed in a familiar and also in a new environment to the mouse. Hippocampal independent memory was however assessed in a new environment on days 1, 3, and 7 on the experiments the hypothesis was tested.

On each day of testing memory, mice were transported to the behavioural room and left undisturbed for at least 60min to allow them to adapt to the new place and settle down from the travelling from the animal house to the behaviour room before placing them into the fear conditioning training chambers.

Analysis was performed by a computer-based system (Freeze-Frame/Freeze-View modes, Actimetrics Software). In brief, the software retrieved the information in a video format recorded by the infrared camera positioned in the chamber. Any lack of movement by the mouse was recognised by the software and recorded as a freezing time. The freezing time is also known as the bout length. The bout length is the time that a single episode of freezing must be equal or exceed. For the experiments in this thesis a single episode of freezing was set at 0.75sec. It means that an episode of freezing was counted when the mouse stopped moving for 0.75sec or more. The percentage of time spent freezing over the total time spent in the chamber to complete the test, was used to score memory.

The threshold for freezing was chosen according to the individual animal motion index histogram (Figure 3.19). The animal index motion histogram illustrates the cessation of movements, which included the absence of fur, vibrissae, and skeletal movement with exception of breathing. Percentage of total time spent freezing was used as an indicator of memory formation during training and retrieval of information after treatment.

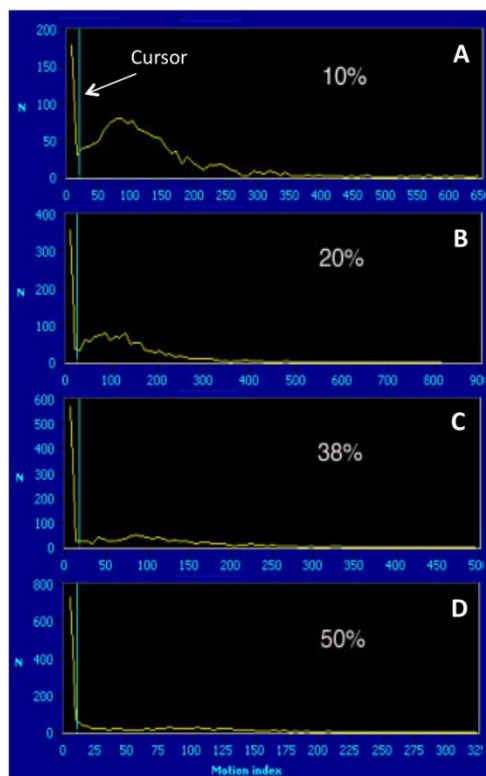


Figure 3.19: This figure illustrates how a freezing threshold is chosen. The freezing threshold is selected by dragging the cursor (blue vertical line) in the histogram. The value is selected by observing the motion index histogram (x axes). When the animals are first introduced to the chamber they show a high broad peak indicating high motion, it basically means “no freezing”. Then there is a second broader peak between 50 and 100 indicating “active movement”. There is also a characteristic trough in between these two peaks in the range of 20-30% of index motion. The threshold is set at the value of the trough. These 4 histograms illustrate the right threshold for different trials. The % in white indicates the percentage of freezing. A) 10% Freezing time. B) 20% Freezing time. C) 38% Freezing time and D) 50% Freezing time. The freezing threshold is set for each mouse individually or it can be set for all trials. The freezing threshold selected for experiments performed for this thesis was the same for all the trials (20% of index

of activity) but individual histograms were analysed when there was a high inter group variability. The histograms were extracted from the Coulbourn instruments manual (Coulbourn instruments, 7462 Penn Drive, Allentown PA, 18106).

3.4.2.b Morris water maze

Spatial memory and memory related to the surroundings is hippocampal dependent and therefore I felt it was essential to test it by using different methods for memory assessment when therapeutic interventions such as statin were introduced.

Therefore to provide further evidence in terms of the memory assessment of the animals, the paradigm of the Morris water maze (MWM) is chosen (Rosczyk, Sparkman *et al.* 2008) (Figure 3.20).

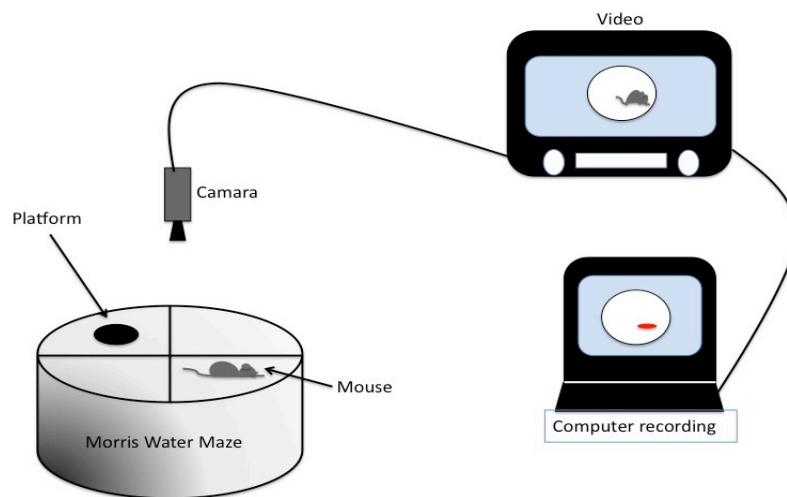


Figure 3.20: Schematic representation of the Morris water maze set up.

The Morris water maze (EthoVision®; Noldus, Wageningen, Netherlands) consists of a pool of water (diameter: 120 cm, height: 40 cm) maintained at a

temperature of $26.5 \pm 0.2^{\circ}\text{C}$ with a hidden submerged platform (1cm below surface) in one quadrant. Mice are placed in the water in a dimly-lit room with various visual clues around the pool. A video camera mounted to the ceiling directly above the centre of the tank in conjunction with a computerised animal tracking system is used to monitor swim speed, escape latency (time taken to find the submerged 'escape platform') and distance swam. Mice undergo daily testing with three trials (cut off time-90 seconds) per day for 5 days before surgery and 7 days after surgery. Each of the swimming trials is begun from a different quadrant. A minimum interval of 10m is allowed between each trial. Additionally, on the 3rd or 7th post-experimental day, the probe trial is performed; this involved the platform being removed before allowing the mouse to swim for 90s. The time spent in the quadrant previously containing the submerged platform was recorded and represented an index of memory. Finally, reversal learning is assessed after the 7th experimental day; this involves cryptically moving the hidden platform. After the animal has been thoroughly trained at one location (the initial position of the platform during training), the platform is moved to a different quadrant of the pool. Because it is hidden, it is not apparent that anything has changed until the animal fails to find the platform in its usual place. The focus is on how the animal reacts to this change and how quickly it learns the new location. All of the trials were completed between 09.00 and 17.00h.

The MWM paradigm for establishing and assessing memory was extracted from previous experiments performed for assessment of memory after hepatectomy (Cao, Ma et al. 2010) and neurodegenerative diseases (Ongali, Nicolakakis et al.

2010). The MWM protocol used in my thesis had been previously used at Chelsea and Westminster campus therefore validation or pilot studies were not rerun before initiation of the experiments.

3.4.2.c Neurological assessment – Garcia Score

Assessment of CNS injury, mainly ischaemic stroke, was performed under general anaesthesia, therefore it is important to establish the degree of motor deficit after surgery. Neurological assessment is used to ascertain if the surgery is successful, to identify differences in motor deficits between groups, whether it correlates with the infarct size and the treatment strategy used to modify the CNS inflammatory response.

The neurological assessment is performed 24h after middle cerebral artery occlusion (MCAO) by using the Garcia scale (Garcia, Wagner *et al.* 1995) which consisted in the assessment of the following 6 domains:

1. Spontaneous activity
2. Symmetry in movement of 4 limbs
3. Forepaw outstretching
4. Climbing
5. Body proprioception
6. Response to light touch

The score given to each mouse at the completion of the evaluation is the sum of each individual score assuming all six variables are assessed. The minimum neurological score is 3 and the maximum score is 18 (normal animal). The

scorer is blinded to the treatment. The Garcia score is used for assessment of the neurological function after middle cerebral artery occlusion.

3.5 Biological assays

3.5.1 *In vitro* experiments

Initially *in vitro* experiment were performed to identify the presence of P2X7 receptors in microglia and astrocytes before exploring the role of the danger signalling mechanisms through the P2X7 receptor in the development of POCD in *in vivo* experiments.

3.5.1.1 Cell culture

3.5.1.1.a Microglia cells (BV2 cells)

These cells are from an immortalized mouse microglia cell line. These cells were grown and maintained in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum and antibiotics (penicillin/streptomycin, 100U/ml). They were plated in a 75cm² cell culture flask (Corning, Acton, MA, USA) under a humidified 5% CO₂/95% air atmosphere at 37°C. These cells were split twice a week and for the experiments the cells were plated on 6-well dishes (1-2 x 10⁶ cells/well).

3.5.1.1.b Mouse cortical astrocytes

Primary astrocyte cultures were prepared from 1-3day old postnatal Swiss Webster mice as previously described (Dugan, Bruno et al. 1995). In brief, the brains were removed from fetal mice, freed from meninges and the cortical hemispheres removed from the rest of the brain under microscopic guidance.

The cortices were minced and treated with 0.09% trypsin for 20m at 37°C. After centrifugation at 4000rpm (40G) for 3m the pellet was re-suspended in 2ml normal media by gentle trituration through a flame-polished Pasteur pipette. The cortical cell suspension was then plated on 24-multiwell Falcon Primaria plates (Becton Dickinson, Lincoln, IL) at a density of 1-2 hemispheres per well in Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% horse serum. The astrocytes were fed weekly and they were confluent by 14 -20 days.

Murine cortical astrocytes were used to ascertain the P2X7 physiological response to stress following challenge with LPS or by cell stimulation with exogenous ATP.

In a second stage of the in-vitro experiments the immortalized microglia cell line (BV2) were used to identify the P2X7 response to stress and then its effect manipulated by using an antagonist of the P2X7 receptor. In vitro experiments were carried out at Professor Rona Giffard's laboratory in collaboration with Dr Yibing Ouyang. Western Blotting (WB) and immunofluorescent staining were performed to ascertain the presence of P2X7 receptors on the cell membrane of BV2 cells and cortical astrocytes.

3.5.1.2 Calcium signalling for P2X7 identification

When cells were ready for identification of P2X7 receptors activity they were washed and rinsed with BSS 5.5 twice and then the cells were loaded with 1µM/well of calcium (Fura-2, 1mM solution, Molecular probe, Inc) and incubated

for 30m at 37°C. After the incubation period the cells were washed once again with BSS 5.5 and taken to the microscope room where the plate was placed on the microscope plate reader. The cells were challenged with 0.5 and 1µM ATP and then a different group of cells were challenged with ATP at the same concentration plus 10µM Brilliant Blue-G (BB-G is a P2X7 antagonist) under direct vision. Six wells were used per condition. There was a non-treated group and also a BB-G alone group that were used for internal control of the experiments.

In a second phase a different group of cells were incubated with 15µl of pro-caspase-1 working solution for 60m (FLICA™) at 37°C. After the incubation period the cells were placed in the microscope plate reader and challenged with ATP, with and without the BB-G.

Baseline pictures plus phase images were taken for all set of experiments (Figure 3.21). Afterwards, pictures were taken every 10s while video-recording the cell response to ATP challenge for 30m (the microscope was turned on/off continuously to avoid bleaching the cells). Cell response was assessed by the percentage change in mean fluorescent intensity (MFI) and plotted over time.

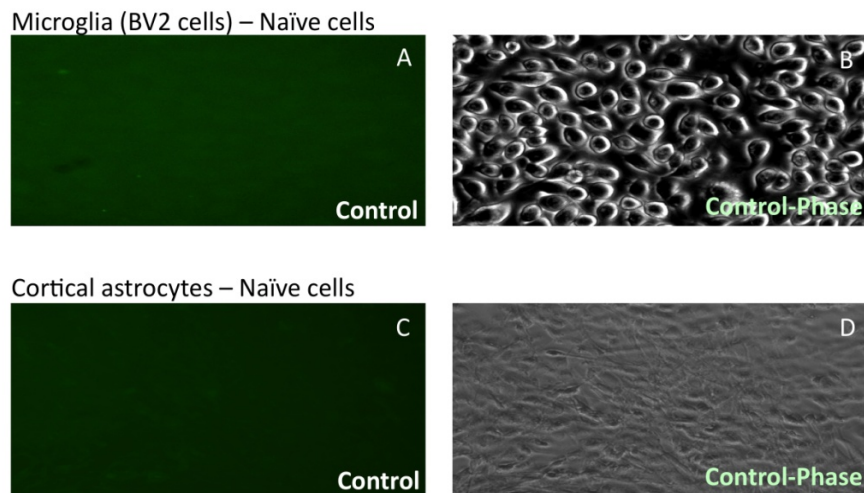


Figure 3.21: This figure illustrates baseline cell networking. A) Microglia cell (BV2) on resting condition without treatment. B) Phase image of microglia cell. C) Cortical astrocytes on resting condition without treatment. D) Phase image of cortical astrocytes.

3.5.2 Immunohistochemistry (IHC)

Direct vision of brain tissues and identification of brain regions is crucial as the studies are trying to identify if areas of the brain involved in memory formation are affected by surgery and anaesthesia. By using immunohistochemistry it should be possible to identify reactivity and/or activation of glial cells after systemic insults such as surgery. If this is the case then it will be possible to identify an association between these changes and the development of POCD. In this section the basic principles of immunohistochemistry (IHC) will be described.

The IHC is a qualitative method that has been in use in the scientific community since 1941. It provides anatomical and morphological information but results

may subsequently be difficult to interpret or reproduce. Although it is a qualitative method the scoring or grading system is performed manually or generated by computing software. The variability of the results may be influenced by the quality of the staining, exposure time and the expertise of the operator performing the scoring. Therefore, the following efforts were made to control variables that could potentially affect the results. These included staining all treated groups at the same time so there were no variation in antibodies' concentrations, incubation times and changes of pH of solutions used for washing up tissues or for tissue fixation. Internal controls without primary antibodies were used for each IHC study. Grading of microglia activation for IHC was based on a previously validated scale for assessment of glial cells activity (Jinno, Fleischer et al. 2007; Lemstra, Groen in't Woud et al. 2007)(Figure 3.22).

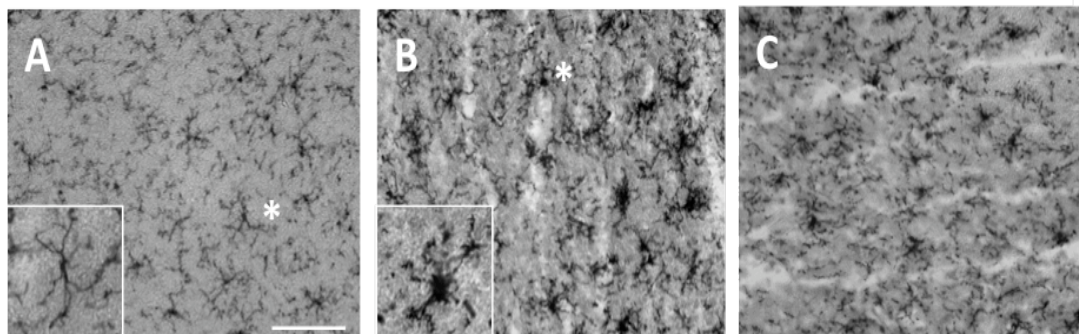


Figure 3.22: These panels illustrate different stages of microglia activation 24h and 72h after stimulation with LPS (1mg/Kg injected intraperitoneally). The microglia cells were stained with CD11b antibodies. Panel A: It shows a normal microglia in hippocampal tissue from a naïve mouse (See x40 magnification on lower left side corner illustrating the fine extensions of a normal or non reactive microglia). A normal microglia is characterised by its fine extensions networking its surroundings. Panel B: It shows an active microglia in hippocampal tissue 24h*

after stimulation with LPS. Active microglia is characterised by its amoeboid shape and short and thick extensions (See x40 magnification on lower left side corner illustrating a typical active microglia with its short extensions*). Panel C: This panel illustrates a reactive microglia 72h after LPS stimulation. Reactive microglia has practically a normal body but still thick extensions. This work was done in collaboration with Niccolo Terrando and Antonio Rei Fidalgo. The characterisation and validation of the immunohistochemistry technique used for assessment of microglia activation after surgery has been published by Cibelli et al (Cibelli, Fidalgo et al. 2010).

The entire process of immunohistochemistry encompasses different steps that are described below (Figure 3.23).

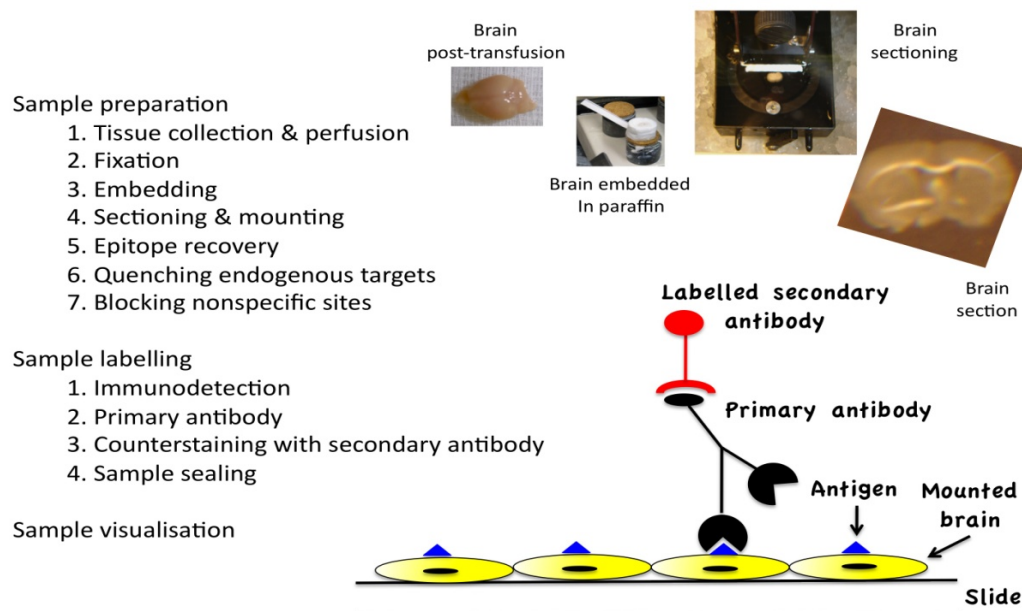


Figure 3.23: Schematic illustration and list of steps required for generation of indirect immuno-staining to identify cells and cells activity in different regions of the brain after surgery and or anaesthesia.

3.5.2.1 Sample preparation

The fixation procedure used for immunohistochemistry was performed immediately after terminal anaesthesia with 5% isoflurane to protect the tissues from its physiological decay and maintain the tissues' anatomical structures and cells morphology. An adequate fixation immobilises antigens, preserves antigen reactivity and allows adequate penetrability of immuno-reagents to the tissues. Animals were killed at different time points after treatment in different cohorts of animals.

Blood samples were taken by direct cardiotomy and perfusion through the left ventricle performed with 20ml of 0.9% sodium chloride to allow exsanguination through the right atrium. The brain tissue was then dissected from the skull and preserved in 4% paraformaldehyde (4% PFA) which has a high degree of tissue penetration and preserves tissue immunoreactivity (Berod, Hartman et al. 1981).

After 24h storage in 4% PFA, tissues were transferred into 30% sucrose where they were kept for 72h or until they sank down to the bottom of the container. The brains were then mounted on a freezing microtome at -19°C (SM2400 Leica, GmbH), using embedding matrix lamb OCT compound (batch; 03661650, BDH) and serial sections cut at 25-30µm thickness.

Coronal sections were collected in 24-well plates containing 1% PBS and stored at 4°C. Groups of sections were then mounted onto Superfrost plus slides (VWR international, Leuven) and after overnight drying on the bench were then stored at -20°C. Approximately 3 days after collecting the sections, the 1% phosphate

buffer solution (PBS) was removed and cryoprotectant solution (150g sucrose, 300ml glycol, 0.05M phosphate buffer in 1000ml distilled water) was added. The sections were then transferred to a -20°C freezer. Approximately 100-200 sections were collected from each brain. The different solutions used for the IHC studies are summarised in table 3.7, appendix 2.

3.5.2.2 Antibody labelling

Antibody labelling is used to identify glial cells within the brain structures to assess the level of activation of this cell after surgery and anaesthesia at different time points in the postoperative period. Antibody staining involves staining cells by a means of antibodies binding to antigens present in cells or tissues of interest. There are 2 main methods of immunohistochemistry antibody staining. A direct method which requires a labelled antibody that reacts with the protein or tissue of interest. This method is quick but lacks amplification signalling and therefore it is not used normally. An indirect method, which consists of 2 stages of labelling. The first step being an identification of the protein/tissue of interest with a primary antibody that reacts with its corresponding antigen. Then a labelled secondary antibody, reacts with the primary antibody that has already formed a complex with its antigen. This reaction of secondary-primary-antigen gives a signal that is amplified depending of the strength and purity of the reaction of the antibodies.

Free-floating and/or glass mounted sections of mouse brain were immunostained for identification of microglia by using CD11b antibody which is

a membrane α -integrin present in microglia. Sections were defrosted and rinsed twice in 0.1% Dulbecco's PBS (GIBCO), permeabilised in 0.1-0.4% Triton X100 (sections were permeabilised with 0.1% triton X100 when CD11b antibody was used for staining) in PBS for 30m and washed in 0.1% Dulbecco's PBS (GIBCO). Permeabilisation buffer was chosen based on the location of the protein of interest. Sections were then incubated with 3% normal donkey serum (CD11b), in 0.1% Dulbecco's PBS (GIBCO) for 1h to permit blocking of non-specific binding. Sections were then incubated with the optimum dilution of primary antibody. This was established from serial dilutions on a few sections prior to the main procedure which had been run either overnight at room temperature or for 72h at 4°C. Following this the test sections were rinsed in 0.1% Dulbecco's PBS (GIBCO, UK) and incubated for 2h with the appropriate secondary antibody: 1:50 donkey anti-mouse Rhodamine (Abcam) (CD11b). Sections were then rinsed in 0.1% Dulbecco's PBS (GIBCO, UK), mounted on gelatinised slides, counterstained with DAPI (Vectorlab H-1200, Vector Laboratories Inc., USA), coverslipped (edges are sealed using nail varnish) and allowed to air dry overnight before viewing under the microscope. Internal controls were used to identify the degree of purity of the antibodies. The internal controls were incubated with the aforementioned blocking serum only (and no incubation with a primary antibody) before incubation with the secondary antibody.

3.5.2.3 Microscopy and image analysis

For each animal, the number of immunoreactive cells in four sub-regions of the hippocampus, CA1, CA2, CA3 and hilus of dentate gyrus (-1.70 to -2.18 mm relative to Bregma) were estimated from photomicrographs with a counting

frame size of 0.4 mm². Sections immunostained for proteins of interest were photographed at 4x, 10x 20x and 40x magnification using a digital camera attached to a Zeiss Axiovert 200M inverted epifluorescence microscope (Zeiss, Jena, Germany). The total numbers of immunoreactive cells were counted in 3 counting frames per region (total 12 frames per animal) using Image J software (USA National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/ij/>). The numbers of cells in the '3 frames per region' were then averaged.

Imaging was performed using a Zeiss Axiovert 200M (Zeiss, Jena, Germany) digital camera attached to an Olympus BX60 inverted epifluorescence microscope (Olympus, UK). The images were then captured using the KS300 (Zeiss, Jena, Germany) software and then analysed and quantified at different magnifications using ImageJ software (National Institute of Health, NIH) as described above (Figure 3.24).

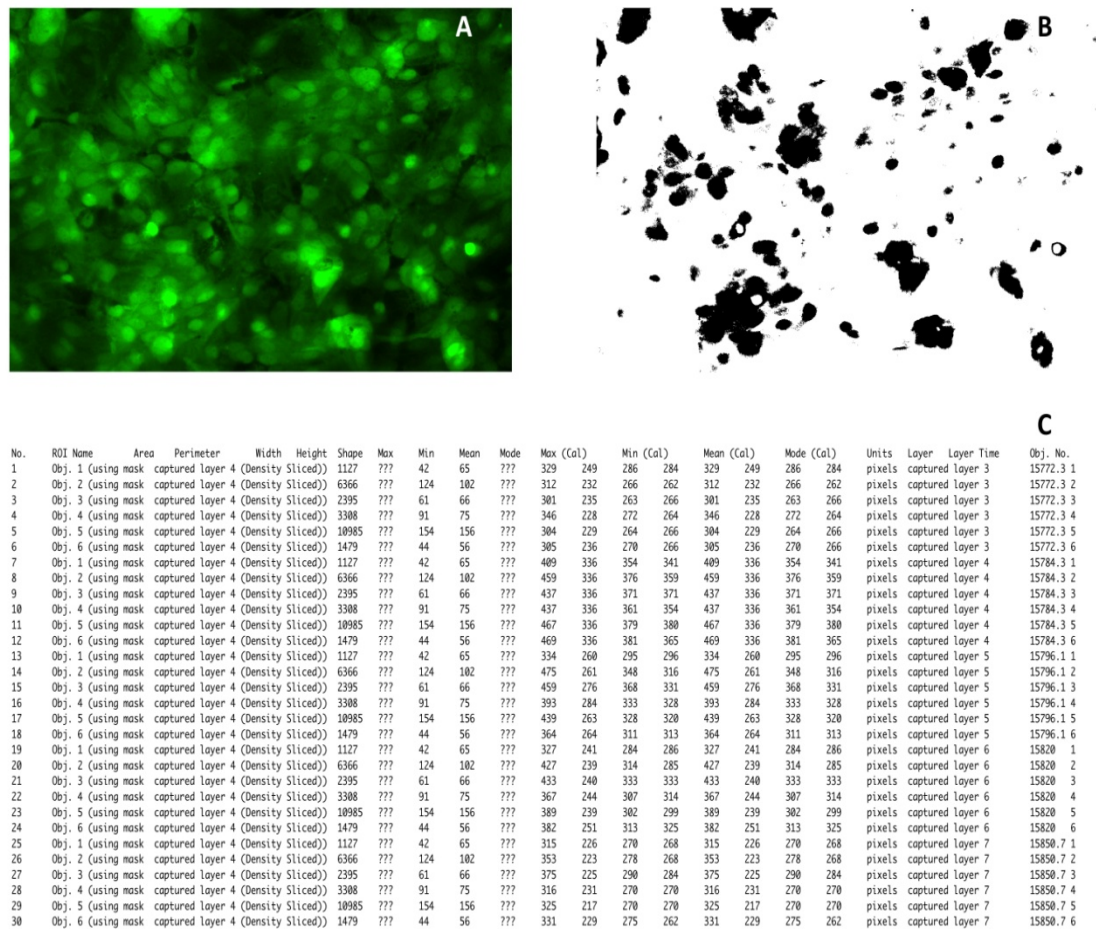


Figure 3.24: This figure illustrates how images were analysed. All images were converted to black and white for analysis, then area, mean, maximum and low intensities were measured for each image and then either composites were created or time line responses was built in some of the in vitro experiments. A) Illustrates cortical astrocytes pre loaded with calcium (Flura-4) and then stimulated with ATP. B) Illustrates the conversion of the image to black and white. The black areas were identified by the imageJ software. C) This snapshot illustrates the output analysis from imageJ software.

3.6 Western blotting (WB)

WB analysis was performed to identify protein levels in specific regions of the brain. The rationale of using a non-cell specific technique was to assess the response of the brain tissue to a systemic major insult such as surgery. This is a non-specific but yet well established technique used worldwide for identification of the level of protein expression. This method helps ascertain if the brain, particularly the area of the brain where memory is formed, is affected by surgery and anaesthesia and if so at what time point in the postoperative period.

The WB analysis is a non-quantitative but very specific method. There are some potential problems. Sometimes, non-intended proteins may react with secondary antibodies resulting in labelling of incorrect proteins. Incidental phosphorylation of the categorical proteins may result in multiple bands after the sample is processed. The transfer of proteins may be affected by methanol resulting in falsely low level of proteins transferred to the membrane. The WB technique does not identify individual cells and/or specific cell populations but it is a valuable tool to assess proteins of interest in tissues of interest.

The WB technique consists of several steps (Figure 3.25) which comprises 1) Retrieval and preparation of tissues. 2) Immunoblotting and tissue labelling with antibodies and 3) Image revelation and analysis.

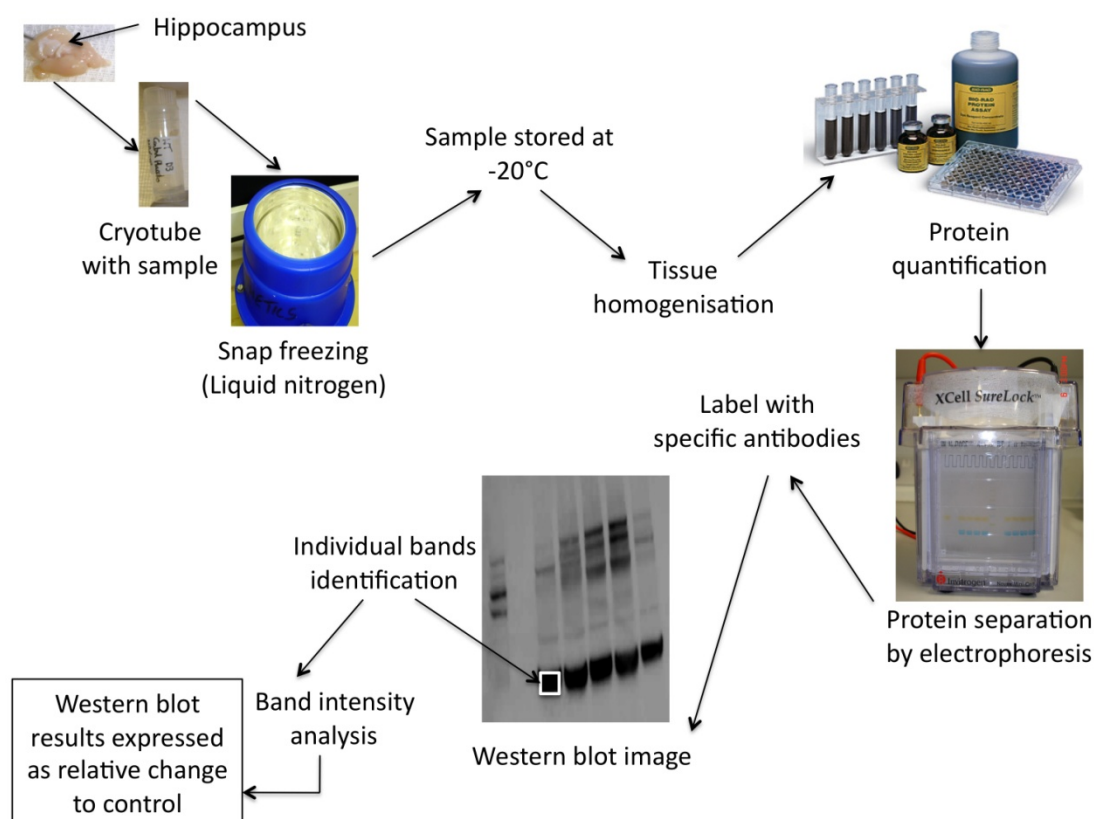


Figure 3.25: This figure illustrates the Western blot steps for identification of protein of interest in tissues after different type of treatments.

3.6.1 Retrieval and preparation of tissues

Hippocampi were homogenised in ice-cold cell lysis buffer (20mM Tris pH 7.5, 1% Triton X-100, 150mM sodium chloride, 1% pyrophosphate, 1% beta-glycophosphate, 1% NP-40, 0.1% SDS, 1mM EDTA, 100µg/ml phenylmethylsulfonyl fluoride, 1µg/ml aprotinin). Homogenates were then centrifuged for 20m at 15,000 rpm (150G) at 4°C. Supernatants were then extracted and protein quantification was carried out using a Bradford protein quantification assay kit (Bio-Rad, Hemel Hempstead, UK). Protein extracts (10µg) from each sample were denatured in NuPAGE LDS sample buffer (Invitrogen, Paisley UK) using a thermomixer at 70°C for 10min. The proteins

were then separated using sodium polyacrylamide gel electrophoresis (NuPAGE, Invitrogen, Paisely, UK) before being transferred to a polyvinylidene fluoride membrane (Invitrogen, UK).

3.6.2 Immunoblotting and tissue labelling with antibodies

Membranes were then blocked in 5% skimmed milk in TBST (50mM Tris-HCl, 150mM sodium chloride, 0.1% Tween 20, pH 7.4). They were then incubated overnight with the primary antibody of interest in TBST at 4°C. This was followed by 2 hours incubation at room temperature with secondary antibody, HRP-conjugated antibody. Membranes were then treated with the ECL chemoluminescence system (Amersham Biosciences, Little Chalfont, UK) and images developed using the Syngene G:Box gel analysis system (Syngene, Cambridge, UK). Membranes were then reprobbed with monoclonal anti- α -tubulin (1:1000, Sigma-Aldrich, Dorset, UK) antibody to calculate the amount of total proteins in each sample. α -tubulin reprobbed serves as the internal control and the analysis of data quality before analysis of proteins of interest. For example, if the loading of proteins in the wells is different between groups then amount of α -tubulin in each sample may vary and therefore differences in the amount of the protein of interest may be seen when they are compared within groups. These artificial differences are easily preventable when samples are normalised to their housekeeping proteins and then the proteins of interest are analysed and compared within groups. For details of antibodies concentration refer to table 3.8, Appendix 2.

3.6.3 Image revelation and analysis

All WB images were developed with the Syngene G:Box gel analysis system (Syngene, Cambridge, UK). The raw volume was initially identified, then the band intensity was calculated. The mean intensity of each band was analysed in relation to the house keeping protein (α -tubulin) process which is also known as 'sample normalisation'. When each sample was normalised to its house keeping protein, they were then compared with other groups or to their respective controls.

3.7 Enzyme-linked immunoabsorbent assay (ELISA)

Identification of level of cytokines in the systemic circulation and also in the CNS as a surrogate of the inflammatory response after surgery and anaesthesia is important as it may help to demonstrate a time response to these changes and identify an association, if any, with the development of POCD.

The ELISA technique consists of several steps which are explained below (Figure 3.26).

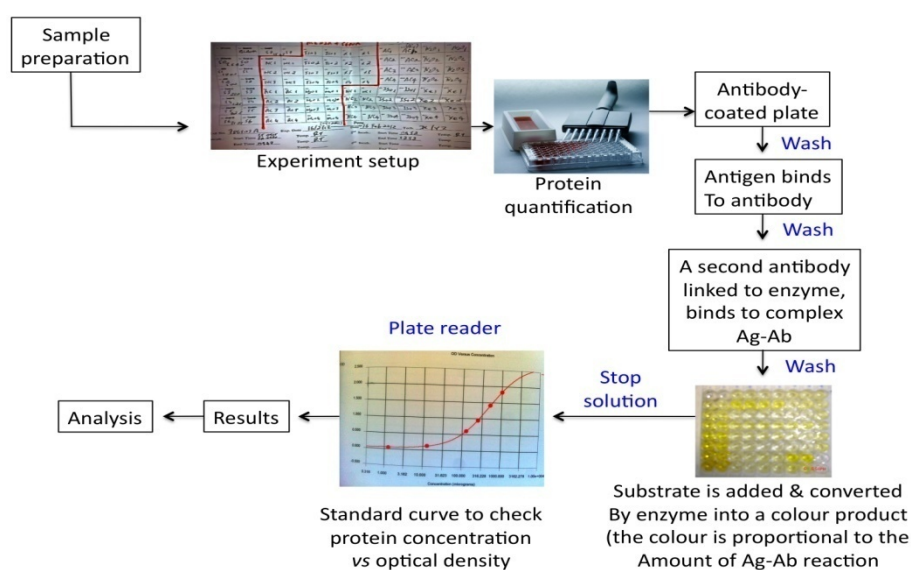


Figure 3.26: This schematic representation illustrates the ELISA steps that were taken to analyse cytokines in plasma and hippocampi after surgery and Imperial College London

anaesthesia. The steps started with sample preparation, experiment setup that involved the creation of a working spread sheet with allocation of samples in duplicate. Protein quantification was followed by the indirect enzyme-linked immuno-assay and so 2 antibodies were used, the latter being an enzyme linked antibody that then allowed the generation of a chemical reaction that resulted in a change of colour of the solution. The change of colour of the solution depends on the amount of antigen-antibody reaction (Ag-Ab). The chemical reaction was then stopped after 20 min and the plate was read and analysed.

3.7.1 Sample preparation for ELISA

The frozen hippocampi samples, which were retrieved at different time points after different interventions, were weighed and sonicated in iced protease inhibitor cocktail (P2714, Sigma, Missouri, USA) before they were centrifuged at 2200rpm (22G) for 20m at 4°C. The supernatant and also the defrosted plasma were then collected and quantification of protein was carried out using a Bradford protein quantification assay kit (Bio-Rad, Hemel Hempstead, UK).

Analysis of individual cytokines, mainly IL-1 β in plasma and hippocampi were performed with enzyme-linked ELISA according to the manufacturer's guidelines (Bender MedSystems® (Ref: BMS6002). The sensitivity of the assay was 1.2pg/ml.

A spectrophotometer (MRX2, Dynex Technologies, Worthing, UK) was used to measure the intensity of colour by absorbance at a wavelength of 495nm. The sensitivity of this assay is 0.05pg/ml as stated by the manufacturer of the

antibodies. Individual cytokines measurements were performed to ascertain the systemic and central nervous system inflammatory response after surgery and anaesthesia.

Multiplex assays on the Luminex 200 IS system were used for measurement of cytokines and chemokines in blood and hippocampal samples. This was to attempt to identify a physiological signature, which could help to characterise the systemic, and central nervous system inflammatory response after surgery and anaesthesia and its ability to discriminate groups prone to develop POCD.

Samples of hippocampi retrieved on day 1 and 7 after surgery and anaesthesia were defrosted and prepared on the day the analysis was performed. All samples were weighed and proteins were quantified before dilution in procarta lysis buffer; 20mg tissue required 120µl lysis buffer (Procarta™ Assay kit 20 ml) and then the hippocampal samples were spun down at 13,000rpm (130G) for 10m at 4°C. The supernatant (130µl) was kept in dried ice until cytokine analysis was performed. The plasma samples were placed in a filter Eppendorf tube (Filter Ultra-free®-MC Centrifugal 0.22µm filter units) and spun down at 13,000rpm (130G) for 20m; the supernatant was collected in Eppendorf tips and kept in dried ice until processed.

Analysis of the multiplex assays was also performed in duplicates in the pg/ml range using the panel from Panomics (Affymetric, 1.877 Panomics). Samples were run in 96-well plates and the cytokine standards were dissolved in serum standard buffer or phosphate buffered solution (PBS). Concentrations of each

cytokine or chemokine were calculated by comparing the median fluorescence intensity (MFI) data to that of its standard curve (Figure 3.27).

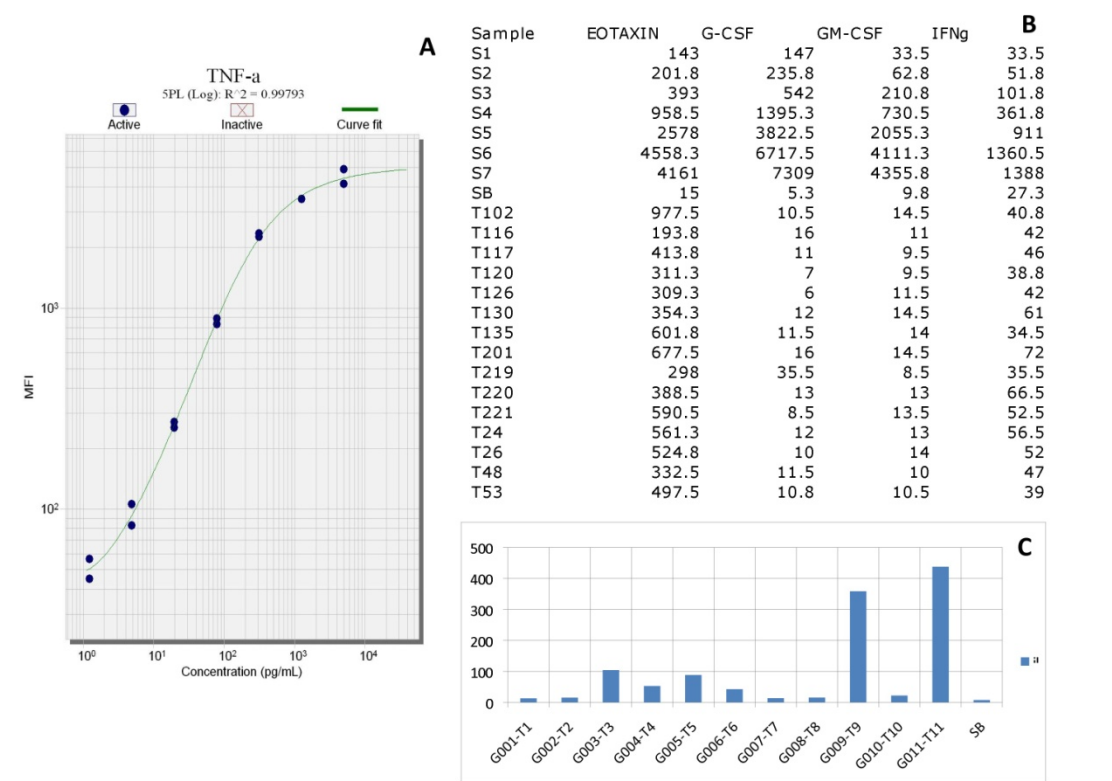


Figure 3.27: This figure illustrates how the raw data is captured after the multiplex assay is read by Luminex. A) This is an illustrative standard curve for a single cytokine. B) This is a snapshot of the raw mean fluorescent intensity (MFI) results and C) Histogram representing the MFI for a single cytokine in a cohort of mice at one time point after interventions.

The MFI background reading is subtracted from each value per cytokine, as there are different backgrounds values for every cytokine and chemokine. A report file was generated for each plate to check the setup of the samples in the plate. A total beads table was produced to show the total number of beads counted in the well for all cytokines combined. The “bead count” shows number of beads read

per well. This table was generated to use for quality control of the assay. Counting of at least 100 beads per cytokine per well is generally required, but a minimum of 50 beads is accepted. This is the minimum amount of beads to be able to state the samples quality is good (Table 3.9).

Table 3.9: This table is a snapshot of the table generated with the number of beads read per cytokine per sample. Checking the number of beads per well is important as it is part of the quality control process before cytokines are analysed. A minimum of 50 beads/well was accepted as a good sample for analysis.

Sample	Well	Name	EOTAXIN	G-CSF	GM-CSF	IFNg	il-10
77	E10	G002-T2	153	173	398	183	237
78	F10	G002-T2	187	230	410	217	259
79	G10	G003-T3	145	134	244	158	183
80	H10	G003-T3	175	163	315	171	205
81	A11	G004-T4	203	209	379	203	239
82	B11	G004-T4	219	231	423	248	314
83	C11	G005-T5	124	136	211	111	139
84	D11	G005-T5	145	100	223	147	180
85	E11	G006-T6	154	169	302	177	184
86	F11	G006-T6	170	151	301	152	213
87	G11	G007-T7	183	185	310	177	193
88	H11	G007-T7	222	227	413	248	276
89	A12	G008-T8	215	334	545	258	390
90	B12	G008-T8	222	347	599	253	400
91	C12	G009-T9	286	243	737	234	486
92	D12	G009-T9	345	300	888	314	619
93	E12	G010-T10	156	134	274	143	199
94	F12	G010-T10	125	110	223	143	146
95	G12	G011-T11	156	129	305	155	203
96	H12	G011-T11	170	142	284	186	188

A standard table was also generated to see how good a correlation was achieved between the Median fluorescent intensity (MFI) values for the standards and the curve/function graph that described them.

Thereafter a curve fitting was performed using the Beadview software®; the software allowed for rejection and recalculation as necessary. “Raw MFI, Results” and “MFI, CV, CON ” and the “MFI, CON” are other ways of expressing the raw data. All data was kept in the report files for preprocessing and analysis.

3.8 Fluorescent-activated cell sorting (FACS)

3.8.1 Basic principles of FACS

Cell involvement in the development of POCD has been identified by immunohistochemistry previously but there is a need to isolate individual cells from the areas of the brain where memory is formed. The identification of the activity of individual cells may help to determine if there is any temporospatial association between cell activation and the development of POCD.

To this end flow cytometry was used and as its name indicates is a means of flow fluorescent activity cell sorting (FACS). A mixture of cells in liquid that are incubated with cell specific conjugated antibodies are run through a computerised system where the labelled cells are identified by a laser system and sorted according to their polarity, shape, granularity and relative fluorescent intensity.

The mixture of cells in a liquid, also known as the fluidic system, carries the cells or particles of interest in a stream to the laser beam for analysis. The laser beam or optical system illuminates the particles in the sample stream and optical filters direct the resulting light signals to the appropriate detectors. The detectors then transduce the light energy to electronic signals that can be

processed by a computer. The electronic system is also capable of initiating sorting decisions to charge and deflect particles. Any suspended particle or cell from 0.2-150µm in size is suitable for analysis. When the cells pass through the laser intercept, they scatter the laser light and any fluorescent molecules present on the cell fluoresce. Appropriately positioned lenses collect both the scattered and fluorescent light and a combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them. List mode data are collected on each event. The characteristics of each event are based on its light scattering and fluorescent properties. The data were collected and stored in the computer for subsequent analysis.

3.8.2 Preparation of cells suspensions

For FACS experiments total hippocampal tissue from different cohort of mice was retrieved at different time points after surgery and anaesthesia. These tissues were homogenised gently in 2ml of 0.5mg/ml collagenase type IV (Sigma) and 1µg/ml DNase (Sigma) using a gentle MACS dissociator (Miltenyi, Biotech). Tissues were then incubated with continual mixing for 15min before pipette trituration, filtered and washed with fluorescent-activated cell sorter (FACS) medium (phosphate-buffered saline, 2% fetal calf serum, 0.1% sodium azide and 5mM ethylenediaminetetraacetic acid). The cell suspension was further dissociated through a 40µm nylon cell strainer (BD Falcon, Oxford, UK) with extensive flushing through of cells with FACS medium. The cell suspension was then centrifuged at 2000rpm (20G) for 5m at 4°C and resuspended with 1ml of

FACS medium and fixed for 5m at room temperature using 1% paraformaldehyde (eBioscience).

To identify the role of the endothelium cells in the inflammatory process and the association with the development of POCD it would be useful to assess measure the activity/concentrations of intracellular proteins e.g. cyclo-oxygenase-2 (COX-2). Inducible COX-2 increases once a cascade of inflammatory events occurs and it appears COX-2 is involved in the initial phase of inflammation and promotes further inflammation (Bazan, Colangelo et al. 2002; Lee, Song et al. 2006). To achieve this, methanol permeabilisation was performed. In brief, cells were resuspended with 800µl of eBioscience fixative for 2m in a 15ml tube following by an addition of 9ml of FACS medium and spinning at 2200rpm (22G) for 5m at 4°C. Then tipped off and resuspended with 150µl FACS medium for methanol permeabilisation. All preparations were stained for identification of endothelium and microglia cells in the hippocampus. The cell suspension was then incubated with COX-2 (1:40, M19, sc-1747 PE, Santa Cruz) antibodies to identify its role in the endothelium cells and the involvement of the endothelia in the inflammatory process associated with the development of POCD. After washing, the nuclear stain 7AAD (1:100; Biolegend) was added to suspensions to exclude myelin debris from analysis and improve the quality of the sample before the analysis. Analysis was performed with the Cyan flow cytometer, ADP, DAKO (Beckman Coulter, High Wycombe, UK).

FACS has been used for cell identification and activity vastly. However, one of the main issues is the recovery of cells from the CNS. In order to improve the

recovery of different CNS cells and then assessment of their activity the FACS technique was modified with the intention to enhance the cell recovery and also to look at the expression of COX-2 in CNS cells. To do this a new method for the simultaneous fixation and homogenisation of CNS tissue was developed during the course of the study. The method employed the Gentle MACS dissociator for homogenisation of tissue suspended in 2ml cell fixative (eBioscience). The lung setting of the homogeniser was used for homogenisation for a 10sec twice. Fixation time of 1min exactly was used for optimal cell identification and reproducibility. Suspension was then sieved as before and washed twice in either FACS medium or a permeabilisation-wash buffer (PBS, 2% FCS, 0.2% saponin). Staining with antibodies was performed in the dark at 4°C for 30min.

3.8.3 Antibody staining of cell suspensions

Antibody staining of cell suspension from hippocampi tissue retrieved after surgery and anaesthesia at different time points was performed to identify different CNS cells and then assessment of their activity was performed by looking at the expression of COX-2. Identifying individual cells should enable a better understanding of which cells are the key players and responsible for the CNS inflammation and therefore contributor with the development of POCD.

The antibodies used for this set of experiments were the following: All preparations were stained for CD31 (1:800, clone Alexa 647, MEC 13.3, Biolegend) for identification of endothelium and CD11b (Clone M1/70) staining for identification of microglia cells in the hippocampus. Then permeabilisation was performed for identification of COX-2. After washing, the nuclear stain

7AAD (1:100; Biolegend) was added to the suspensions to remove cell debris and myelin to improve the samples' quality (Figure 3.28).

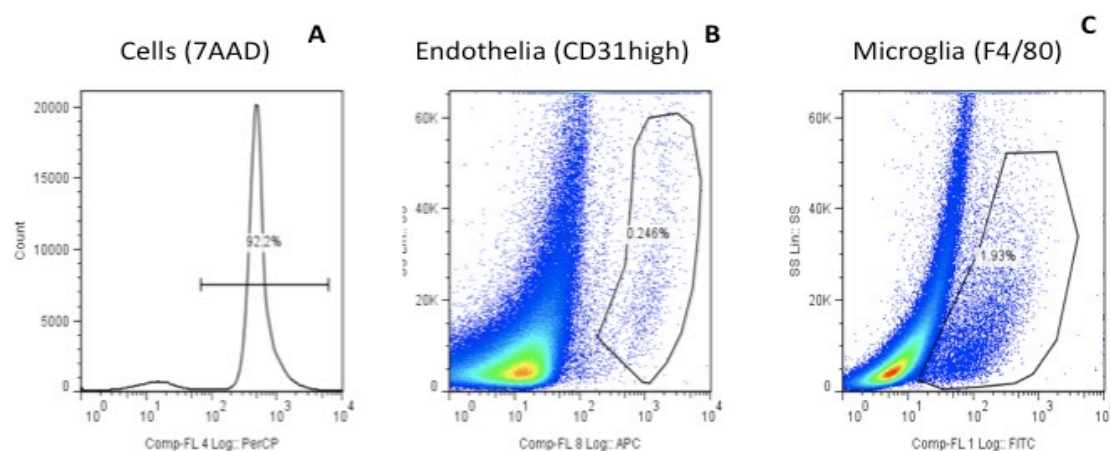


Figure 3.28: This figure illustrates A) The identification of cells of interest under the curve. B) Identification of endothelia and C) Identification of microglia by FACS.

3.8.4 FACS data analysis

FACS Analysis was performed with the Cyan flow cytometer, ADP, DAKO (Beckman Coulter, High Wycombe, UK) by using flowJo software (Tree star, Ashland, OR, USA). A *P* value of less than 0.05 was taken to be statistically significant.

The areas that needed to be developed were plotting and gating for identification of individual cells. Antibodies were allocated to different channels and 1 channel left blank for internal control. Data is displayed as frequency in the “Y” dependent axis against intensity that is plotted in the “X” or independent axis.

By plotting 2 levels of intensity to identify the number of cells labelled by 2 colours should helped to quantify cells by identifying their relative density of dots rather than individual dots. The intensity was expressed as mean fluorescent intensity (MFI).

3.9 Renal function tests

Assessment of renal function and cholesterol level was performed after nephrectomies to rule out renal impairment as cause of POCD. It was also used to ascertain if a short course of statin in the preoperative period affected cholesterol level in mice.

Renal function and cholesterol levels were assessed on day 3 after nephrectomy. Creatinine ($\mu\text{mol/l}$), urea (mmol/l), and cholesterol (mmol/l) were measured in plasma by calorimetric methods using clinical chemistry analyser (Abbott, architect) at the biochemistry department at Chelsea and Westminster campus. Creatinine level was measured by Jaffe's reaction (kinetic/rate method) and urea by urease method again by kinetic/rate method.

Reported normal values for C57BL6 mice were(Boehm, Zur et al. 2007):

Creatinine: 28-29 $\mu\text{mol/l}$; Urea: 13.2-13.3 mmol/l ; Cholesterol: 1.09-1.12 mmol/l .

3.10 Data presentation and analysis

Western blotting results are presented as % change relative to control. Immunohistochemistry data is presented as changes of density per unit area and illustration are presented on 4, 10, 20 or 40 times magnification. Mean fluorescent intensity is presented as maximum variation and range. A *P* value was used to demonstrate whether the difference between groups was likely to be due to chance. A *P* value < 0.05 was considered to be statistically significant.

3.10.1 Softwares

3.10.1.1 GraphPad®

The analysis of the data was completed by using the statistical software system GraphPad Prism for Mac OS X version 5.0a, 1992 – 2008. (GraphPad Software Inc. San Diego CA, USA).

3.10.1.2 Statistical Isolinear Multicomponent Analysis (SIMCA®)

The SIMCA programme is a well developed statistical programme for analysis of multiple data with multiple dependent and independent variables which makes analysis difficult by conventional statistical programmes in current use. SIMCA is often used by the industry but a relatively novel method in biological sciences.

Multivariate data analysis enables the extraction of data from large files and by using the principle of projection, the data can be presented in few simple graphs. Furthermore a model can be created based on the findings to tests if the results could be reproducible.

In brief, statistical analysis of multiple cytokines results from different groups at different time points after surgery and anaesthesia was carried out to discriminate between the groups using Principal components analysis (PCA) and orthogonal partial least squares (OPLS) using the SIMCA 12 package (Umetrics®). The main aim of this type of analysis was to identify a discriminatory cytokine that might help explain why some mice develop POCD and other not.

Prior to analysis all cytokine values were logarithmically transformed to remove skewedness allowing all cytokines to have equal influence on the analyses. Under stable conditions, any process or system varies around its mean and stays “within control limits; ± 3 standard deviations in 99.4% of the observations”. Any experiment is influenced by noise “variability” hence transformation was needed to provide an interpretable model. There were also variables (in this case cytokine concentrations) with substantially different ranges, which are a problem as they will skew the data. Logarithmic transformation should facilitate analysis of the samples.

PCA identifies a sequence of derived summary variables called 'principal components' (PCs) or scores, which are weighted linear combinations of variables (cytokines). The first PC accounts for the greatest variation within the whole dataset and succeeding PCs account for decreasing amounts of the residual variation. Scores plots, plot of the leading two PCs, representing each individual sample in the new co-ordinate space were generated to provide an overview of the samples, highlight clustering and identify outliers. PCA is an

unsupervised technique in that PCA does not involve knowledge of group membership of each sample.

Data was analysed by orthogonal partial least squares discriminative analysis (OPLS-DA) (Kang, Choi *et al.* 2008) which is a variant of Partial least squares discriminative analysis (PLS-DA). OPLS-DA and PLS-DA were supervised techniques in that the group membership of each sample was known to the method which again produces weighted linear combinations of variables, scores, that both explain the variation among the cytokines, as does PCA, and also that discriminated between groups. Thus the discriminatory score produced could be viewed as a physiological signature. It therefore relates the cytokines to the group membership of the sample, allowing the identification and visualisation of cytokines responsible for differences between groups. The OPLS-DA method was a combination of partial least squares discriminative analysis (PLS-DA) incorporating the data filtering technique of Orthogonal Signal Correction (OSC). Whereas PLS-DA produces a series of components each of which both explain variation in cytokines and discriminates between groups, OPLS-DA in contrast, separates scores into two groups, some that explain variation (orthogonal) and others that discriminate between groups. This removes variation in the cytokine variables that were not directly related to the physiological conditions being studied, and to minimise the possible influence of inter-sample variation. Each cytokine provided a weight in the OPLS-DA calculation of the discriminatory and explanatory scores. These are also known as loadings and the loading of each cytokine in the discriminatory score provided an estimate of the importance of that cytokine in discriminating the groups.

OPLS-DA was used to both assess the ability of the primary score or discriminating physiological signature to discriminate between the two groups. It is well known in model validation that if the same data is used to both construct the model, in this case to derive the physiological signature, and to assess its performance, the estimate of its discriminating performance is over-optimistic. An optimal approach to assess the metabolic signature is by taking a cut-off to predict group membership and then compare predicted and actual group membership on future experiments. In practice this was done in one of two ways: data can be divided into a training subset, typically 2/3 of the data, to estimate the physiological signature, and then use the actual and predicted group membership of the remaining 1/3 to assess the rule in terms of sensitivity and specificity. An alternative method, within SIMCA, can be used to obtain an unbiased assessment of discrimination called cross validation whereby, each (or several) samples in turn were removed from the data and their groups predicted from a signature estimated from the remaining samples.

Repeating this process by removing each sample in turn allows an unbiased estimate of the predictive ability of a physiological signature from that data to be obtained either in terms of the proportion of correct predictions, sensitivity or specificity or by use of measure called Q^2 . Q^2 is a cross-validated estimate of the fraction of the variation of the response that can be predicted by the discriminatory score. A final score or signature is then obtained using all the data (SIMCA 7-fold CV). Statistical analysis of plasma samples was performed on day 1 and 7 and of hippocampus tissue on day 1 and 7 after surgery.

There were 3 groups; control group, anaesthesia group and tibial fracture surgical group and for each of the 4 analyses we attempted to discriminate group 1 (control) from group 2 (anaesthesia) and then group 1 from group 3 (surgery). To achieve this analysis 3 models were created. Model 1; control and anaesthesia were selected and surgical observations were excluded, Model 2; control and surgery were selected and anaesthesia was excluded and Model 3 compared anaesthesia to surgery and control group was excluded.

Finally, if there was significant prediction in both discriminations as assessed via Q2, the Shared and Unique Structure plot (SUS-plot) was used. This combines information from a number of 2 class models having the same reference and thereby allows comparison of the effects of the variables in the two discriminations.

3.10.1.3 Statistical Analysis

3.10.1.3.a t-test

The t-test was performed to compare the mean of 2 groups when the mean values were assumed to follow a Gaussian distribution or the median in groups with not normal distribution. When an unpaired t test was used the F test was used to confirm that the 2 samples analysed came from populations that have identical standard deviations. If the variance of the sample was big a Welch analysis was performed.

3.10.1.3.b One-way ANOVA

The one-way ANOVA was used to compare the means of 3 or more unmatched groups when the sample followed a Gaussian distribution. For non-parametric data the Kruskal-Wallis post-test analysis was used.

3.10.1.3.c Two-way ANOVA

The 2-way ANOVA was used to assess the response or how a response is affected by 2 factors. E.g. genotype effect and treatment effect on behavioural response.

Chapter 4:

Characterisation of the inflammatory response by using multiple-cytokines analysis in plasma and CNS

There is a body of evidence that suggests inflammation is implicated in memory impairment after surgery (Cibelli, Fidalgo et al. 2010; Fidalgo, Cibelli et al. 2011). CNS involvement has extensively described in acute infection (Dantzer 2004), surgery (Wan, Xu et al. 2007) and burns (Reyes, Wu et al. 2006) as well as in patients with extensive organ injury (Liu, Liang et al. 2008; D'Mello, Le et al. 2009). It has been described in more indolent inflammatory states such as rheumatoid arthritis (RA) (Emmer, van der Bijl et al. 2009).

There is also an association of CNS inflammation with neurological and psychological diseases. These include chronic neurodegenerative diseases such as Alzheimer's diseases (AD) (Akiyama, Barger et al. 2000), multiple sclerosis (MS) (Prat, Biernacki et al. 2002) and Parkinson's disease (PD) (Hirsch and Hunot 2009). It has also been associated with depression (Loftis, Huckans et al. 2010).

The CNS inflammatory reaction is considered an integral part of the reaction to the primary cause of the disease but can be exacerbated by peripheral insults that cause inflammation (Perry, Newman et al. 2003) although this involvement of the CNS in a systemic inflammatory responses would appear to contradict the traditional view of the BBB as an impermeable membrane (Medawar 1948; Barker and Billingham 1977).

In this chapter the inflammatory responses in plasma and the hippocampus after surgery and anaesthesia were assessed. To achieve this a novel approach to analyse biological signalling has been used in an attempt to identify a

physiological signature that can discriminate those with POCD from those without. The physiological signature would be the cytokine profile. Hence the intention is to try to identify and use the profiles to see if the method can discriminate between untreated mice that developed POCD from treated mice that have preserved their memory after the surgical intervention and anaesthesia.

4.1 Methods

The intention was to assess cytokine profiles in plasma and hippocampal tissues at intervals of 1 and 7 days after the interventions, comparing control mice with those with just anaesthesia and those with anaesthesia and surgery.

Adult male mice (wild type C57BL/6) were randomly allocated to one of three groups: group 1 (control=C), group 2 (anaesthesia=A) and group 3 (surgery=S).

For method details about surgery, anaesthesia and measurement of cytokines refer to chapter 3, sections 3.1, 3.1.2, 3.1.3.a, 3.2.1.a, 3.7.1 and 3.10.1.2.

4.1.1 Cytokine analysis

The statistical isolinear multicomponent analysis (SIMCA) was performed to attempt to discriminate group 1 (control) from group 2 (anaesthesia) and then group 1 from group 3 (surgery).

The above groups were called 'class' and each mouse in the groups was called 'component'. Cytokines were called variables or scores. All variables or scores with zero values were defined as 1 and then logarithmically corrected to normalise the variables in each component.

For group analysis 3 models were created; each model was formed from 2 groups. Each model is 2 classes and all components from each class.

Model 1 (M1) contained Control and Anaesthesia groups.

Model 2 (M2) contained Control and Surgery groups

Model 3 (M3) contained Anaesthesia and Surgery groups.

Once the models were created a multicomponent analysis was performed for plasma and hippocampal tissue on day 1 and 7 after the event as follows:

Identification of outliers was performed by using a Principal Component Analysis (PCA). All components of each group were included for the analysis. The results were presented on a cumulative Ellipse Hotelling T2 (The Ellipse Hotelling T2 is the equivalent to t-test for univariate analysis and it is proportional to the F distribution).

Identification of groups separation by discriminatory cytokines using the models described above. The variability of cytokines level in each component was assessed and then compared to the rest of the components of the respective group and this variability of the variables (cytokines) was called orthogonal. First, second and so on orthogonal were referred to as the orthogonal that

contribute to the separation between components in each group and then used to assess the corresponding model.

Group separation in each model was assessed by using an orthogonal partial least squares (OPLS) plot and referred to as score plot through out the results section. These are presented as Q2 and R2 values. A Q2 value is a cross-validated estimate of the fraction of the variation of the response that can be predicted by the discriminatory score (cytokine) and it has a predictive ability. A R2 is the least square of the class and it represents a singular value decomposition that identifies variability between components in a class. This cross validation discriminatory score was used for verification of the groups (class) separations. Good Q2, R2X and R2Y values are ≥ 0.5 while poor Q2, R2X and R2Y values are ≤ 0.2 . The R2X value explains variance related to class separation. The R2Y values explain variation within groups.

The x-axis represents the separation (variability) between groups while the y-axis represents the variability within groups. The cross validation analysis has a predictive power therefore the analysis requires partitioning of the sample of data into complementary subsets, performing the analysis on 1 subset (called the training subset) and validation the analysis on the other subset (called the validation set or testing set).

1. A loading Bi-plot was used to assess how close the cytokine that discriminated (if any) the groups in each model was to a particular group in the model.

2. A share and Unique Structure plot (SUS-plot) was created when there was a significant prediction assessed via Q2 ($Q2 \geq 0.5$). A SUS-plot combines information from at least 2 models having the same discriminatory cytokines in order to assess the effect of these cytokines in each component of the groups of these models.

4.2 Results

13 mice were studied each with a total of 26 variables (cytokines). There were 4 mice as controls, 4 mice had just anaesthesia and 5 mice had anaesthesia and tibial surgery. The data set consisted of a total of 13 observations (mice) and 26 variables (cytokines). The observations were divided in three groups; control, anaesthesia and surgery (classes and each mouse in the group is component).

4.2.1 Plasma samples - day 1

On samples taken on day 1, a principal component analysis (PCA) analysis was performed. It included the 3 groups to identify if there were any outliers with a cumulative Ellipse: Hotelling T2 (0.95). The scores plot indicated no major outliers (Figure 4.1), indicating that the quality of the samples were good.

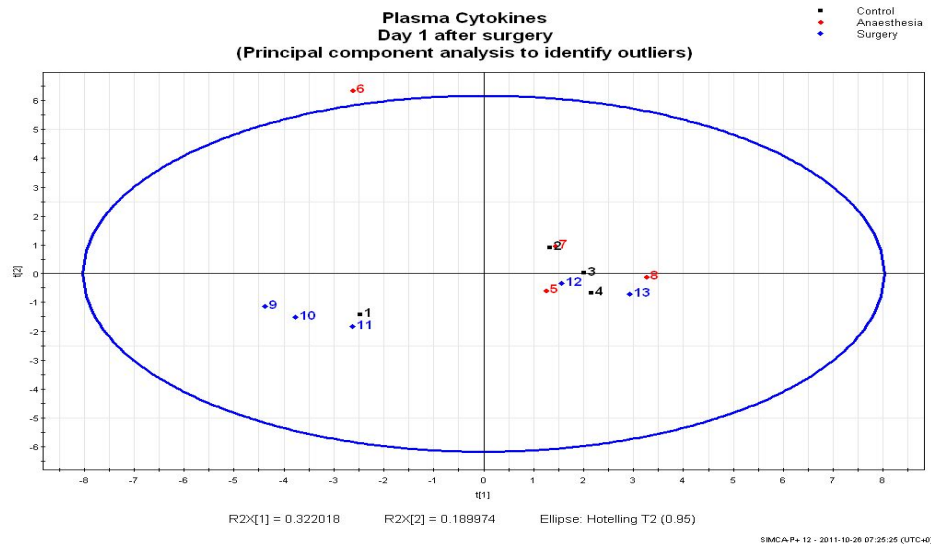


Figure 4.1: Scores for PCA to identify outliers. Control group is represented in black (4) Anaesthesia group in red (4) and Surgery group in blue (5). Component n6 in red is an outlier.

With these data from day 1, three models were created: *Model 1*; the control and anaesthesia groups were selected and surgical observations were excluded. *Model 2*; control and surgery groups were selected and anaesthesia was excluded and *Model 3*; anaesthesia and surgery groups selected and control group was excluded. Variables were defined as single Y-variable. All the cytokines (variables) were logarithmically transformed.

There was no class separation between the control and anaesthesia groups. Therefore Model 1 did not have a predictive ability as there were no significant differences between the cytokines (variables) selected that could discriminate control from anaesthesia group, Q2: -0.344 with 1 discriminatory component and adding orthogonal components did not improve the discrimination. In the score-plot IL-1 α , IL-1 β and MCP-1 showed a pattern, which might trend towards

discrimination, but was not significant with these numbers.

In model 2, control versus surgery group there were no outliers by Ellipse Hotelling T2 (0.95) (Figure 4.2), and there was a good class separation with a Q2 of 0.851 where it was fitted 1 discriminatory cytokine and further 6 cytokines were different between groups (orthogonal). This model has a predictive ability to discriminate between the control group and the surgical group.

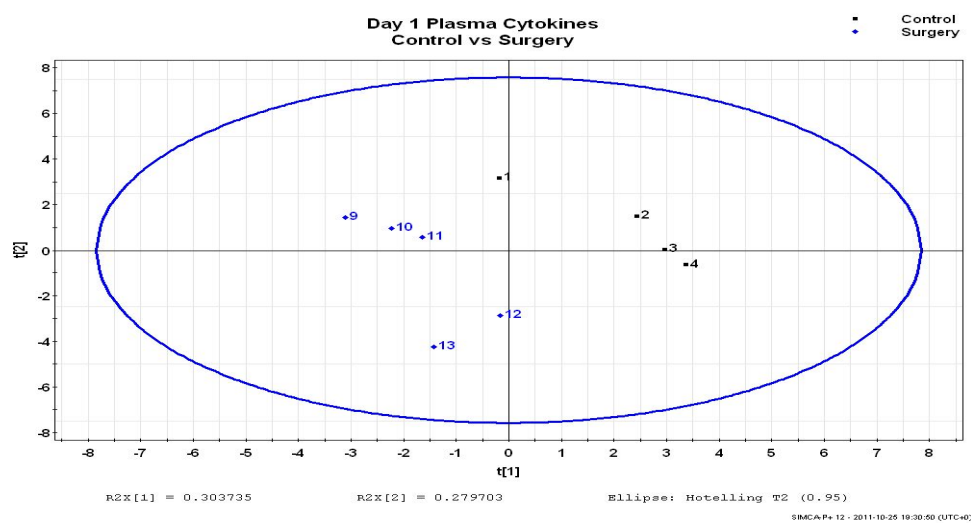


Figure 4.2: Scores for PCA. Control group is represented in black (1–4), and Surgery group in blue (9–13).

The cross-validated discriminatory score was plotted (Figure 4.3). It was produced for each 1/7 mice sample of the data (training set) using the remaining 6/7 mice samples (testing set), against the first orthogonal component (cytokines). Adequate discrimination was confirmed by the horizontal separation of the control and surgery groups by the cross-validated discriminatory component (R2X: 0.31).

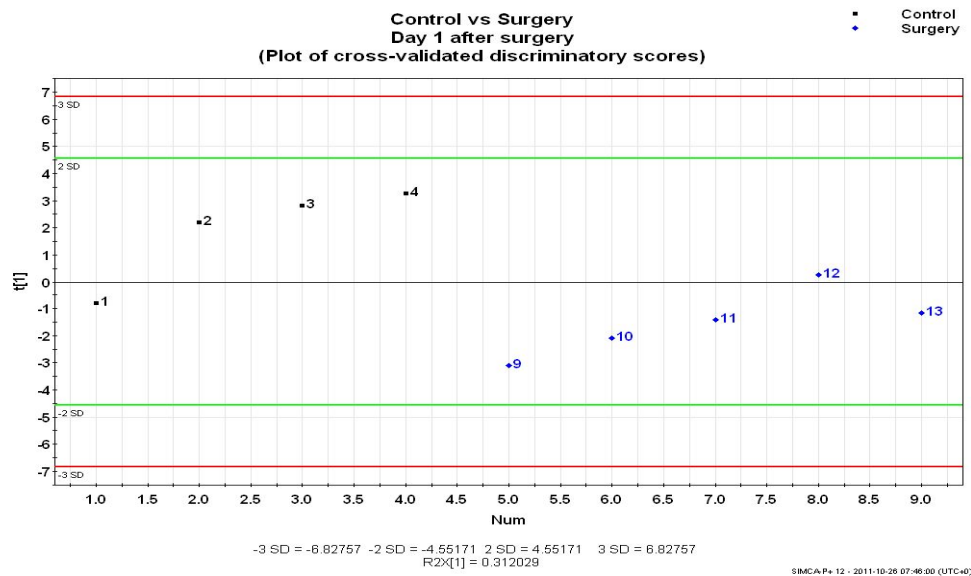


Figure 4.3: Plot of cross-validated discriminatory scores for identification of class separation; control group in black (1–4) and surgery group in blue (9–13).

The cytokines responsible for discrimination between groups (control and surgery) in the Model 2 were IL-12p40 which had a large positive value with the 95% CI not encompassing the x-axis and hence indicating higher values in the control samples. Cytokines IL-1 α , IL-1 β , MCP-1 and MCP-3 had negative values indicating higher values in the surgery group. 12% of the variation in the x-axis was related to the separation between control and surgery (R2X: 0.315) and 75.8% was a systemic variation unrelated to the group separation. These findings indicate that the variation in the x-axis is associated with the level of the different cytokines in each mouse.

The cross validated score plot was confirmed by using a loading Bi-plot. The loading Bi-plot showed how close the variables were to each mouse in the groups (observations). The changes in IL-1 α , IL-1 β , IL-6 and MCP-3 values were closely associated with the surgical group.

The *Model 3*; anaesthesia versus surgery showed class separation and high predictive ability (Q^2 : 0.88 and R^2X : 0.607). IL-6, IL-1 α , KC and TNF- α were identified as discriminatory cytokines between anaesthesia and surgery groups in the loading column plot.

4.2.2 Hippocampal samples - day 1

A PCA analysis was performed. The principal component analysis included the 3 groups to identify if there were any outliers. The cumulative Q^2 was 0.527. The scores plot indicated no major outliers, Ellipse Hotelling T^2 of 0.95 (Figure 4.4). This suggests that the quality of the samples were good.

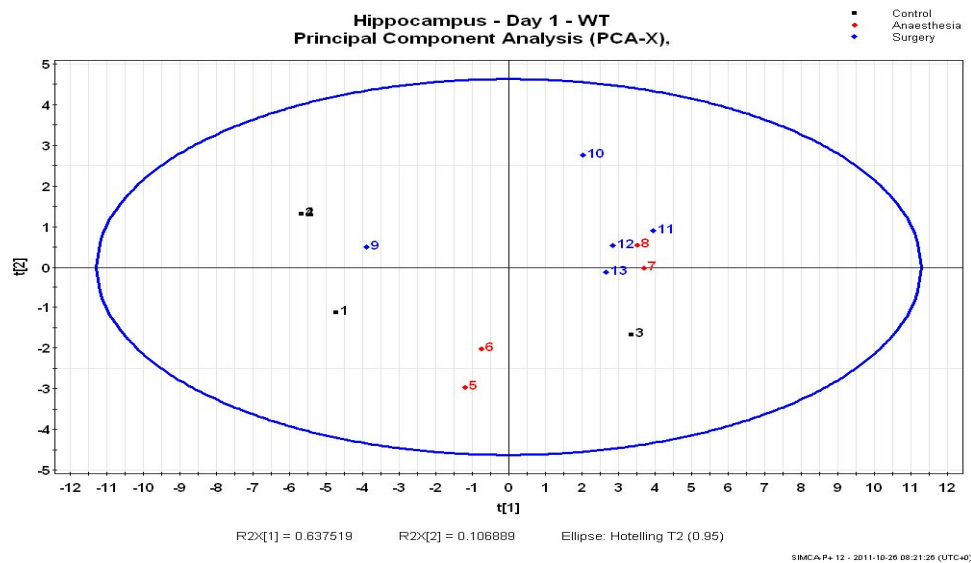


Figure 4.4: Scores for PCA to identify outliers. Control group (1–4) is represented in black, Anaesthesia group (5 – 8) in red and Surgery group (9–13) in blue.

Class separation in each group. In model 1, control compared to anaesthesia, there was no class separation. Model 1 showed a pattern of class separation but the model lacked predictive ability (Q^2 : -0.0271 and R^2Y : 0.177) with just 1

(discriminatory) component and adding orthogonal components reduced the Q2 further. In the loading column plot VEGF, RANTES, IP-10 and IL-12p40 all had putative discriminatory power but none of the variables had the predictive ability to separate between groups.

In Model 2, control compared to surgery there was class separation (group). The first principal component (mouse of the group) had a Q2 of 0.54 and R2X of 0.64 (Figure 4.5) and fitted 1 discriminatory component and 2 orthogonal (cytokines). This is therefore predictive.

A cross-validated discriminatory score was plotted against the first orthogonal component (cytokine) where discrimination was confirmed.

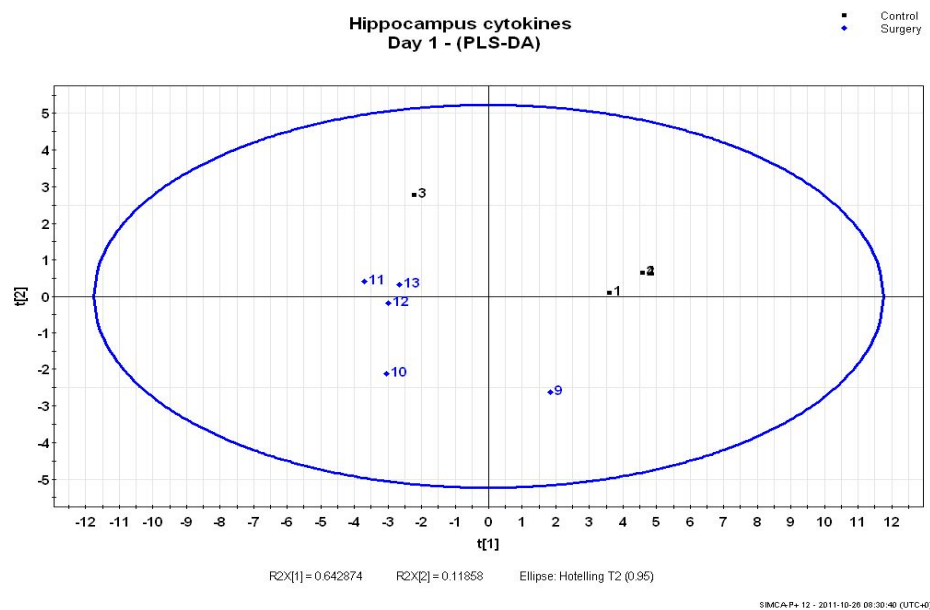


Figure 4.5: Scores for identification of class separation in hippocampal tissue on day 1 after surgery; control in black (1–4) and surgery in blue (9–13).

For the control versus surgery groups, the loadings weights for the discriminatory component (mouse) for each variable (cytokine) were plotted.

MCP-3 had negative values indicating higher values in surgery group while IL-13, IL-17, RANTES, IL-23 and to least extent IL-2, IL-10, IL-12p40 and GM-CSF had positive values (95% CI did not including the x-axis) indicating higher values in control samples (Figure 4.6). The pro-inflammatory cytokines and GM-CSF were low and MCP-3 high in the surgery group relative to control group in the hippocampi on day 1 after surgery.

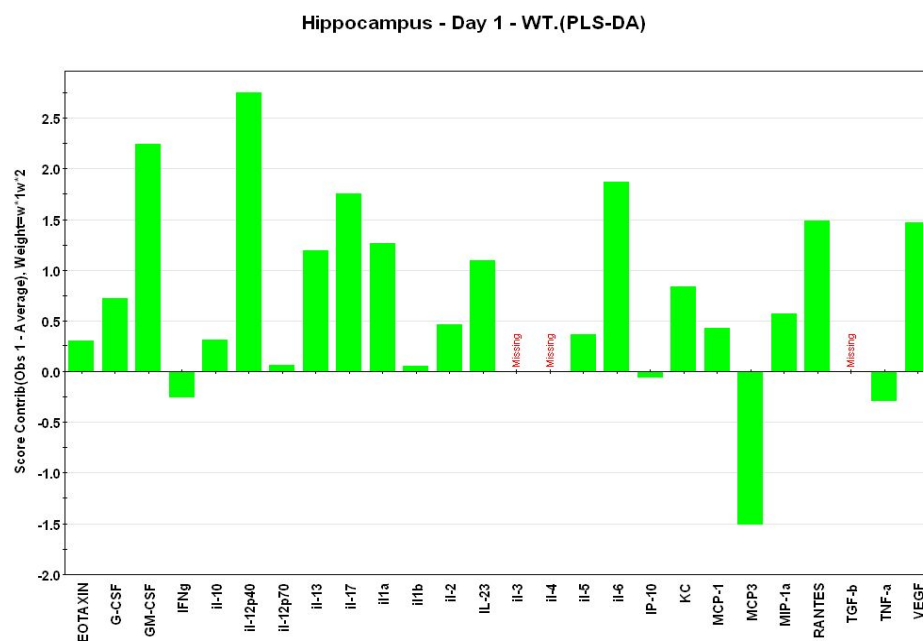


Figure 4.6: Loading column plot for discriminatory cytokines in hippocampal tissue at day 1 after tibial fracture surgery to compare control versus surgery.

Model 3. Anaesthesia was modelled against the surgical group and class separation (group) was identified. The principal orthogonal component displayed an RY2 of 0.837 with a Q2 of 0.630. This suggested a difference in cytokine levels in hippocampi on day 1 after surgery when compared with the anaesthesia group.

A share unique structure plot (SUS-plot) was created to combine results from Model 2 and 3 as they share the same discriminatory cytokines. The SUS-plot could show the effect of these cytokines in each component of the groups of these models. The IFN γ , MCP-3 and GM-CSF were brought up when SUS-plot was applied to the models 2 and 3 for analysis of cytokines in hippocampi on day 1 after surgery under general anaesthesia.

The discriminatory cytokines on the models created for assessment of plasma and hippocampi inflammatory response to tibial fracture surgery and anaesthesia on day 1 are summarised in table 4.1.

Table 4.1: This table summarises the discriminatory cytokines in plasma and hippocampus on day 1 after tibial fracture surgery and anaesthesia.

Models	Plasma	Hippocampus
Model 1 (Control vs Anaesthesia)	No class separation	No class separation
Model 2 (Control vs Surgery)	IL-12p40, IL-1 α , IL-1 β , MCP-1, MCP-3	MCP-3, GM-CSF, IL-13, IL-17, RANTES, IL-23, IL-2, IL-10, IL-12p40
Model 3 (Anaesthesia vs Surgery)	IL-6, IL-1 α , KC, TNF α	MCP-3, GM-CSF, IFN γ

4.2.3 Plasma samples - day 7

On plasma samples taken from a different cohort of wild type C57BL/6 mice on day 7. 12 mice were studied each with a total of 26 variables (cytokines). There were 4 mice as controls, 4 mice had just anaesthesia and 4 mice had anaesthesia and tibial surgery. The data set consisted of a total of 12 observations (mice) and 26 variables (cytokines). The observations were divided in three groups; control, anaesthesia and surgery (classes or components).

A principal component analysis (PCA) analysis was performed. It included the 3 groups to identify if there were any outliers with a cumulative Ellipse: Hotelling T2 (0.95). The scores plot indicated no major outliers (Figure 4.7) suggesting that the quality of the samples were good.

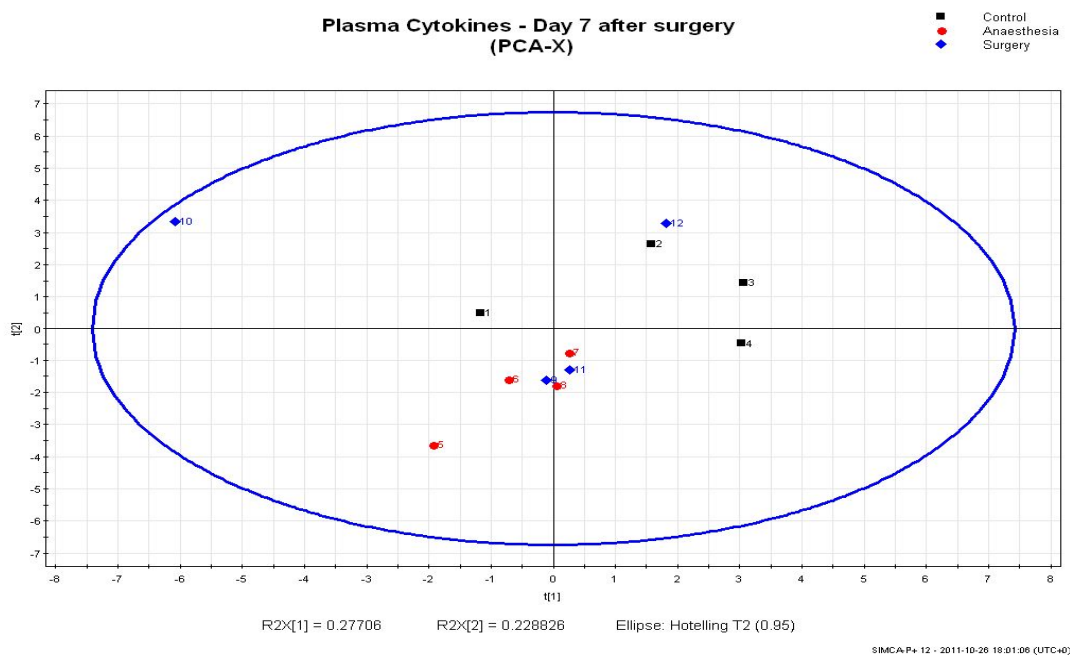


Figure 4.7: Scores for PCA to identify outliers. Control group is represented in black (1–4), Anaesthesia group in red (5–8) and Surgery group in blue (9–12).

With these data from day 7, three models were created as it was done for day 1

analysis: Model 1; the control and anaesthesia groups were selected and surgical observations were excluded. Model 2; control and surgery groups were selected and anaesthesia was excluded and Model 3; anaesthesia and surgery groups selected and control group was excluded.

In Model 1, control versus anaesthesia group there was no class separation (Q2: 0.325) (groups separation) but there was small variability within components in each group (R2Y: 0.783). TNF- α , IFN γ and IL-12p40 were potentially the discriminatory cytokines between these 2 classes (control *versus* anaesthesia group) but there was no statistical differences.

In Model 2, control versus surgery group there was a good class separation with a Q2 of 0.502 and R2Y of 0.45. MCP-1, IFN γ were identified as the cytokines responsible for the discrimination between control and surgery. The cross-validated discriminatory score plotted identified IL-12p40 as the highest cytokine in the control group.

In Model 3, anaesthesia versus surgery there were no outliers identified by the ellipse Hotelling T2 (0.95). There was a good class separation with a Q2 of 0.95 and R2Y of 0.37. However, the loading column plot did not identify a discriminative cytokine between anaesthesia and surgery which suggested a large variability within and between the components in each group. These data suggested that there were no difference in cytokine levels in plasma between surgery and anaesthesia groups on day 7 after intervention.

4.2.4 Hippocampi samples - day 7

A PCA analysis was performed. The PCA selected 6 principal components with a cumulative Q2 of 0.566. The scores plot had no detected outliers (Figure 4.8).

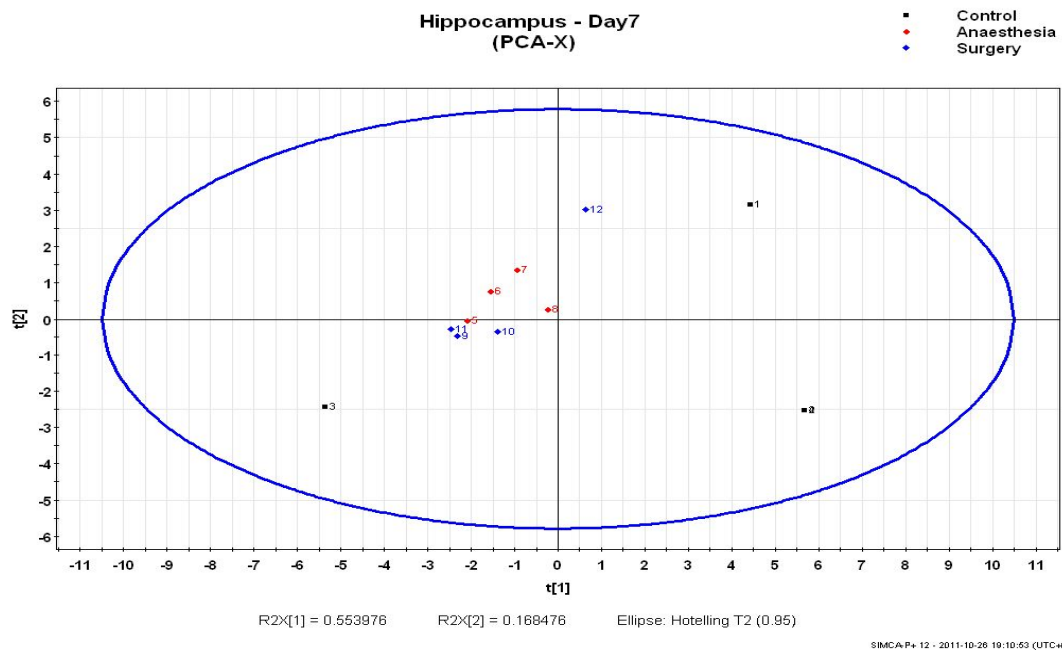


Figure 4.8: Scores for PCA to identify outliers. Control group is represented in black (1–4), Anaesthesia group in red (5–8) and Surgery group in blue (9–12).

In Model 1, control versus anaesthesia there were not outliers identified by the ellipse Hotelling T2 (0.95) and there was not a class separation by cross validation analysis.

In Model 2, control versus surgery there was a good class separation with a high predictive ability, Q2: 0.61 and small components variability, R2Y: 0.872. High levels of cytokines were detected in hippocampus. GM-CSF, IL-12p40, IL-5, and IFN γ were those that separated surgical group from control group (Figure 4.9).

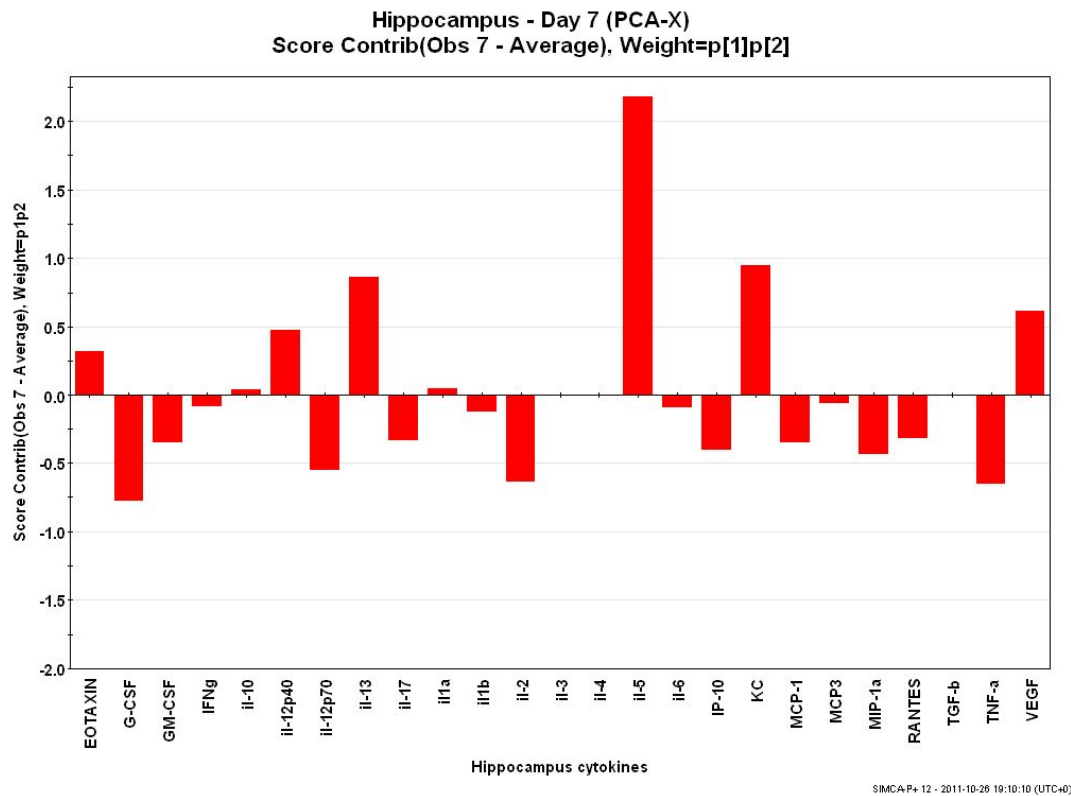


Figure 4.9: Loading column plot for discriminatory cytokines in hippocampi on day 7 after surgery.

In a loading Bi-plot TNF- α , IL-1 β , and IL-3 were close related to the surgical group and it was well apart from the control group in the hippocampi on day 7 after tibial fracture surgery.

In Model 3, anaesthesia *versus* surgery there was a good class separation and the model had also a good predictive ability (R²_Y 0.961 and Q² 0.903) (Figure 4.10). In the loading Bi-plot GM-CSF, IL-3, IFN γ and IL-5 were close related to the surgical group and it was well separated from the anaesthesia group.

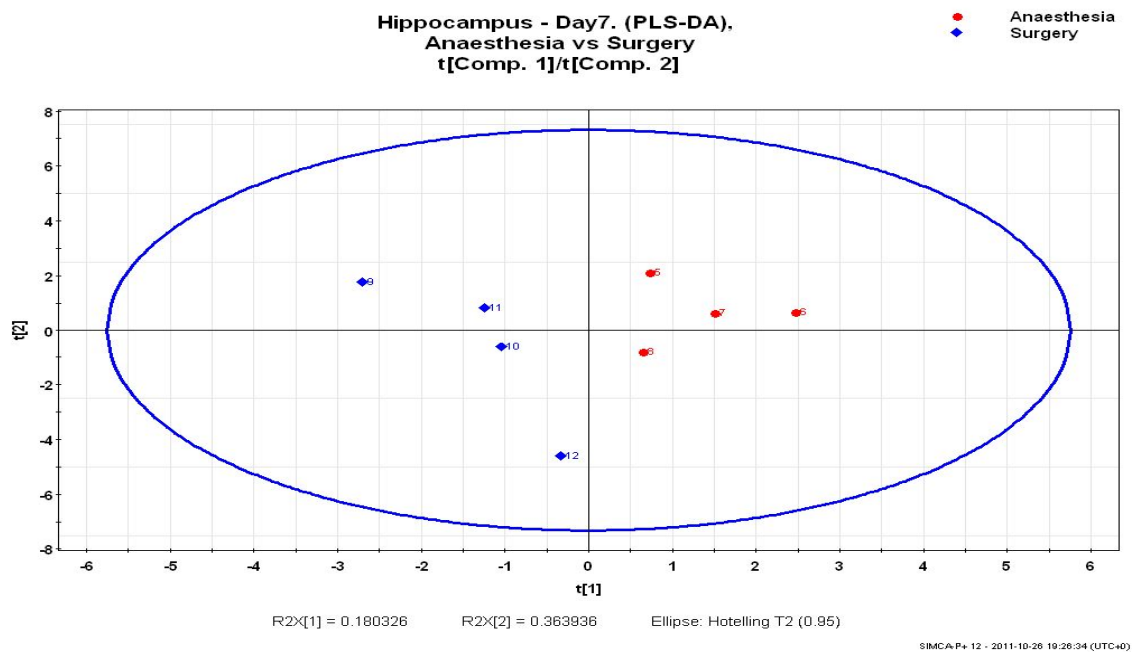


Figure 4.10: Plot of discriminatory scores for identification of class separation in hippocampal tissue at day 7 post-surgery; anaesthesia in red and surgery in blue.

Since Model 2 and Model 3 discrimination scores were good judged by Q2, a shared unique structure plot (SUS-plot) was produced to see if the same cytokines were predictive in the 2 models. In the SUS-plot the loading for the 2 models were plotted against each other. GM-CSF, IFN γ and IL-5 had the same effect in both models.

The discriminatory cytokines found in the models created for assessment of plasma and hippocampi inflammatory response to tibial fracture surgery and anaesthesia on day 7 are summarised in table 4.2.

Table 4.2: This table summarizes the discriminatory cytokines in plasma and hippocampus on day 7 after tibial fracture surgery and anaesthesia.

Models	Plasma	Hippocampus
Model 1 (Control vs Anaesthesia)	No class separation	No class separation
Model 2 (Control vs Surgery)	IL-12p40, MCP-1, IFN γ	GM-CSF, IL-3, IL-5, IFN γ , IL-12p40
Model 3 (Anaesthesia vs Surgery)	GM-CSF, IL-3, IFN γ , IL-5	GM-CSF, IFN γ , IL-5

4.3 Discussion

In summary the statistical isolinear multicomponent analysis showed a good separation between the surgery group and its respective controls on day 1 and 7 after tibial fracture surgery under general anaesthesia.

Plasma level of cytokines identified on day 1 and 7 after surgery allowed good class separation within the models but was not good enough to allow the creation of a SUS-plot to predict the inflammatory response in plasma on these days after tibial fracture surgery under general anaesthesia.

The hippocampus level of cytokines on day 1 and 7 after tibial fracture surgery under general anaesthesia had a clear signal and discriminative ability between

groups in each model and they had good Q2 allowing the creation of SUS-plot for assessment of reproducibility and predictability of the neuroinflammatory response on days 1 and 7 after surgery. IFN γ , GM-CSF and MCP-3 were identified as the key discriminative cytokines in hippocampi on day 1 after surgery whilst the IFN γ , GM-CSF and IL-5 were identified as the key discriminative cytokines in hippocampi on day 7 after surgery.

This is a new tool and therefore it is important to understand the real and potential limitations of the study. There are several limitations with this approach for the identification of the physiological signature for the development of POCD.

Firstly, the mice used for these experiments were young and therefore these results may not represent the inflammatory response in old mice as the physiological decay may interfere with the normal immunological response to stress. Secondly, the sample size may be too small to develop a model that can reliably predict a biological response. Thirdly, the samples used for assessment of cytokines were stored at very low temperature then brought back to room temperature for processing and therefore it is difficult to ascertain if some proteins were potentially denatured.

Lastly, the statistical isolinear multicomponent analysis is a useful tool as it not only allows the assessment of multiple variables at any time point but also their influence on the model and also the degree of variability within samples. It is a system of linear equations with repeated measurements of physical phenomena

to attempt to compensate for experimental errors. This statistical software may have some limitations when it is used for analysis of small samples as the data may be over fitted and therefore increase the false discovery rate - type I error. The cross validation analysis rotates the sample over the x-axis by using predictors which are normally one of the variables that separates different classes within a model. It shows how accurately a predictive model will perform in practice.

Given these limitations this method is potentially an exciting new technique for looking at complex mechanisms. The conclusions are limited but these findings suggest that even though there is a systemic and CNS inflammatory response after tibial fracture surgery under general anaesthesia, it is difficult to pin point a single cytokine as the key player and main contributor for the development of POCD. It is evident that the inflammatory response is complex and the cytokines released in the periphery after surgery may trigger a CNS inflammatory response, they may be involved in the process of neuronal connectivity disruption.

Chapter 5:

Identification of the cell response to surgery by flow cytometry analysis

This is an approach for analysis of cells in areas of the brain where memory is formed, to attain a better understanding of how these cells may be involved in the CNS inflammatory response after non-neurological surgery.

The inflammatory response has been suggested as one of the key components in the development of postoperative decline (Wan, Xu et al. 2007; Cibelli, Fidalgo et al. 2010; Terrando, Monaco et al. 2010). The implication of the systemic inflammatory response is well-established in *in-vivo* studies (Terrando, Rei Fidalgo et al. ; Barrientos, Higgins et al. 2006) and several attempts have been made to understand the role of inflammation in the central nervous system following major non-neurosurgical insults (Dantzer, Bluthé et al. 1998; Dantzer 2001; Thomson and Sutherland 2005).

In particular glial cells have been amply studied and it is generally accepted that the microglia is activated following remote surgery (Barrientos, Higgins et al. 2006; Wan, Xu et al. 2010). An association has also been described between microglia activation and memory decline after surgery (Wan, Xu et al. 2010).

The role of the endothelial cells in the blood brain barrier is less well understood but it is thought that they act as a bridge or inter-phase between the periphery and the central nervous system (Swiergiel and Dunn 2007; Teeling, Felton et al. 2007). The interaction of the endothelium cells with neurones are less well understood when it comes to their contribution to the development of POCD.

To dissect out the activity of any such inter-cellular pathways during their transmission of the systemic response, quantitative analysis of key mediators

and responses would be appropriate. Flow cytometry has been used routinely to monitor the state of inflammatory cells within the blood or immune organs (spleen, thymus etc), but less so in solid non-immune tissue. In the CNS, the abundance of myelin debris in enzymatically digested tissue has been a particular hindrance. To get around this problem, a prior cell enrichment step has been a pre-requisite for flow cytometric analysis, and this usually involves density gradient centrifugal separation (Ford, Foulcher et al. 1996) optimised for a particular population of interest (microglia). The advent of higher capability flow cytometers may reduce the impact of sample 'contamination' simply through collecting more sample 'events' at a higher speed.

The aim of this chapter is to attempt to identify CNS cells, specifically from the hippocampus with the aim of optimising the flow cytometry technique for isolation of central nervous system cells (CNS) and hence gain a more precise understanding of the inflammatory response associated with the development of POCD after non-neurological surgery.

5.1 Methods

A flow cytometric method was chosen to perform these experiments but this can be challenging as cell recovery from small tissues can be difficult. Initially the aim was to obtain sufficient cells from a single mouse hippocampus for multi-parameter analysis of different populations. Following this, pilot studies for validation were conducted.

Animal handling, termination and surgery were explained in chapter 3, sections:3.1.2,3.2.1.b and 3.3.2. The preparation of cell suspensions and staining were explained in section 3.8.

The steps used to optimise and validate the flow cytometry studies for hippocampal cells are described in Table 5.1

Table 5.1: This table illustrates the steps taken for optimization and validation of the flow cytometry studies for isolation and identification of CNS cells in the hippocampi of mice.

Steps	Experiments
1	Analysis of microglia in hippocampus single cell suspensions prepared by standard enzymatic digestion
2	Development of a fixation-homogenisation method for preparation of single cell suspensions from hippocampus
3	Development of a direct method for removal of myelin debris during data acquisition and analysis
4	Proof of concept: Measurement of CD11b expression changes after surgery

5.1.1 Analysis of microglia in hippocampus single cell suspensions prepared by standard enzymatic digestion.

This step consisted of the isolation of microglia cells from the hippocampus of a wild type C57BL/6 mouse without treatment, that was terminated by schedule 1.

To develop the flow cytometry based method for analysis of multiple cell types in the mouse hippocampus, a recently published method for the identification of microglia within collagenase-prepared total single cell suspensions from rat spinal cord, was explored for suitability (Blackbeard, O'Dea et al. 2007).

In brief, the cells were isolated by a standard enzymatic reaction which consisted of collagenase digestion and then microglia were identified on the basis of CD11b expression and low CD45 expression. This distinguished them from vascular CD11b-positive cells which display distinctly higher CD45 levels (Ford, Goodsall et al. 1995). The final step consisted of microglia enrichment by density gradient separation that removes other cell types and myelin debris (Ford, Goodsall et al. 1995).

5.1.2 Development of a fixation-homogenisation method for preparation of single cell suspensions from the hippocampus

This step consisted in isolating astrocytes and endothelium and microglia from the hippocampus of a wild type C57BL/6 mouse without treatment that was terminated by schedule 1.

Due to the potential for modifications of surface and intracellular antigen levels

during enzyme digestion (37°C for 15m), a novel method for preparation of single cell suspensions (O'Dea, Dokpesi et al. 2011) was used. A fixation-based approach for preparation of the single cell suspension from hippocampus and preservation of their *in vivo* phenotype was investigated.

In brief, hippocampus tissue was suspended in 1% paraformaldehyde base fixative and immediately homogenized (Gentle MACS, Miltenyi) for a short period (1 min), and then allowed to fix for a further 10 minutes. An optional step in this method is cell permeabilisation using saponin-based buffers, in order to stain for intra-cellular antigens. Cell suspensions were then processed and stained with antibodies as before.

5.1.3 Development of a direct method for removal of myelin debris during data acquisition and analysis

This step consisted in excluding the myelin debris from analysis of the hippocampus of a wild type C57BL/6 mouse without treatment that was terminated by schedule 1.

To support these results and validate the method for removal of myelin debris during data acquisition and analysis the same methods were performed using hippocampi from newborn mouse pups where minimal myelination takes place (Simons and Trotter 2007).

In brief, physical and immune-magnetic bead (Miltenyi) methods exist for removing myelin debris from CNS cell suspensions, but these add additional steps to the procedure and may not be compatible with the fixation technique and the very small amounts of tissue available. To clearly distinguish between cells and myelin debris, fluorescent labelling of DNA to positively identify cells was investigated. Just before acquiring cells, the fluorescent DNA dye 7AAD (1:100; Biolegend) was added to samples in which cells had been permeabilised after fixation.

Modifications to the cell homogenization and fixation protocol were introduced at this time, with a slower homogenization speed and reduced fixation period (1 min instead of 10min) which produced more consistent staining patterns and yield.

5.1.4 Measurement of CD11b expression changes after surgery

To implement the optimised method, both CD11b and CD68 expression in hippocampi at 24h after nephrectomy were determined in a proof of concept experiment.

A cohort of wild type C57BL/6 mice were divided into 2 groups; Group 1: Control which received no treatment (n=3) and Group 2: Surgery which had left nephrectomy under general anaesthesia (n=3).

The microglia were identified by the combination of CD68 and CD45 markers to quantify CD11b, or CD11b and CD45 markers to quantify CD68.

5.2 Results

5.2.1 Analysis of microglia in hippocampus single cell suspensions prepared by standard enzymatic digestion

As shown in figure 5.1, microglia could be identified in hippocampus from a single mouse, but they were a relatively rare population when considered as a percentage of total events acquired ($\sim 0.2\%$).

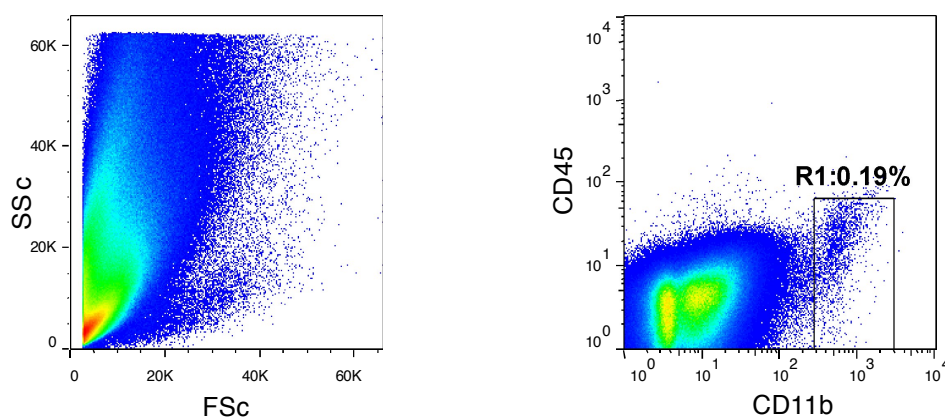


Figure 5.1: A single cell suspension from the hippocampus of a single mouse was prepared by standard collagenase digestion method. The forward scatter (FSc) and side scatter (SSc) profile is depicted. Staining with fluorescence-conjugated CD11b and CD45 antibodies identified microglia cells (R1: CD11b+, CD45low).

5.2.2 Development of a fixation-homogenisation method for preparation of single cell suspensions from hippocampus

Microglia (CD11b+, CD45low) were clearly identified using this method. Further validation was performed using the anti-CD68 myeloid marker antibody. The suitability of this method for identification of other cells types was also

demonstrated for astrocytes by using the intracellular GFAP marker (GFAP versus CD11b). Combination of GFAP with the CD31 (Platelet endothelial cell adhesion molecule-1 - PECAM-1) marker identified cells that could be either GFAP-positive endothelia/platelets or a CD31-positive astrocyte subpopulation (Figure 5.2). Further markers in addition to GFAP may be required to delineate astrocytes by flow cytometry in view of the high sensitivity of this method (compared to the standard IHC visualization of GFAP+ astrocytes) and therefore the potential to pick up low-GFAP expressing non-astrocyte cells.

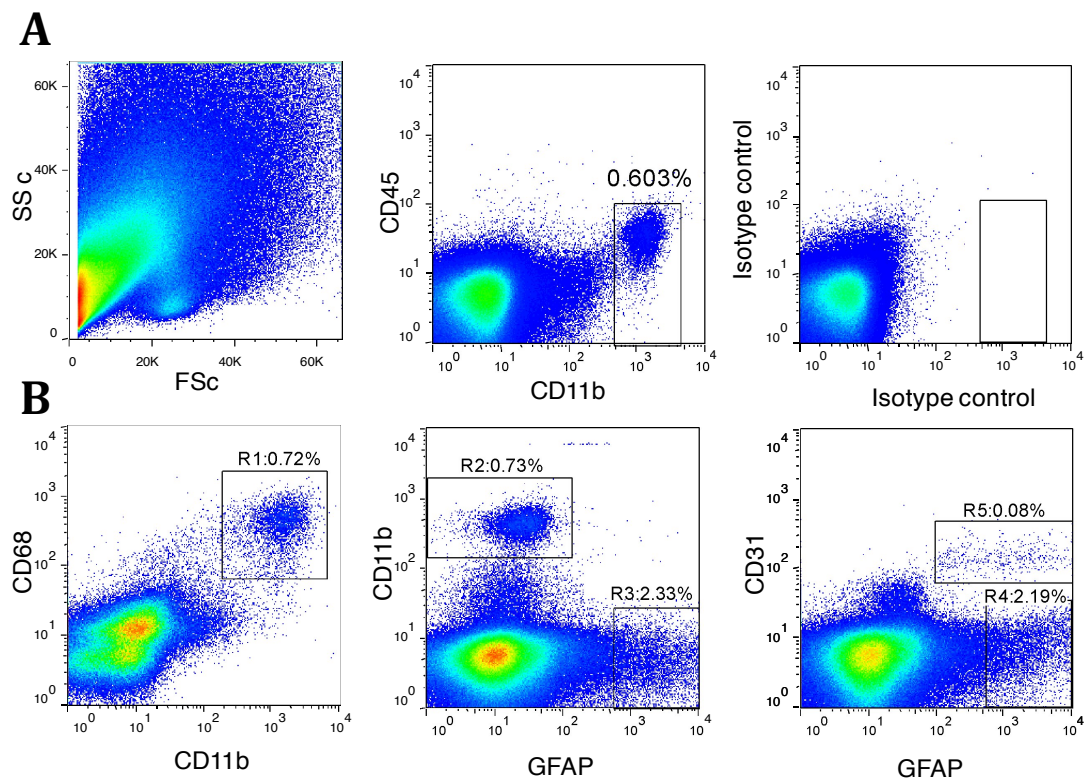


Figure 5.2: Cell identification in fixed-homogenised mouse hippocampi. A. microglia were identified as CD11b+, CD45low. Isotype-matched control antibody stained is shown for comparison. B. Additional markers were used to identify microglia (R1:CD68+, CD11b+), and to discriminate GFAP positive cells (R3 and R4) from CD11b and CD31 positive cells. A CD31+, GFAP+ population (R5) was also identified.

5.2. Development of a direct method for exclusion of myelin debris during data acquisition and analysis

The fix-homogenise method appeared to produce a clearer identification of microglia than in collagenase prepared cell suspensions. However, plots of event size (FSc) against granularity (SSc) in comparison with the normal profile of cells (data not shown) suggested considerable contamination with non-cellular material. This was likely to be myelin debris.

7AAD staining produced a clear separation of DNA containing events/cells, excluding ~80% of events (Figure 5.3). Limiting analysis to 7AAD gated events, revealed a higher proportion of CD11b and GFAP positive cells, at proportions closer to that expected in the CNS. Cell counts produced a yield per mouse hippocampus of $\sim 6.5 \times 10^4$ microglia and $\sim 1.5 \times 10^5$ GFAP+ cells. This is sufficient to run multiple stained samples from one mouse.

Consistent with 7AAD negative events being derived from myelin debris, the proportion of total events dropped from ~80% in adults to less than 15% in neonate hippocampi (Figure 5.3). With the technique validated, it was possible limit the acquisition of data to the cell component by setting the flow cytometer threshold for event recognition and acquisition of only 7AAD positive events.

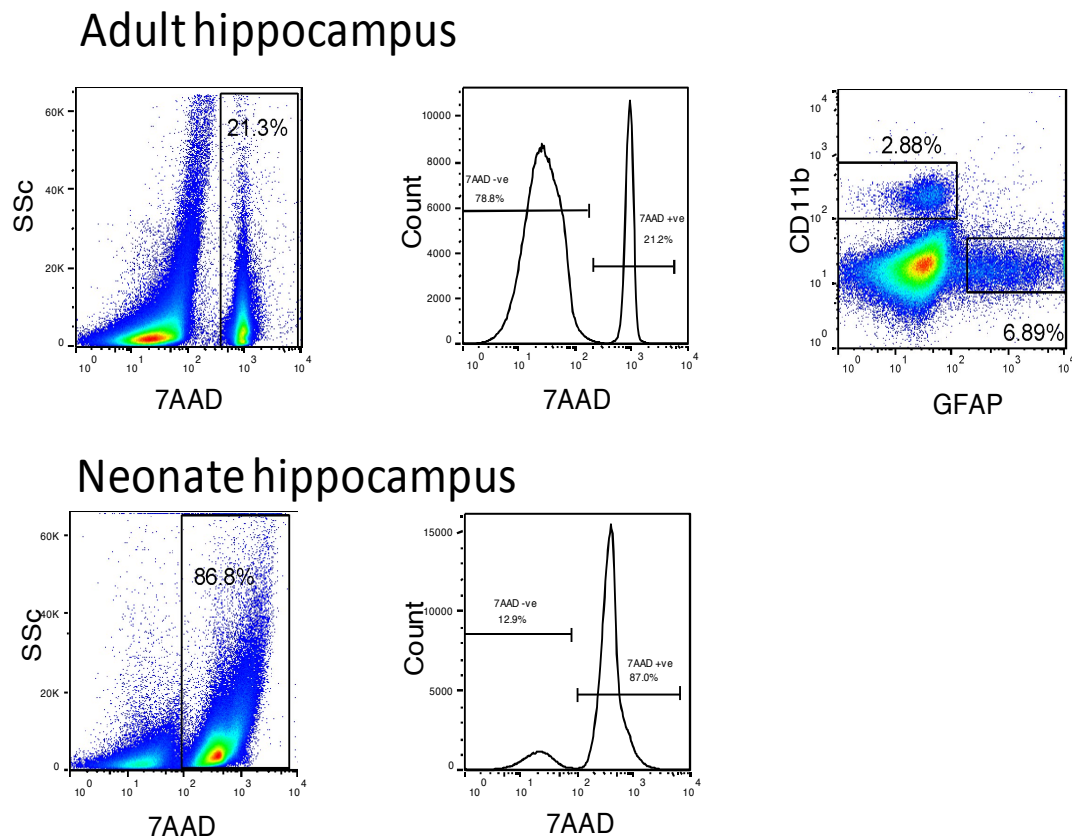


Figure 5.3: Cells in fixed-homogenised hippocampi suspensions identified using the fluorescent DNA stain, 7AAD. Cells were identified as 7AAD positive in adult hippocampi (dot plot against SSC and histogram shown for comparison). 7AAD gated cells with CD11b and GFAP staining are shown. Neonate hippocampi displayed a much higher proportion of 7AAD positive events consistent with lack of myelination.

5.2.4 Measurement of CD11b expression changes after surgery

A significant increase in CD11b resulted in a mean fluorescence intensity of 470 ± 85 after surgery compared to controls ($p < 0.01$, $n = 3$) (Figure 5.4). This proof of concept experiment demonstrated the validity of the new method to quantify absolute changes in antigen expression. This is in contrast to visualizing

antigen density changes that can be affected by alterations in cell morphology.

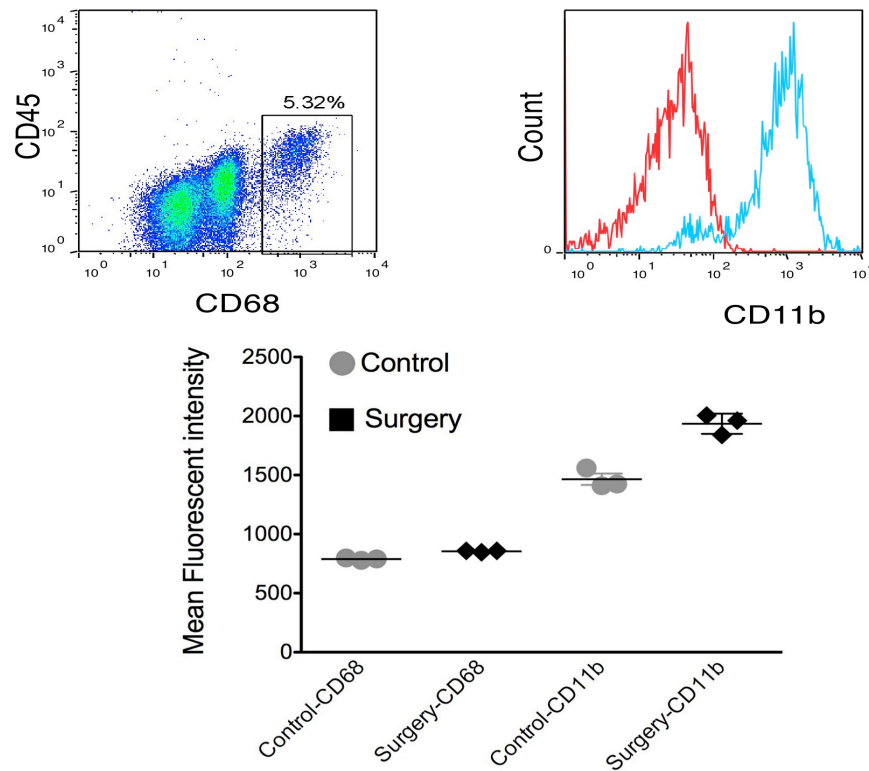


Figure 5.4: Microglia expression of CD11b and CD68 at 24h post-surgery. Hippocampus from nephrectomised mice (+24h) were processed by fixation, homogenisation and cells gated on 7AAD. Histogram shows microglia (CD68+, CD45^{low}) staining (blue) compared to isotype-matched control antibody (red). A significant increase in CD11b expression was observed in microglia at 24 hours post-surgery ($p < 0.01$, $n = 3$).

5.3 Discussion

By implementing a method of simultaneous fixation and homogenisation the microglia were clearly identified in sufficient numbers to perform multiple parameter analysis of cells from a single mouse hippocampus tissue. The

fixation step enabled quantification of different antigens with minimal alterations in their levels in contrast to the results that might be expected with traditional CNS tissue disaggregation methods.

The main techniques currently used to assess hippocampus inflammation include ELISA, Western blot and IHC.

The enzyme-linked immunoabsorbent assay (ELISA) for instance is a good quantitative method but the immunoreaction of the antibodies maybe adversely affected. Cross reactivity may occur with the secondary antibodies resulting in non-specific signal. The ELISA method does not detect individual cell response or the specific cell population and the signal amplification may be diluted.

The western blotting (WB) analysis is a non-quantitative method but it is very specific. There are potential problems in that sometimes, non-intended proteins may react with secondary antibodies resulting in labelling of incorrect proteins. The transfer of proteins may be affected by methanol resulting in falsely low level of proteins transferred to the membrane. As with the ELISA the WB technique does not identify individual cells and or specific cell populations.

The immunohistochemistry (IHC) is an excellent qualitative method. It provides anatomical information and morphology but sometimes lacks specificity. Results may be difficult to interpret or reproduce. Even though it is a qualitative method the scoring or grading system is performed manually or generated by computing software. The variation of the results may be related to the quality of the

staining, exposure time and the operator performing the scoring. In addition, the IHC is not a quantitative method and therefore is not sensitive enough when cell activity needs to be measured.

The aim of this study was to develop a flow cytometry method that could be used routinely to distinguish different cells types in the hippocampus in sufficient numbers and accuracy to quantify key markers of activation. This would be of benefit over existent methods because it will complement IHC findings by identifying individual cells response to different insults.

In this set of experiments microglia activation was identified after surgery by FACS with a higher yield of cells. This is in line with previous immunohistochemical findings reported in the literature (Cibelli, Fidalgo et al. 2010). The FACS analysis permits the assessment of different cell lineage, at different time point, from specific areas of the brain. This could, therefore, facilitate more complex experiments aiming to identify cellular communication during ongoing inflammatory processes.

However, there were at least two major challenges. A considerable challenge was presented by the mouse hippocampus because of its size. The other major technical challenge presented by CNS tissue for direct flow cytometric analysis was presented by the overwhelming contamination of sample with myelin debris. Isolation of cells partly addresses this problem, but may modify the composition of cells populations normally present in tissue and may not be realistic for analysis of small tissue samples, such as the hippocampus. To

address this issue, DNA dye (7AAD) was used, successfully, to reduce or eliminate myelin debris from analysis and hence reduce its impact on data acquisition. Therefore, a simple and economic method was identified to enable direct flow cytometric analysis of multiple samples obtained from small CNS tissue samples.

There are some disadvantages to using flow cytometry, particularly in comparison to IHC. These are principally the inability to detect anatomical differences in a single cell population (e.g. microglia) and the loss of morphological indication of cell activation. This is a problem because this may not allow individual cell characterisation and identification of interaction with other adjacent cellular structures. A benefit of the result obtained here is that the CD11b expression post surgery highlights an important issue relating to absolute changes in antigen expression versus apparent changes that can result from altered cell geometry and distribution within the tissue. These findings may highlight that when the cell activity increases there were morphological changes. These could well have been the initial changes seen after cell activation even before mobilisation and the subsequent increase in the number of cells in the injured areas of the brain or those areas of the brain that were affected by remote insults. In this respect flow cytometry offers a complementary approach to IHC, measuring both antigen expression quantitatively and also absolute cell numbers that may change as a result of cell proliferation or death.

Future directions for this work includes identification of additional populations and validation of methods for measuring additional inflammatory markers, both

membrane and intracellular.

If it is possible to identify specific cell populations from specific regions of the brain at different time points it should provide a novel approach with which to unravel the mystery of cell communication.

Chapter 6:

The role of P2X7 nucleotide receptor in the development of POCD

The theme that the inflammatory response to surgery is linked to the development of POCD makes the DAMPs relevant as a potential mechanism of signalling. DAMPS effectively trigger signalling through purinergic receptors (P2X7) and so the role of these receptors will be explored in this chapter.

The purinergic receptors, P2X7, activate microglia *via* IL-1 β and IL-18 synthesis (Solle, Labasi et al. 2001; Lopez-Castejon, Young et al. 2007; Skaper, Debetto et al. 2010). These cytokines, in turn, stimulate the inflammatory response through further production of IL-6, COX-2, IFN γ , MCP-1, superoxides and MMPs. There is also a feedback loop that increases the sensitivity and expression of P2X7 (Guan, Buckman et al. 1998; Parvathenani, Tertyshnikova et al. 2003; Narcisse, Scemes et al. 2005). An increase in pro-inflammatory cytokines was associated with an up-regulation of P2X7 receptors and with the cognitive decline that occurs with advancing age, ischaemia and neurodegenerative diseases including Alzheimer's, multiple and lateral amyloid sclerosis (Hartlage-Rubsamen, Zeitschel et al. 2003; Parvathenani, Tertyshnikova et al. 2003; Franke, Gunther et al. 2004; Yiangou, Facer et al. 2006; Crain, Nikodemova et al. 2009).

The role of P2X7 has also been tested in models of neuronal injury. P2X7 nucleotide receptor antagonists in mice improve functional recovery and neuronal survival after spinal cord injury (Wang, Arcuino et al. 2004). Similarly, recovery from spinal cord injuries and neuropathic pain improved significantly after the use of a selective inhibitor of P2X7 purinergic receptor (Nieber, Eschke et al. 1999; Chessell, Hatcher et al. 2005) Peng et al. 2009).

If antagonising the effect of P2X7 receptor is beneficial then its activation may be a mechanism of POCD. Therefore the main aim of this chapter is to ascertain if the activation of P2X7 receptor contributes to the development of POCD.

6.1 Methods

To characterize the role of P2X7 receptor in POCD a series of *in vitro* experiments were performed. Subsequently 3 *in vivo* models with specific insults and interventions were developed (Table 6.1).

Cell culture, surgeries, behavioural studies and laboratory techniques performed in this chapter were described in details in chapter 3, section: 3.1.2, 3.1.3.a, 3.1.3.b, 3.2.1.a, 3.2.1.c, 3.4, 3.5.1, 3.5.2, 3.6 and 3.7.

Table 6.1: This table summarises the experiments performed in this chapter to ascertain the contribution of the P2X7 receptor to the development of POCD.

	Experiments	Technique
<i>In vitro</i>	Identification of P2X7 receptor in astrocytes and microglia	- Western Blotting and Immunohistochemistry
	Calcium signalling for identification of P2X7 activity after ATP challenge	- Confocal microscopy (analysis of MFI)
	Characterisation of the role of P2X7 in sickness behaviour	- Fear conditioning training
<i>In vivo</i>	Characterization of the role of P2X7 in POCD	- Behavioural studies (Fear conditioning training) and analytical biochemical assay (ELISA)
	Characterization of the role of P2X7 in ischaemic stroke	- Behavioural studies (assessment of the motor score) and calculation of the volume of the infarct area

6.1 *In vivo* experiments

6.1.1 Characterization of the role of P2X7 receptor in sickness behaviour

To assess if the P2X7 had an impact on learning (acquisition) in sick mice the LPS mouse model was used. In this preliminary work, a lipopolysaccharide (LPS)

challenge was performed to assess learning (acquisition) and memory in mice treated with P2X7 antagonist. This was used to assess proof of concept

Adult wild type C57BL/6 male mice were allocated into one of the following groups:

Group 1: Naïve or no treatment group (n=3).

Group 2: LPS group: 1mg/Kg injection of LPS i.p was given 24h after training and memory was assessed on day 3 after treatment (n=3).

Group 3: LPS + P2X7 antagonist: 1mg/Kg injection of LPS i.p and 60mg/Kg suramin also i.p were giving simultaneously 24h after training and memory was assessed on day 3 post treatment (n=3).

All groups were trained by fear conditioning (FC); for those having LPS it was 24h after the LPS challenge. A cohort of mice was used for assessment of learning by association. Another cohort of mice was used for assessment of hippocampus dependent and independent memory on day 3 after the LPS challenge with and without P2X7 antagonist.

6.1.2 Characterization of the role of P2X7 receptor in POCD

The characterization of the role of P2X7 receptor in POCD was performed by assessment of memory impairment by fear conditioning after tibial fracture under general anaesthesia. The systemic inflammatory response was also assessed by measuring the pro-inflammatory cytokines, IL-1 β in plasma on day 1 and 7 after corresponding interventions.

Assessment of memory was performed by fear conditioning training after the insult in wild type C57BL/6 adult male mice (C57BL/6-Wt) and in P2X7 knock-out (P2X7-KO) adult male mice. The mice were allocated to one of the following groups:

Group 1: Control: naïve animals without treatment (C).

Group 2: 1.5% Isoflurane anaesthesia (A).

Group 3: Tibial fracture surgery under general anaesthesia (S).

Different cohorts of mice were used for assessment of memory on day 1, 3 and 7. Thirty mice were allocated to each group (There were 3 C57BL/6-Wt and 3 P2X7-KO groups). A total of 180 mice were used for these experiments. There was 1 fatality and 1 termination by schedule 1 on day 1 after surgery.

Baseline response to pain, motor activity and ability to learn was assessed in a different cohort of C57BL6-Wt and P2X7-KO before commencing these experiments (for details in baseline differences between these 2 type of mice please refer to table 3.4 appendix 1).

6.1.3 Characterization of the role of P2X7 receptor in ischaemic stroke

Focal brain ischaemia was performed to characterize the role of P2X7 receptor in the reactive microgliosis seen after transient ischaemic stroke. The characterization of the P2X7 receptor was performed at 24h after the left middle cerebral occlusion (MCAO) by assessing the neurological deficit and infarct size with and without the P2X7 antagonist, BB-G.

Wild type adult C57BL/6 mice were allocated to one of the following groups:

Group 1. Left middle cerebral occlusion (MCAO) + 500µl normal saline i.p (S-P) (n=8).

Group 2: Left middle cerebral occlusion (MCAO) + 125µg BB-G in 500µl normal saline i.p (BB-G) (n=8).

(There were no negative controls in this set of experiments as the aim was to assess motor activity and infarct area)

6.2 Results

6.2.1 *In vitro* experiments

6.2.1.a Identification of P2X7 receptor in astrocytes and microglia

The presence of P2X7 receptor in astrocytes and microglia (BV2 cells) was confirmed by western blotting (Figure 6.1). As can be seen in Figure 6.1 the astrocytes and microglia clearly show P2X7 bands.

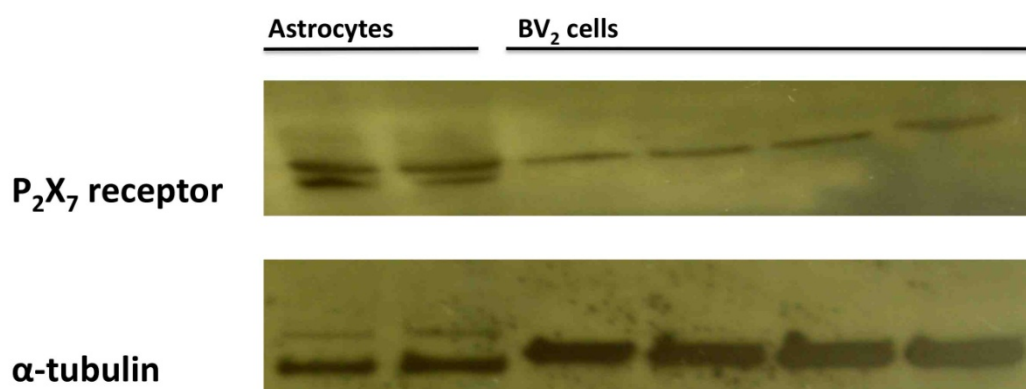


Figure 6.1: Illustrative western blotting images to show the presence of positive bands when immuno-blotted with P2X7 receptors antibodies on the cells used for in

vitro studies (Top panel). The presence of α -tubulin was used for internal control of the samples (Low panel) to illustrate the load of proteins in each well.

The presence of P2X7 receptor in microglia was also confirmed by immunohistochemistry (Figure 6.2).

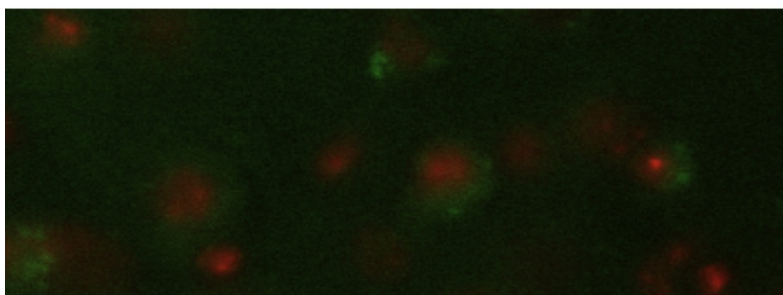


Figure 6.2: Representative illustration of microglia (BV2 cells) stained with FITC for identification of P2X7 receptors. The green immunostaining illustrates the localization of P2X7 receptors on the microglia cells. The cell nuclei stain red.

6.2.1.b Calcium signalling for identification of P2X7 activity after ATP challenge using confocal microscopy

The mouse cortical astrocyte cells showed an immediate response to 1.0 μ M ATP challenge as assessed by the increase in the mean fluorescent intensity (MFI). The maximum response was seen at 5m after ATP stimulation. A gradual decay of the MFI was observed afterwards, reaching baseline levels 20m post ATP stimulation (Figure 6.3).

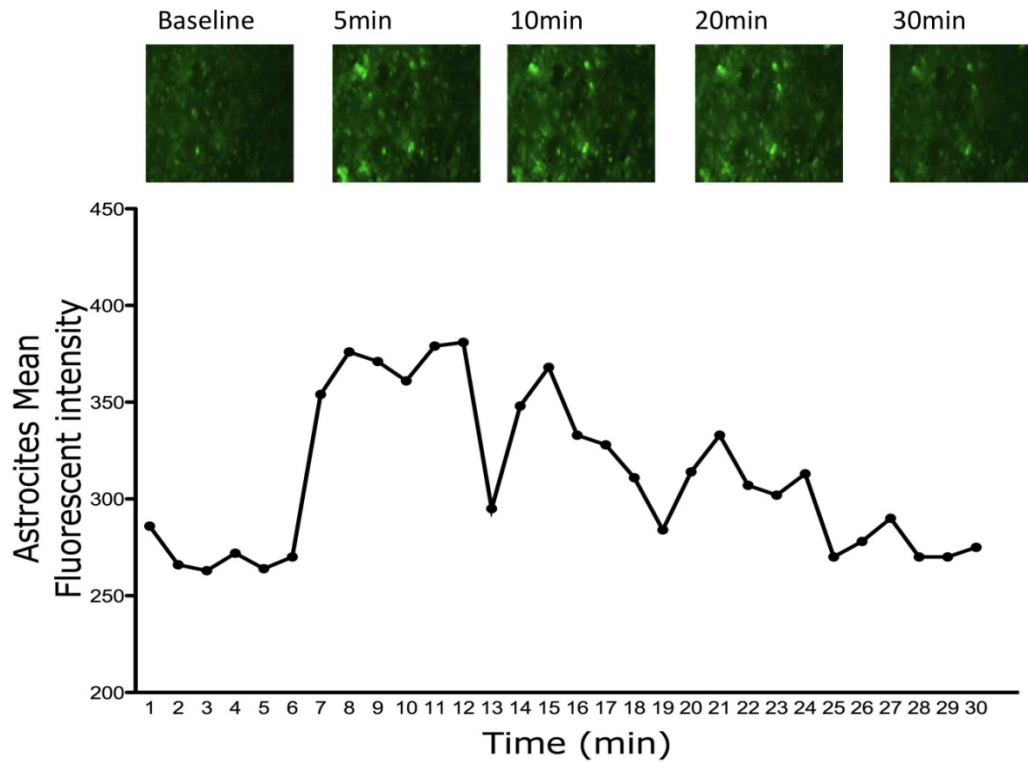


Figure 6.3: Representative images of 20d old cortical mouse astrocyte at baseline and then representative images up to 30m after ATP stimulation. Response was measured by mean fluorescent intensity.

The microglia showed an immediate response to 1 μ M ATP challenge as assessed by an increase in the MFI and then a steady increase in the MFI with a maximum response at 13m. The decay phase mirrored the cortical astrocyte response (Figure 6.4).

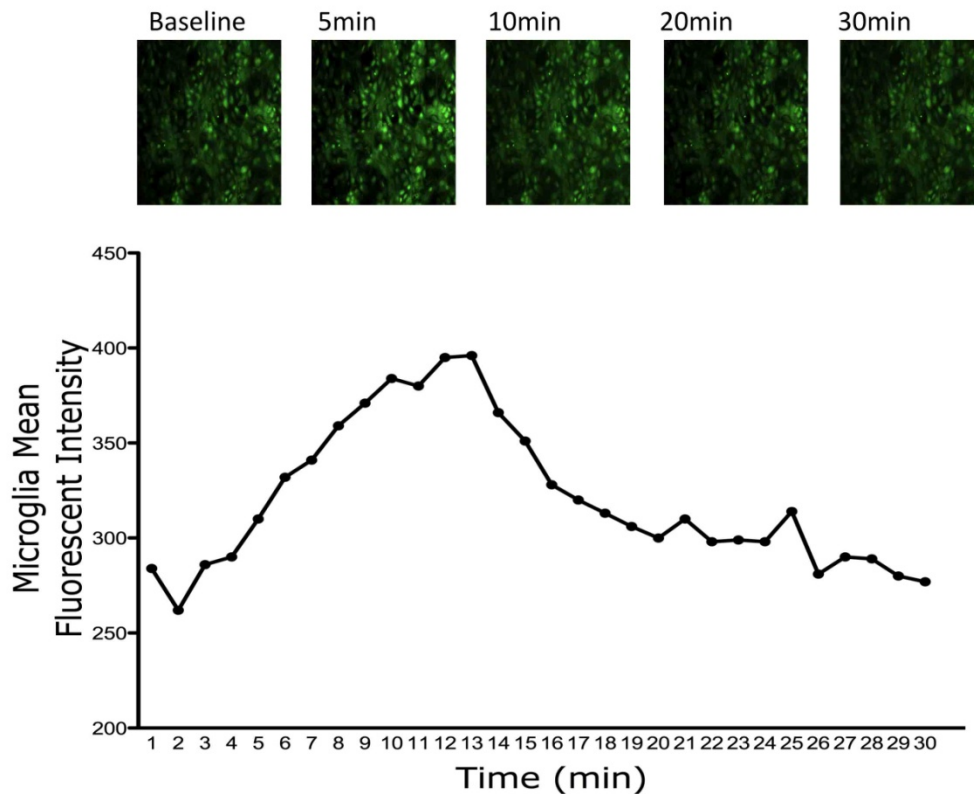


Figure 6.4: Representative images of immortalised microglia cells (BV2) at baseline and at different time points up to 30m after ATP stimulation. Response was measured as mean fluorescent intensity (MFI).

The same experiments were conducted with hippocampal and microglia astrocytes (Figure 6.5). Unfortunately, the cell recovery from the hippocampus was very difficult therefore this was a solo experiment conducted with wild type C57BL/6 mouse hippocampal cells.

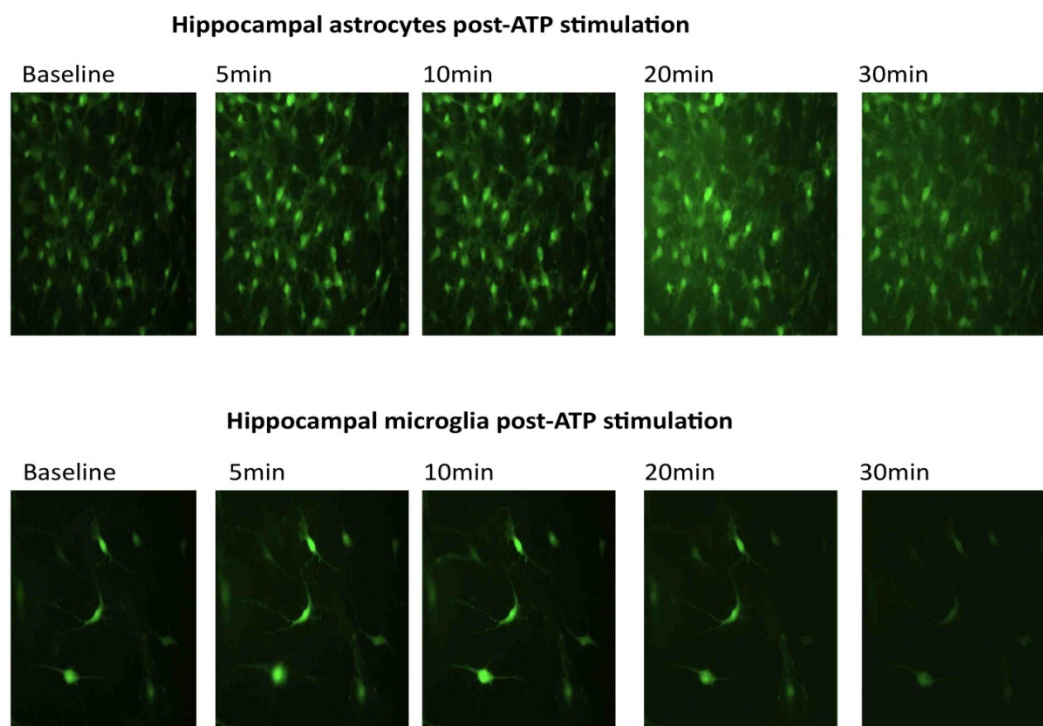


Figure 6.5: This figure illustrates the response of hippocampus astrocytes (top panel) to ATP stimulation at different time points (5, 10, 15, 20 and 30m). The microglia response to ATP stimulation is illustrated in the low panel at different time points (5, 10, 15, 20 and 30m). It clearly shows hippocampal and microglia activation after ATP stimulation. (This is a representative image of 1 well stimulation with ATP in real time).

The following step in the calcium signalling after stimulation with ATP study, was to modify the cells activation by adding an antagonist of the P2X7 receptor to the medium where the cells were kept. The antagonist was BB-G at a concentration of 10 μ M.

The cortical astrocyte cells response to ATP stimulation was reduced in intensity when the cells were exposed to the P2X7 receptor antagonist (Figure 6.6).

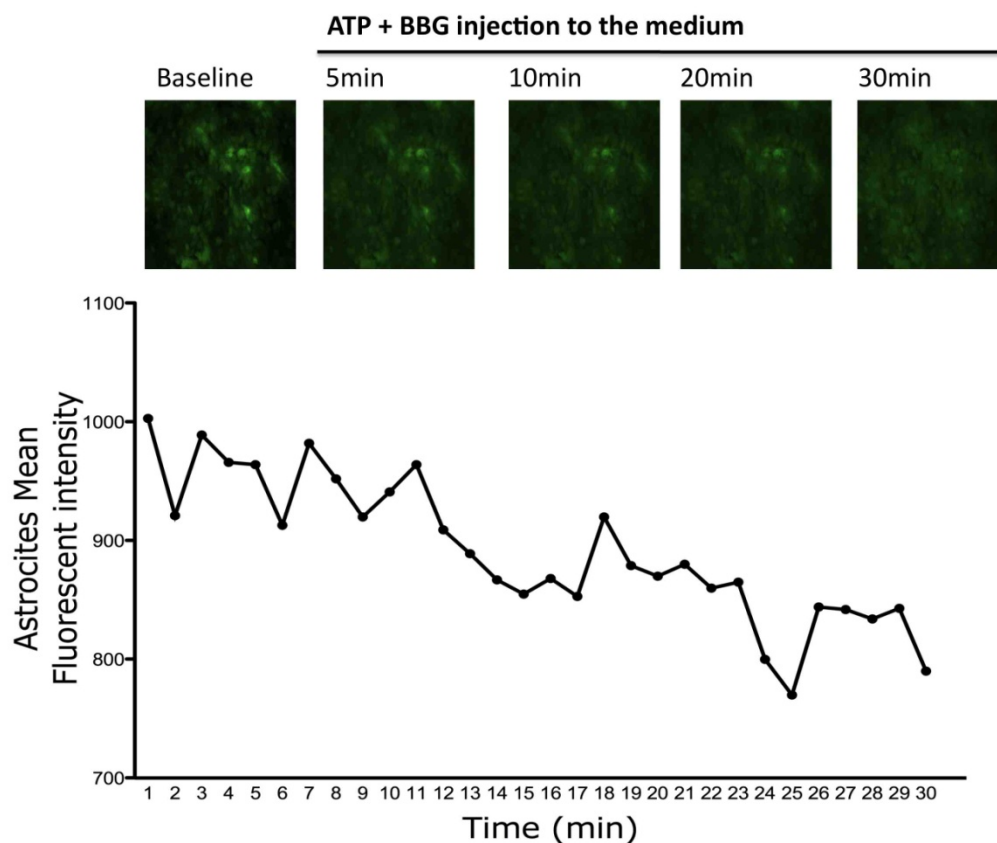


Figure 6.6: Representative images of mouse cortical astrocytes at baseline and at different time points up to 30m after ATP stimulation and P2X7 receptor antagonism with BB-G. The response was measured as mean fluorescent intensity(MFI). There is a clear reduction in MFI after the addition of the P2X7 antagonist (BB=G).

The final step of the calcium signalling after ATP challenge study, was to assess the activation of the procaspase-1 by looking at the cell morphological changes. The procaspase-1 is converted to caspase-1 after the activation of the P2X7 receptor. This is the enzyme responsible for cleaving the pro-IL-1 β to the active IL-1 β .

This was explored by using confocal microscopy for assessment of MFI and cell morphology after incubating the cortical astrocytes and microglia with procaspase-1 (FLICA™) and 1 μ M ATP for 60m.

The cell morphology changed after the cells were stimulated with ATP and there was clear evidence of activation of the proscapse-1 enzyme as is shown in Figure 6.7. Under the microscope the cortical astrocyte displayed long and extended ramifications, while the body became more prominent. The astrocytes developed new extensions. The new extensions were similar to dendritic cell processes. The cells seemed to “network” actively. The bodies started detaching from each other and the cells looked very active. Some of them had an amoeboid body while other cells ramified after ATP stimulation and started releasing microvesicles (Figure 6.7 and see video 1 in appendix 3).

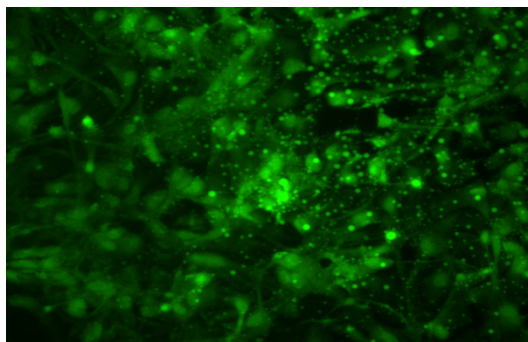


Figure 6.7: Illustration of microvesicles released by cortical astrocytes 18min after stimulation with ATP. This photography was taken during the video recording of the astrocytes stimulation with ATP in the confocal microscope. It shows a marked effect on cells morphology and activity. Activity defined by cells ability to release microvesicles. Cells are stained with fluorescein isothiocyanate (FITC).

The microglia (BV2 cells) also showed significant morphological changes after 60m incubation with ATP and procaspase-1. The microglia released microvesicles 18m after exposure to ATP. This effect was suppressed when the ATP was co-administered with the P2X7 receptor antagonist (BB-G) (Figure 6.8).

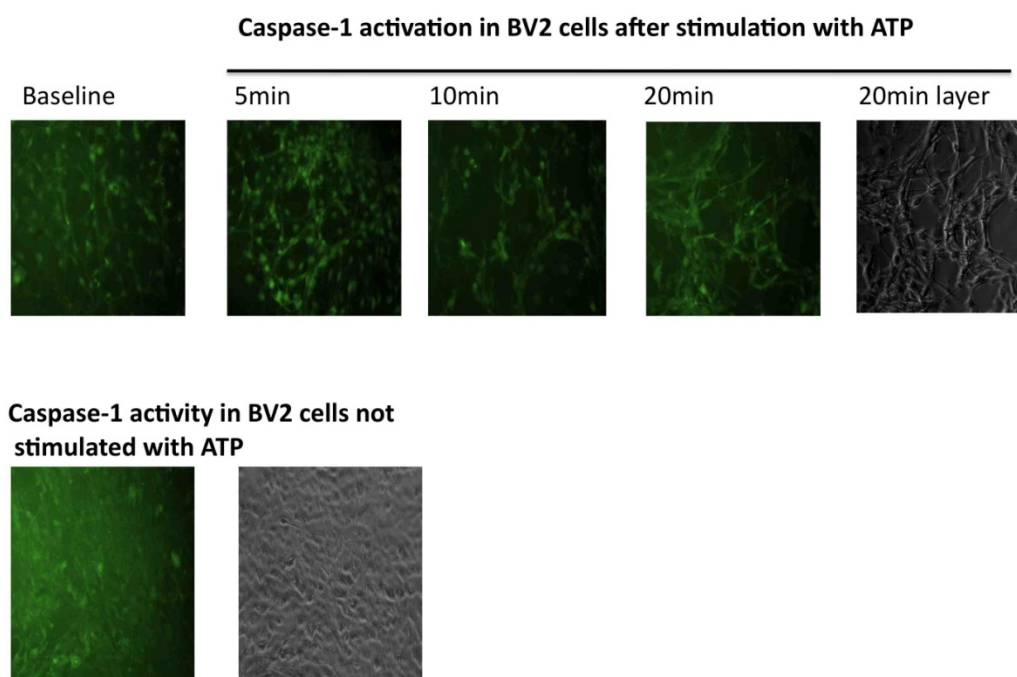


Figure 6.8: Representative images of caspase-1 activity in BV2 cells taken on the confocal microscope at different time points to illustrate the microglia response to ATP stimulation. The top panel illustrates the microglia morphological changes at different time points after stimulation with ATP (baseline, 5, 10, 15 and 20m). The low panel illustrates the microglia morphology without stimulation with ATP.

In summary the *in vitro* experiments demonstrated the presence of P2X7 receptors in the astrocyte and microglia cells. They also confirmed the response to both ATP stimulation and activation of pro-caspase-1. Given these findings it was reasonable, therefore, to conduct *in vivo* experiments to characterise the role

of P2X7 receptors initially in sickness behaviour and then in the development of POCD.

6.2.2 *In vivo* experiments

6.2.2.1 Characterization of the role of P2X7 in sickness behaviour in mice

6.2.2.1.a Learning abilities

The ability to learn by fear conditioning was assessed 24h after either LPS challenge or LPS plus P2X7 antagonist and in the control group which did not receive any treatment. All mice in the control group learned by association as indicated by their ability to recall after fear-conditioning training. Ability to recall by fear conditioning training was defined as the percentage of freezing after training or time of immobility apart from respiration observed after the delivery of the conditional stimulus. The group exposed to LPS after training showed deterioration in the ability to learn (acquisition) expressed as a median/IQ/range (24/21-43/21-43) when compared with their control (non treated group) (50/45-66/45-66). The group that received the antagonist of the P2X7 receptor in conjunction with the LPS did not show deterioration in the ability to learn by fear-conditioning training (37/36-48/36-48) when compared to the control group (Figure 6.9). There was a clear trend towards the prevention of learning impairment as assessed by fear conditioning training after the administration of P2X7 antagonist. Unfortunately, the numbers were too small to realistically perform statistical calculations.

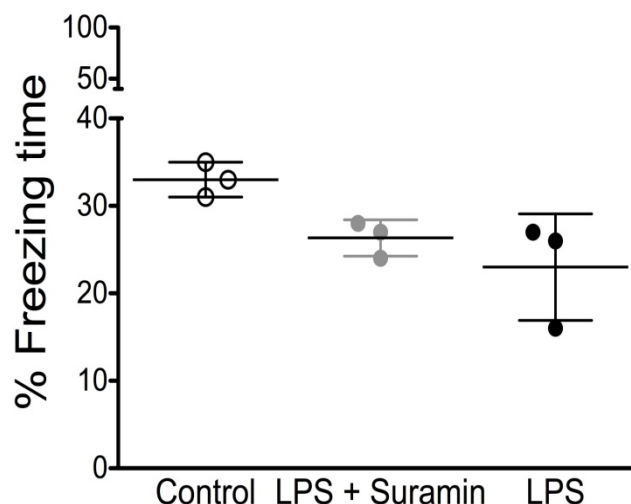


Figure 6.9 This scatter histogram represents acquisition after training. % Freezing time represents the degree of recall. LPS was administered i.p 24h before the training. There were 3 groups; Control (Open dot), LPS + Suramin (light gray dot) and LPS (black dot). N3 per group, data presented as mean/IQ/range.

6.2.2.1.b Hippocampal dependent memory

The hippocampal dependent memory was assessed on day 3 following challenge with either LPS or LPS and P2X7 antagonist (suramin).

Following LPS challenge, mice treated with the P2X7 antagonist, showed preservation of hippocampal-dependent memory (54/44-64/44-64) when compared to the LPS alone group (24/19-53/19-53). The LPS alone group showed a clear difference in memory recall when compared to control (54/44-64/44-64) (Figure 6.10).

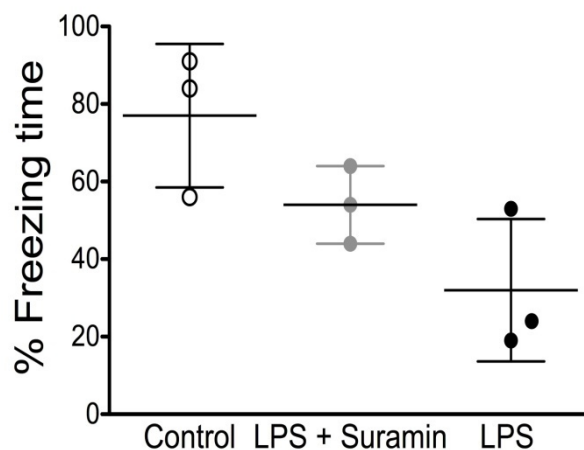


Figure 6.10: This scatter histogram represents hippocampal dependent memory after treatment. % Freezing time represents the degree of recall. LPS was administered i.p 24h before the training. There were 3 groups; Control (Open dot), LPS + Suramin (light gray dot) and LPS (black dot). N3 per group, data presented as median/IQ/range.

6.2.2.1.c Hippocampal independent memory

The hippocampal independent memory was also assessed on day 3 after mice were challenged with LPS i.p or LPS plus suramin i.p, the P2X7 antagonist.

Mice exposed to LPS showed a reduction in freezing time. However, there was no statistical difference in hippocampal independent memory on day 3 after LPS challenge (24/21-43/21-43) when compared to LPS + suramin group (37/36-48/36-48) or control group (50/45-66/45-66) (Figure 6.11).

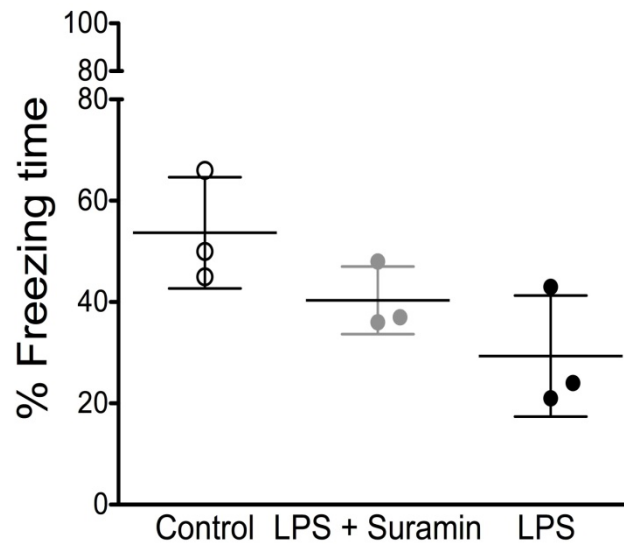


Figure 6.11: This histogram represents hippocampal independent memory after treatment. % Freezing time represents the degree of recall. LPS was administered i.p 24h before the training. There were 3 groups; Control (Open dot), LPS + Suramin (light gray dot) and LPS (black dot). N3 per group, data presented as median/IQ/range.

6.2.3 Characterization of the role of P2X7 in POCD

6.2.3.a Learning abilities

Acquisition of memory during training 24h before surgery was similar between C57BL/6-Wt and P2X7-KO mice. Maximal performance was achieved by the end of the 6th training session (Figure 6.12) indicating that memory was acquired and established after learning by association.

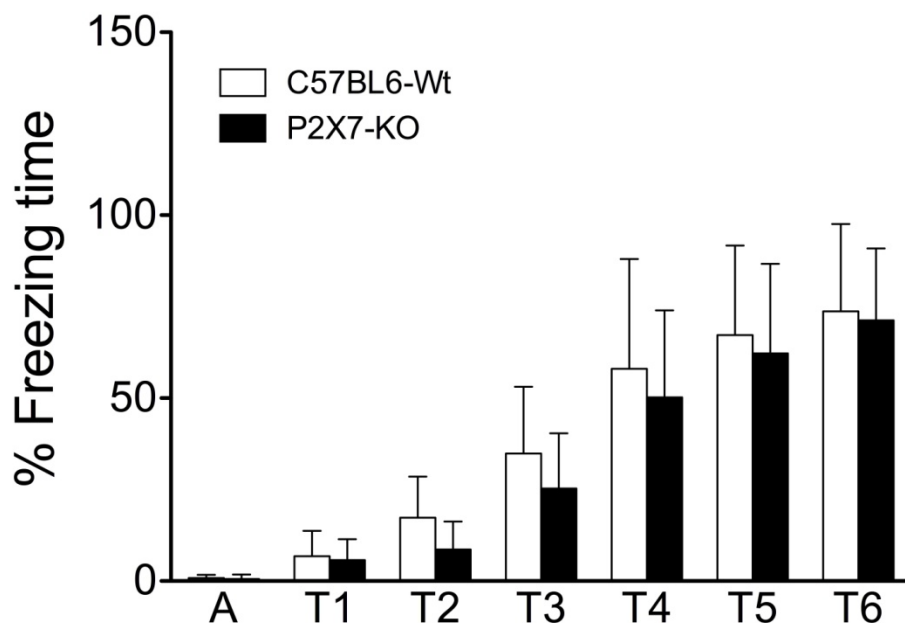


Figure 6.12: Acquisition of memory during the training phase of the fear-conditioning protocol is similar in C57BL/6-Wt and P2X7-KO mice. Both mouse strains achieved maximal performance by the 6th training session. No differences in establishment of long term memory were found between C57BL/6-Wt (open bar) and P2X7-KO mice (black bar). Values are mean $\pm$ SD for $n=30$ mice per group. (The y-axis scale up to 150 to illustrate the top end of the error bars as there were few mice that showed a % 100 freezing time).

6.2.3.b Hippocampal dependent memory

The contextual response a form of hippocampal dependent memory was not affected by tibial fracture surgery under general anaesthesia on day 1 after intervention. There was no treatment effect by 2-way ANOVA analysis on day 1, 3 and 7 ($P=0.09$, $P=0.35$ and $P=0.71$ respectively).

There was a genotype effect on day 3 with a $P=0.041$ but, there was not genotype effect on day 1 and 7 ($P=0.21$ and $P=0.87$ respectively) (Figure 6.13).

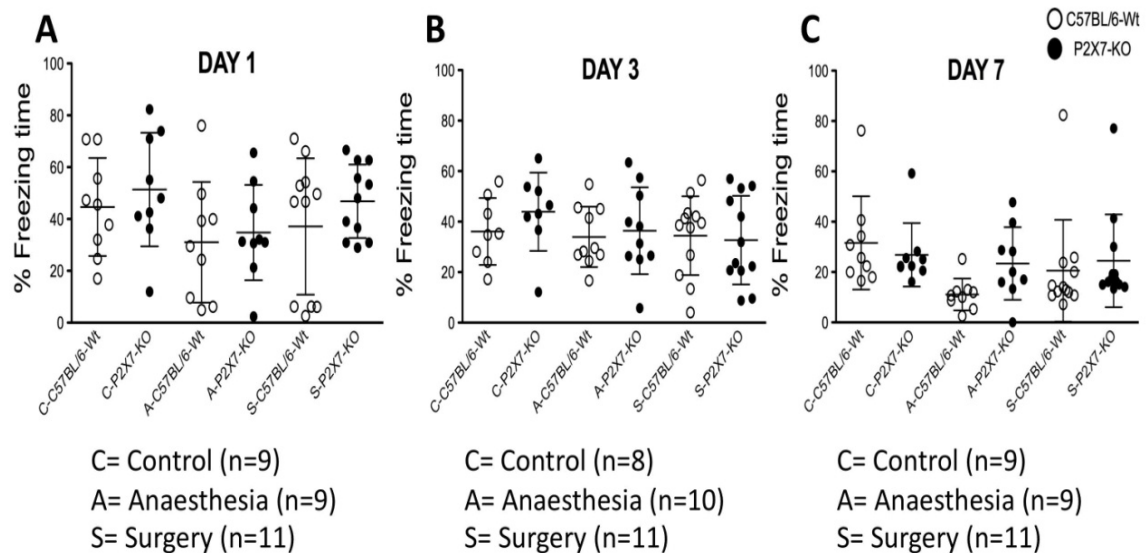


Figure 6.13: Hippocampal dependent memory assessed by contextual response showed a genotype effect on day 3. Contextual response (CR) a form of hippocampal dependent memory was assessed as % freezing time on days 1, 3 and 7 after surgery. Groups were Control, Anaesthesia and Surgery. (A) Shows % freezing time on day 1. (B) Shows % freezing time on day 3. (C) Shows % freezing time on day 7. Values are median $\pm$ range. Black dots represent C57BL/6-Wt groups and white dots represent P2X7-KO groups.

Contextual conditional response (CCR) another form of assessment of hippocampal dependent memory, showed neither treatment nor genotype effect on day 1; $P=0.07$ and $P=0.20$ respectively, on day 3; $P=0.37$ and $P=0.43$ respectively and on day 7; $P=0.63$ and $P=0.53$ respectively post-treatment by 2-way ANOVA analysis (Figure 6.14).

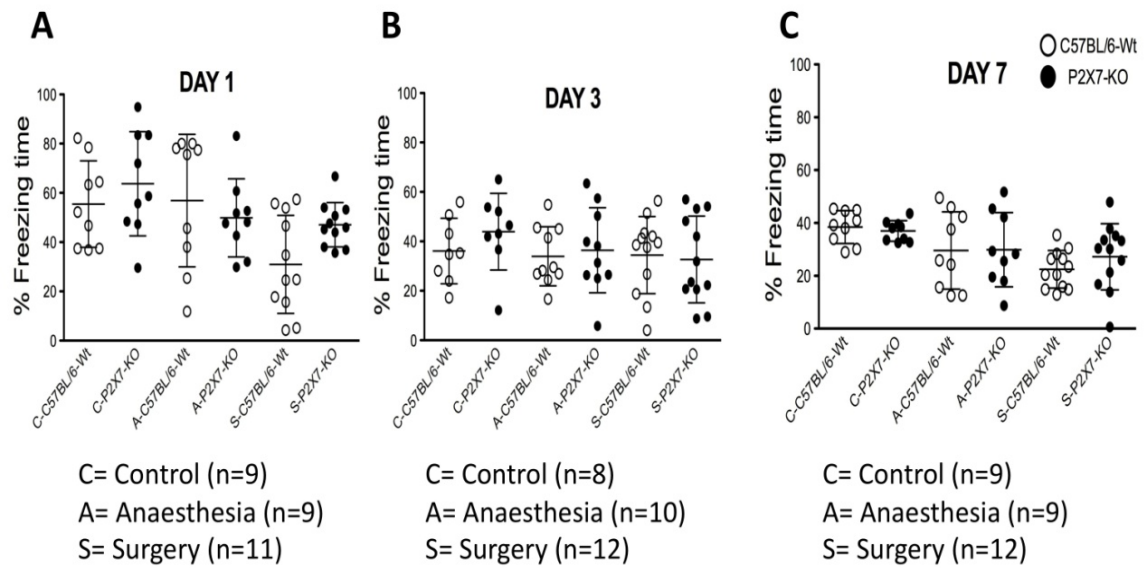


Figure 6.14: Hippocampal dependent memory assessed by conditional contextual response showed neither genotype nor treatment effect on any of the days assessed. Contextual conditional response (CCR), a form of hippocampal dependent memory, was assessed as % freezing time on days 1, 3 and 7 after surgery. Groups were Control, Anaesthesia and Surgery. (A) Shows % freezing time on day 1. (B) Shows % freezing time on day 3. (C) Shows % freezing time on day 7. Values are median $\pm$ range. Black dots represent C57BL/6-Wt groups and white dots represent P2X7-KO groups.

6.2.3.c Hippocampal independent memory

The effect of anaesthesia and surgery on hippocampal independent memory was assessed on day 1, 3 and 7 after corresponding treatment.

Neither genotype nor treatment effect was observed on day 1, $P=0.06$ and $P=0.15$ and on day 7, $P=0.84$ and $P=0.21$ respectively after treatment. There was a genotype effect on day 3, $P=0.045$ (Figure 6.15).

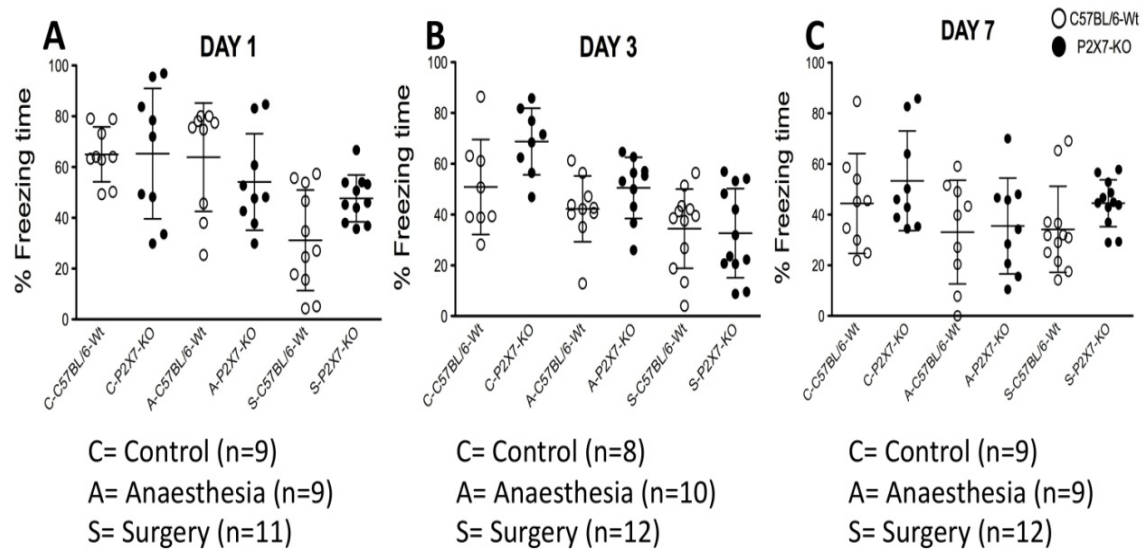


Figure 6.15: Hippocampal independent memory assessed by response during delivery of the conditional stimulus. There was genotype and marginal treatment effect on day 3. Conditional response a form of hippocampal independent memory was assessed as % freezing time on days 1, 3 and 7 after surgery. Groups were Control, Anaesthesia and Surgery. (A) Shows % freezing time on day 1. (B) Shows % freezing time on day 3. (C) Shows % freezing time on day 7. Values are median $\pm$ range. Black dots represent C57BL/6-Wt groups and white dots represent P2X7-KO groups.

6.2.4 Assessment of the systemic inflammatory response by cytokine values

6.2.4.a Plasma level of IL-1 β after surgery

The characterization of the systemic inflammatory response after tibial fracture under general anaesthesia was performed by assessing the plasma level of IL-1 β

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on day 1, 3 and 7 after the corresponding interventions. Different cohorts of mice were used for assessment of IL-1 β level in plasma on day 1, 3 and 7.

There was neither genotype effect nor treatment effect by 2-way ANOVA analysis in plasma IL-1 β after tibial fracture and anaesthesia on day 1, 3 and 7 (Table 6.2).

Table 6.2: This table summarises the 2-way ANOVA analysis performed for assessment of IL-1 β in plasma after tibial fracture surgery under general anaesthesia. Genotype effect refers to C57BL/6-Wt group and P2X7-KO group. The treatment effect refers to Control, isoflurane anaesthesia and surgery groups. Day 1, 3 and 7 refers to days after surgery and anaesthesia.

	Day 1	Day 3	Day 7
Genotype effect	P = 0.70	P = 0.77	P = 0.06
Treatment effect	P = 0.24	P = 0.61	P = 0.66

The level of IL-1 β in plasma after tibial fracture on day 1 was high in the surgery and anaesthesia groups when compared to their respective control groups (Figure 6.16).

The plasma level of IL-1 β remained high in both surgical groups and in the C57BL/6-Wt Anaesthesia group on day 3 and 7 (Figure 6.16).

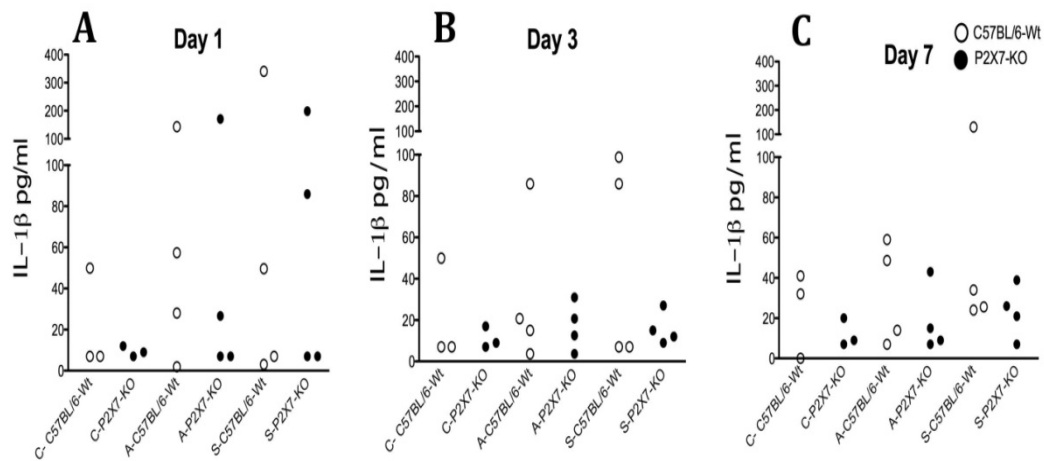


Figure 6.16: This figure illustrates the plasma level of IL-1 β on day 1, 3 and 7 after treatment (Scatter plots A, B and C respectively). The experimental groups were Control (C-C57BL/6-Wt, C-P2X7-KO), Anaesthesia (A-C57BL/6-Wt, A-P2X7-KO) and Surgery group (S-C57BL/6-Wt, S-P2X7-KO), (n=3-4/group).

6.2.4 Characterization of the role of P2X7 in ischaemic stroke

6.2.4.1 Neurological assessment

Neurological assessment was performed 24h after the MCAO using the Garcia scoring system which takes into consideration spontaneous activity, symmetry in movements of 4 limbs, climbing and forepaw outstretching. There was no difference in neurological deficit between mice that received placebo (11.71 ± 0.52) and mice treated with the P2X7 antagonist, BB-G (10.71 ± 0.52) $P=0.10$ on day 1 after MCAO.

6.2.4.2 Infarct size assessment

The infarct size was calculated by measuring the volume of the infarction. There was no difference in the infarct volume on day 1 after MCAO between animals

that received placebo (400 ± 200 - $800 \mu\text{l}$) when compared with mice treated with the P2X7 antagonist after MCAO (600 ± 200 - $800 \mu\text{l}$) (Figure 6.17).

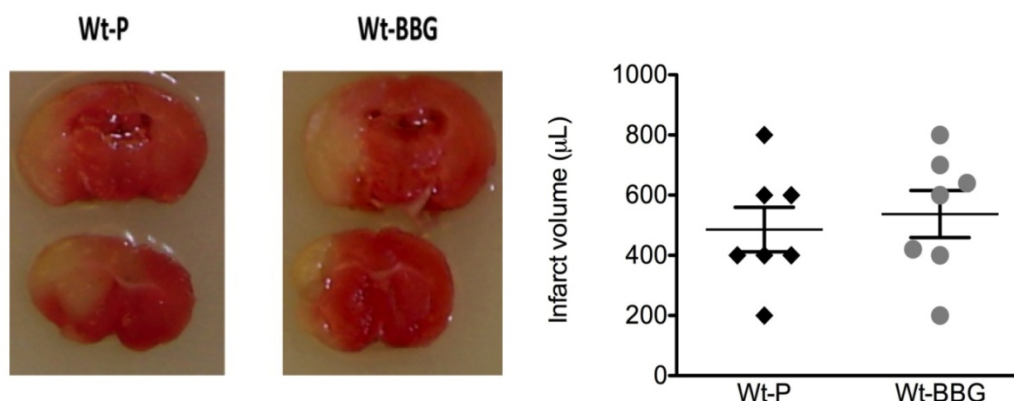


Figure 6.17: Representative illustration of the ischaemic infarction after transient MCAO in a mouse treated with placebo (Wt-P) and in a mouse treated with P2X7 antagonist, BB-G (Wt-BBG). The histogram shows the infarct volume in the 2 groups. There were 7 animals per group. Data presented as median $\pm$ range.

6.3 Discussion

In this chapter the presence and activity of P2X7 receptor in microglia and astrocyte cells has been demonstrated. While it suggests the possibility of an influence of P2X7 receptor on sickness behaviour, POCD could not be prevented in mice by blocking P2X7 after tibial fracture surgery under general anaesthesia.

In a 2 hit systemic LPS-surgery model, Fidalgo et al showed the priming effect of low-grade systemic inflammation on hippocampus memory acquisition after surgery (Fidalgo, et al. 2011). These findings were linked to enhanced IL-1 β in the hippocampus as a result of LPS pre-treatment. The synthesis and release of IL-1 β heavily relies on active P2X7 receptors (Solle, et al. 2001). Interestingly

potentiating P2X7 receptor function, through its enhanced expression after local administration of LPS was described by Choi et al as a possible mechanism for the inhibitory effect of oxidized ATP reducing inflammation and neuronal damage (Choi, Ryu et al. 2007). There is also some evidence that when P2X7 receptor antagonist is administered systemically there is a reduction in the infarct size after MCAO and improvement in the neurological outcome in mice (Melani, Amadio et al. 2006).

From this background investigation of the P2X7 receptor appeared justified but the role of the P2X7 receptor in a mouse model of POCD would appear to be more contentious. The limited set of experiments described above, failed to demonstrate a clear role in preventing POCD in the surgical groups. There were some indications of a protective effect in relation to memory acquisition in the anaesthesia group.

There are some limitations of this set of experiments. In most parts of the study the numbers of animals used was very limited and this might influence the strength of signal. Nevertheless where there was no obvious signal it might be reasonable to suppose there is either no signal or an extremely weak signal.

The use of P2X7-KO mice which lack the receptor since birth might be a problem. The effects of P2X7-KO receptor in macrophages may not suffice to ameliorate the systemic inflammatory response and CNS disturbances associated with non-neurological surgery, which manifest with memory impairment when surgery is performed under general anaesthesia. It could be that the P2X7-KO mice have

developed chronic compensatory mechanisms by up-regulating the expression of P2X4 receptors as was recently demonstrated in a mouse model of Parkinson disease (Hracsko, Baranyi et al. 2011). Alternatively or even additionally, it could be that the fear-conditioning paradigm used for assessment of memory was not sensitive enough to pick up changes on memory after surgery. It might be worth assessing more robust tests such as the Morris water maze and the smart cage to assess memory in the postoperative period.

In summary, even though the P2X7 receptor has been linked to the prevention of memory decline after an infectious stimulus, in these studies the lack of P2X7 receptor in macrophages does not prevent POCD after tibial fracture under general anaesthesia in mice as assessed by fear conditioning. If there is a role or mechanism for the P2X7 receptor in POCD then either more work with more highly selective P2X7 antagonists could be performed or alternatively more sensitive testing methods should be sought, or both. It is hard to dismiss the possibility that there is no role for P2X7 but these studies provide little evidence to support the role of P2X7 receptor in the development of POCD.

Chapter 7:

The expression of Hsp72, a cytoprotective mechanism and POCD

The aim of this chapter is to explore whether a high level of expression of the stress inducible cytosolic heat shock protein 72 (Hsp72) can prevent the development of POCD using the tibial fracture surgery under general anaesthesia in the mouse model. This is based on the evidence suggesting that it is the level of stress response, which itself depends on the type of insult and its duration, which contributes to the development of POCD (Kalman, Juhasz et al. 2006; Marcos, Aisa et al. 2008).

It has been reported that in aged rodents there is reduced induction of heat shock proteins in response to stress (Tandara, Kloeters et al. 2006) and that these reduced levels of Hsp72 may be insufficient to prevent the injurious effect of stress and hence the development of POCD (Okaya, Blanchard et al. 2005; Tandara, Kloeters et al. 2006; Rumora, Lovric et al. 2007). In other circumstances Hsp72 appears to be protective. In the setting of acute neurological injury, stress inducible Hsp72 appears to confer neuroprotection (Hoehn, Ringer et al. 2001). Similarly, it has been shown in animal and cell models of stroke that the lesion is reduced both in whole animals and in brain cells over expressing Hsp72 (Sun, Ouyang et al. 2006).

From a different perspective, Hsp72 has also been implicated in learning and plasticity (Pizarro, Haro et al. 2003) and changes in Hsp72 have been noted in the hippocampus of animals in which different learning paradigms were used (Ambrosini, Mariucci et al. 2005). A set of experiments was conducted to investigate whether Hsp72 over expression could prevent or reduce memory decline following surgery in mice.

7.1 Methods

To characterize the role of Hsp72 in POCD, in vivo experiments were performed for assessment of memory after tibial fracture under general anaesthesia and also assessment of microglia activation in areas of the brain where memory is formed.

The tibial fracture surgery, anaesthesia, behavioural studies and laboratory techniques performed in this chapter were described in details in chapter 3, section: 3.1, 3.2.1.b, 3.4, 3.5.2, 3.6 and 3.7.

7.1.1 Characterisation of the role of Hsp72 over expression in POCD

Adult wild type C57BL/6 (C57BL/6-Wt) and Hsp72 over expressing transgenic (Hsp72-Tg) mice were allocated into one of the following groups:

Group 1: Control (naïve mice)

Group 2: Isoflurane anaesthesia alone

Group 3: Tibial fracture surgery under isoflurane anaesthesia.

All groups were trained 24h before surgery using a fear-conditioning protocol consisting of paired non-painful (tone/sound) and painful (foot-shock) stimuli. Mice were then assessed in their training environment and in a novel context on post-treatment days 1, 3, and 7.

Different cohorts of mice were used for assessment of memory on day 1, 3 and 7 after surgery and anaesthesia. A total of 252 mice were used for these

experiments (220 mice for behavioural experiments and 32 mice for IHC). There were 4 fatalities in the surgical groups (2 C57BL/6-Wt and Hsp72-Tg).

Baseline response to pain, motor activity and ability to learn was assessed in a different cohort of C57BL/6-Wt and Hsp72-Tg mice before commencing these experiments (for details in baseline differences between these 2 type of mice please refer to table 3.4, Appendix 1).

7.1.2 Assessment of microglia activation after tibial fracture surgery under general anaesthesia

Microglia activation was assessed in the hippocampus by immunohistochemistry (IHC) analysis on day 1 and 7 after the intervention in 2 different cohorts of mice that were not trained by fear conditioning.

7.2 Results

7.2.1 Physiological variables

No statistical differences were detected among monitored physiological variables between groups. Mice ages were 3 to 14m with a median of 6 (range: 3–14) for C57BL/6-Wt and 7 (3–14) for Hsp72-Tg group. Mice weighed from 21 to 51g with a median of 37.2 (range: 23–51) for C57BL/6-Wt and 32.0 (21–49) for Hsp72-Tg group. Rectal temperature during surgery and anaesthesia were $35.9^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ in C57BL/6-Wt mice and $36.1^{\circ}\text{C} \pm 0.68^{\circ}\text{C}$ in Hsp72-Tg (normal range: $35.5\text{--}37.1^{\circ}\text{C}$). Respiratory rate was between 20 and 30 breath per min in both groups during maintenance of anaesthesia with 1.4–1.6 % MAC of isoflurane.

Major bleeding was observed in 3 mice (1 C57BL/6-Wt, 2 Hsp72-Tg). These were removed from the study and killed immediately. One C57BL/6-Wt mouse was found dead one day after surgery.

7.2.2 Characterization of the role of Hsp72 over expression in POCD

7.2.2.a Learning abilities (acquisition)

Acquisition of memory during training 24h before surgery was similar between C57BL/6-Wt and Hsp72-Tg. Maximal performance was achieved by the end of the 6th training session (Figure 7.1) indicating that memory was acquired and established after learning by association.

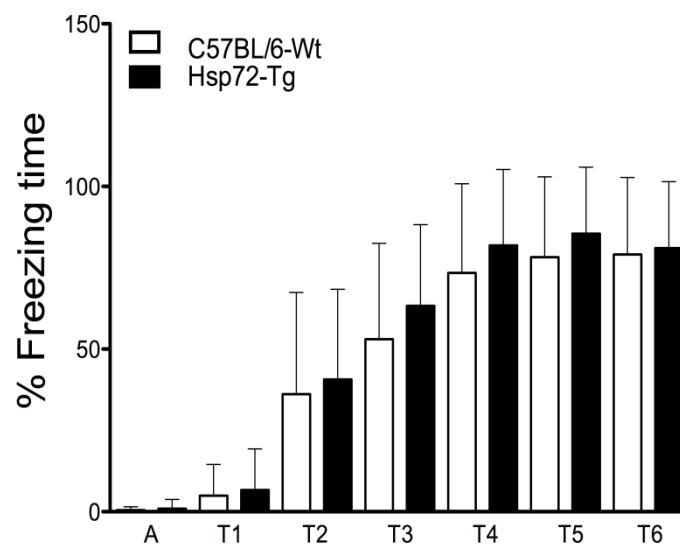


Figure 7.1: This bar histogram illustrates the acquisition of memory during the training phase of the fear-conditioning protocol was similar in C57BL/6-Wt and Hsp72-Tg mice. Both mouse strains achieved maximal performance by the 6th training session. No differences in establishment of long-term memory were found between C57BL/6-Wt (open bar) and Hsp72-Tg mice (black bar). Values are median $\pm$ SD for $n=34$ mice per group. The scale in the y-axis is up to 150% to

illustrate the top end of the error bars as there were few mice that had 100% freezing time.

A= Acclimatisation. T1, T2, T3, T4, T5 and T6= Trial 1, 2, 3, 4, 5 and 6.

7.2.2.b Hippocampal dependent memory

The contextual conditional response a form of hippocampal dependent memory was affected by tibial fracture surgery under general anaesthesia on day 1 and 3 after corresponding intervention.

Treatment effect: C57BL/6-Wt mice showed reduced memory based on treatment on day 1 (n=14) and 3 (n=11) after anaesthesia alone; mean $\pm$ SD (95% CI): 58.83 $\pm$ 18 (48–69.6) and 46.5 $\pm$ 16 (35.7–57.2) respectively and following surgery also on days 1 (n=14) and 3 (n=12); 53.4 $\pm$ 20 (40.6–66.2) and 44.1 $\pm$ 23 (28.7–59.5) respectively ($P<0.05$ and $P<0.001$ respectively) when compared to controls (n=14) 78.78 $\pm$ 17 (68.6–88.8) and 63.4 $\pm$ 20 (49.5–77.4) respectively, but not on day 7 (Figure 7.2 A, B, C). Hsp72-Tg mice showed no difference by treatment on any of the days (Figure 7.2 A, B, C). On day 3 the genotype had a strong effect on the contextual conditional response ($P<0.0001$). Whilst on day 7, no differences between groups were observed (Figure 7.2 C). A sub-analysis to identify any possible age-related differences on memory on day 1 and 3 after surgery and or anaesthesia was performed and no age-related statistical difference were found (figure 7.3 A, B).

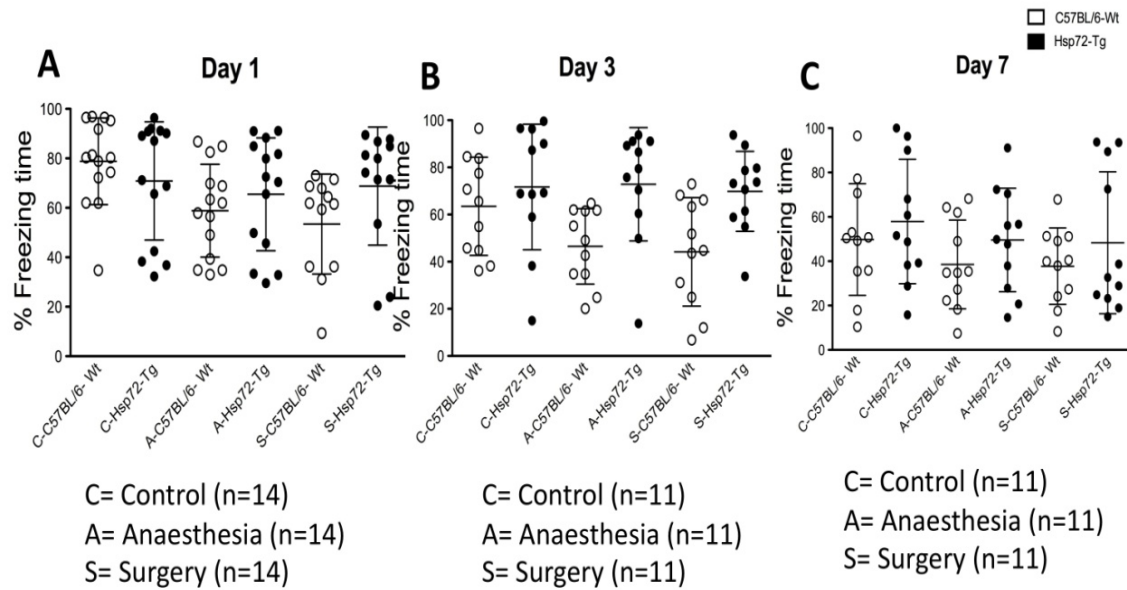


Figure 7.2: Hippocampal dependent memory was impaired following anaesthesia or surgery. Contextual conditional response (CCR) a form of hippocampal dependent memory was assessed as % freezing time on days 1, 3 and 7 after surgery. Groups were Control (C), Anaesthesia (A) and Surgery (S). (A) Shows % freezing time on day 1. (B) Shows % freezing time on day 3. (C) Shows % freezing time on day 7. Values are median $\pm$ range from n=11-14 mice per group.

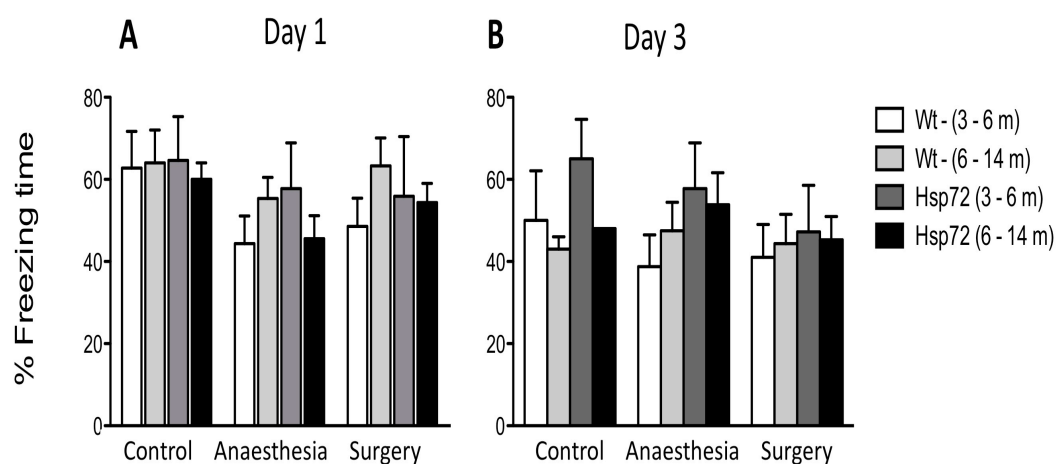


Figure 7.3: Hippocampal dependent memory was impaired following anaesthesia or surgery but, there was no age-related differences. Contextual conditional

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response (CCR) a form of hippocampal dependent memory was assessed as % freezing time on days 1, 3 after surgery. Groups were Control (C), Anaesthesia (A) and Surgery (S). (A) Shows % freezing time on day 1 ($n=8-11$). (B) Shows % freezing time on day 3 ($n=3-10$). Values are median $\pm$ range. The data passed the Shapiro-Wilk normality test. P values were not statistically significant.

7.2.2.c Hippocampal independent memory

Freezing behaviour during the conditional stimulus assesses hippocampal independent memory, e.g. prefrontal cortex and amygdala. The dependence of the non-conditional response (Tone) on either treatment or genotype was assessed. *Treatment effect:* It was found that the response of Hsp72-Tg mice was not affected by treatment on any day whilst C57BL/6-Wt mice showed reduced memory following surgery on day 1 ($n=12$) and 3 ($n=11$); mean $\pm$ SD (95% CI): 31.9 $\pm$ 18.2 (20.3–43.5) and 36.6 $\pm$ 20 (23.7–50.0) respectively and day 3 after anaesthesia 37.35 $\pm$ 11.1 (29.5–45.2) ($P<0.05$) when compared to the control group $n=14$; mean $\pm$ SD (95% CI): 49.2 $\pm$ 18.9 (38.4–60.2) or anaesthesia alone group $n=14$; mean $\pm$ SD (95% CI): 46.2 $\pm$ 13.2 (38.5–53.8) on day 1, while those exposed to anaesthesia alone differed from control only on day 3. *Genotype effect:* It was found that on day 1, the non-conditional response was strongly dependent on genotype after surgery ($P<0.001$). On day 3 the genotype effect was significant after anaesthesia alone and surgery ($P<0.05$). Such dependence was still significant on day 7 for the surgery group by two-way ANOVA analysis ($P<0.05$) whilst the anaesthesia alone C57BL6-Wt mice did not differ from control (Figure 7.4 A, B, C). No age-related difference on day 1, 3 and 7 after surgery and anaesthesia were found.

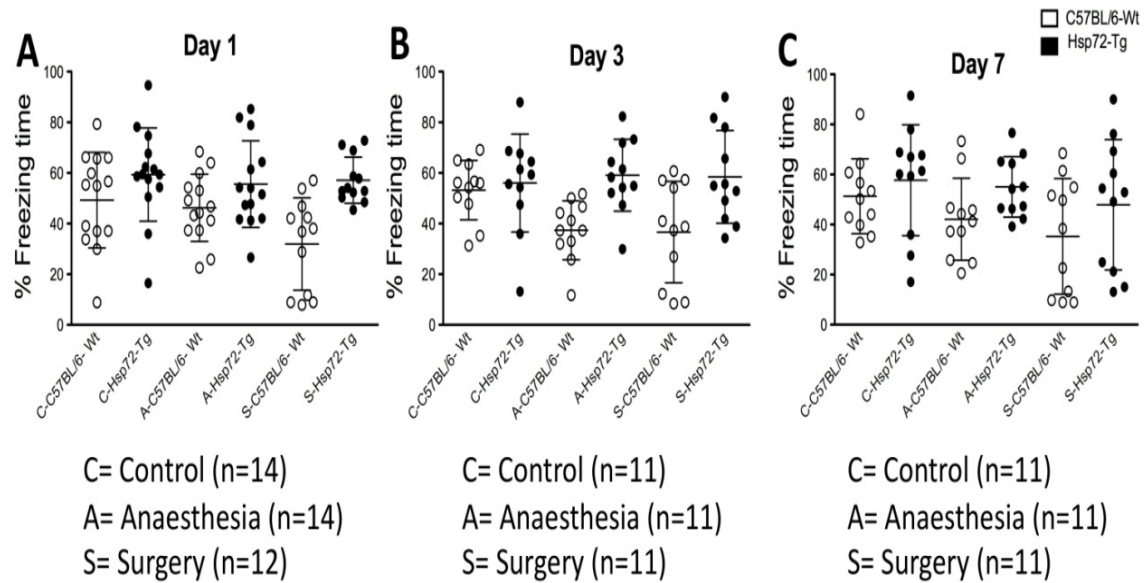


Figure 7.4: Hippocampal independent memory was impaired after anaesthesia or surgery. Conditional response (CR), a form of hippocampal independent memory assessed as % freezing time following the tone on days 1, 3 and 7 after treatment. Groups are Control (C), Anaesthesia (A) and Surgery (S). (A) Shows % freezing time on day 1. (B) Shows % freezing time on day 3. (C) Shows % freezing time day 7 after surgery. Values are mean $\pm$ SD.

7.2.3 Assessment of microglia activation after tibial fracture surgery under general anaesthesia

All brains studied showed differences in the numbers of activated microglia with treatment assessed by CD68 immunostaining, a cellular marker of microglia activation. Activated microglia were more prominent in mice that underwent surgery and these were observed throughout the brain, but there were also some region specific differences between treatment groups. The numbers of CD68 positive cells in the CA1, CA2, CA3 and hilus of dentate gyrus, subsections of the

hippocampus formation were counted and numbers of microglia compared between regions.

On day 1 Hsp72-Tg mice showed no statistical difference in numbers of activated microglia in the CA3 region of the hippocampus following surgery when compared to the control group and it; mean $\pm$ SEM (95% CI): 25.9 $\pm$ 3.61 (-0.2–15.4) and 22.8 $\pm$ 1.1 (-8.52–7.0) respectively. In contrast, C57BL6-Wt showed an increase in activated microglia after surgery 32.7 $\pm$ 1.6 (-5.7–5.9) or anaesthesia 27.0 $\pm$ 1.34 (-7.0–6.1) that was statistically significantly different from the control group 23.6 $\pm$ 1.2 (-5.7–5.7) (P <0.05 and P <0.001 respectively; Figure 7.5 and Table 7.1). Numbers of activated microglia were different in CA1 and CA3 hippocampal sub-regions by genotype (P <0.05 in CA1, CA3; Figure 7.5 and Table 7.1).

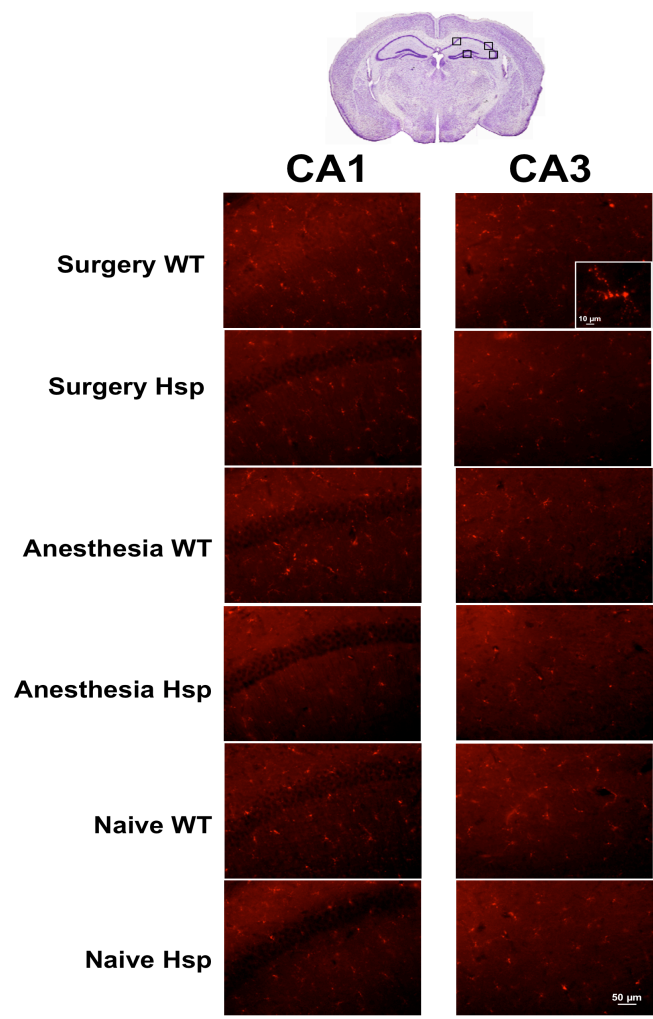


Figure 7.5: Microglia activation was assessed by immunoreactivity for CD68 and cell counting. The sub-regions within the hippocampus assessed are indicated by squares on the brain section at the top. Representative photomicrographs of the CA1 and CA3 sub-regions of the hippocampus immunostained for CD68 on day one are shown for each of the conditions. Scale bar, 50μm. In the insert, the scale bar represents 10μm.

Table 7.1: Number of CD68 immunopositive cells in CA1 and CA3 on day 1 and day 7. Values are number of immunopositive cells within a 0.4mm² counting frame,

median±Sd from n= 4-6 mice per group. Significant differences from the control of the same genotype and day are indicated by * $P<0.05$.

Condition	CA1		CA3	
	Day 1	Day 7	Day 1	Day 7
C - C57BL/6-Wt (n=4)	24.7±1.36	24.7±1.4	23.58±1.3	23.58±1.3
C - Hsp72-Tg (n=4)	26.507±1.7	24.7±2.7	22.8±1.1	22.1±2.52
A - C57BL/6-Wt (n=6)	31.3±2.5*	25.2±2.8	27.0±1.3	26.5±2.2
A - Hsp72-Tg (n=6)	24.1±2.0*	30.3±0.9*	21.1±1.9*	29.1±0.9*
S - C57BL/6-Wt (n=6)	32.0±2.2*	34.7±1.3*	29.0±1.6*	32.6±1.7*
S - Hsp72-Tg (n=6)	26.58±3.7	33.3±0.7*	25.9±3.6	33.5±1.0*

On day 7 there was an overall increase in the number of CD68 positive microglia on CA1 and CA3 hippocampal regions in C57BL/6-Wt and Hsp72 mice after surgery; median±SD (95% CI): CA1: 34.7±1.36 (-6.9–6.8) and 33.3±0.7 (-1.3–14.8) respectively, CA3: 32.7±1.7 (-5.7–5.7) and 33.5±1.05 (-0.2–15.4) respectively; this was statistically different compared to their respective controls CA1: 24.1±1.3 (-6.79–6.79) and 24.6±2.7 (-9.9–6.3) respectively, CA3: 23.6±1.3 (-5.69–5.69) and 22.1± 2.5 (-8.5–7.02) respectively) ($P<0.0001$; Figure 7.5 and

Table 7.1). Furthermore, the count depended, significantly on treatment on day 7 in all 4 regions ($P < 0.0001$ in CA1, CA3 and $P < 0.05$ in CA2 and hilus of dentate gyrus; Table 7.1 and 7.2).

Table 7.2: Number of CD68 immunopositive cells in CA2 and hilus of dentate gyrus on day 1 and day 7. Number of cells are presented as median $\pm$ SD.

Condition	CA2		Hilus of dentate gyrus	
	Day 1	Day 7	Day 1	Day 7
C - C57BL/6-Wt (n=4)	24.7 $\pm$ 3.50	24.6 $\pm$ 3.50	25.4 $\pm$ 4.64	24.51 $\pm$ 4.64
C - Hsp72-Tg (n=4)	22.9 $\pm$ 2.53	24.0 $\pm$ 6.30	19.7 $\pm$ 5.00	20.7 $\pm$ 4.66
A - C57BL/6-Wt (n=6)	25.4 $\pm$ 8.50	27.2 $\pm$ 5.11	23.0 $\pm$ 7.13	25.2 $\pm$ 5.0
A - Hsp72-Tg (n=6)	23.7 $\pm$ 5.40	27.6 $\pm$ 4.56	21.5 $\pm$ 5.45	27.1 $\pm$ 3.37*
S - C57BL/6-Wt (n=6)	30.2 $\pm$ 3.80	35.1 $\pm$ 3.94*	25.5 $\pm$ 4.44	31.7 $\pm$ 4.13*
S - Hsp72-Tg (n=6)	24.7 $\pm$ 7.24	33.1 $\pm$ 3.90*	21.7 $\pm$ 5.40	31.8 $\pm$ 2.75*

7.3 Discussion

This chapter showed the reproducibility of the paradigm developed in chapter 6 for assessment of postoperative memory decline following anaesthesia and tibial

fracture surgery, in which fear conditioning training was performed before anaesthesia and surgery. This model is sensitive to changes induced by exposure to anaesthesia alone, as well as changes due to the combined effects of anaesthesia and surgery. Mice over expressing Hsp72 showed no memory impairment following the same exposures.

Whilst both hippocampal dependent and independent memory was impaired by both anaesthesia and surgery, the effect of anaesthesia alone was most notable on day 3. By day 7 no treatment showed a difference in hippocampal dependent memory, while the C57BL-Wt surgical group still showed significant hippocampal independent memory deficit at 7 days.

Anaesthesia has been shown to have an influence on learning. Specifically, isoflurane has been reported to suppress learning in a dose-dependent fashion (Dutton, Maurer et al. 2001). It has also been demonstrated that isoflurane interferes with anterograde but not retrograde memory for Pavlovian fear conditioning (Dutton, Maurer et al. 2002; Culley, Baxter et al. 2003). In that study, fear conditioning training took place during delivery of anaesthesia. In the study described here, study mice were trained before surgery and anaesthesia which in effect removed the influence of the acquisition phase on assessment of memory in the postoperative period.

There was an increase in the number of activated microglia in C57BL-Wt mice on day 1 compared to Hsp72 over-expressors. On day 7 when there was only a difference in hippocampal independent memory in C57BL/6-Wt mice exposed to

surgery, but not in Hsp72 mice, there was no difference in numbers of activated microglia; numbers were significantly increased in both genotypes. This suggests either that the memory deficit is not correlated with numbers of activated microglia, or that the relationship is complex, and only directly correlated on day 1.

This could reflect the possibility that microglia can play a positive role in plasticity, as well as contribute to memory impairment in some settings. Conceivably the early activation is deleterious, but the late activation may not be, and in contrast may contribute to plasticity and the reestablishment of connectivity.

Previously Hsp72 was shown to be lower in animals that had learned a radial maze, (Pizarro, Haro et al. 2003; Ambrosini, Mariucci et al. 2005) while in contrast heat shock cognate protein (Hsc70), from the inportin beta family was increased in animals learning the Morris water maze (Pizarro, Haro et al. 2003). The exact relationship between plasticity and increased expression levels of Hsp72 is not yet clear. In this set of experiments there were no difference in acquisition of fear conditioning between C57BL/6-Wt and Hsp72-Tg mice that chronically over expressed Hsp72 in all cell types.

Retrieval of information was fully protected in Hsp72-Tg mice up to day 7, though the sensitivity is reduced at this point as all mice show reduction in freezing time with time following training. These findings suggest that Hsp72 is important either to memory retrieval or protection from a process that degrades

memory retrieval following anaesthesia or surgery. Hsp72 is known to inhibit inducible nitric oxide synthase (iNOS) gene transcription by the inhibition of activation of its transcription factor NF κ B (de Vera, Kim et al. 1996; Zheng, Kim et al. 2008). Hsp72 also stabilises mitochondrial membrane potential and ameliorates ROS production (Ouyang, Xu et al. 2006). Whether the observed protection of memory involves reduced oxidative stress such as from inhibition of induction of iNOS, or inhibition of cytokine production, or both remains to be evaluated.

One of the limitations of this set of experiments is that the transgenic mice over expressing Hsp72 may have also developed chronic adaptive mechanisms that are less well known. Therefore although the high expression of Hsp72 appears protective, it needs further investigation. Acute induction in the perioperative period may be an area that could be explored in order to ascertain the reproducibility of the effect of high level of inducible cytoplasmic Hsp72 on the development of POCD. Another potential limitation of this set of experiments is animal age. There was a wide age range in this set of experiments and even though there were no memory differences within the age range a new set of experiments where a narrow age range of mice was included would be appropriate in demonstrating the reproducibility of the study.

In summary, Hsp72 over-expression in mice is associated with full maintenance of memory following isoflurane anaesthesia and surgery as compared to wild type mice. However, whether the mechanism of this improved memory performance in Hsp72 mice compared to wild type mice reflects direct effects on

processes in the pathways serving memory establishment and retrieval or alternatively it could be due to modulation of inflammation, or both, which in turn affects memory still remains to be elucidated.

Chapter 8:

The role of xenon in POCD

The aim of this chapter is to investigate whether xenon pretreatment could prevent POCD following tibial fracture surgery under general anaesthesia in a mouse model.

Xenon pre-treatment has been shown to have an ameliorating effect on systemic inflammation by reducing the levels of pro-inflammatory cytokines (Hein, Roehl et al. 2008; Saravanan, Exley et al. 2009). It is thought it may be associated with the enhancement of cytoprotective mechanisms by activating the PI3-kinase pathway (Weber, et al. 2005) and also by increasing the expression of the inducible cytoplasmic Hsp72.

In chapter 7, it has been showed that over expression of Hsp72 was associated with prevention of POCD following tibial fracture surgery under general anaesthesia. Several anaesthetics including xenon and isoflurane have been shown to have effects on cellular signalling and it is postulated that these effects may mediate potential or real organoprotection (Kelly, Zhang et al. 2002).

Xenon has been found to confer neuroprotection in animal models of cerebral ischaemia (Limatola, Ward et al. 2010), cardiopulmonary bypass (Ma, Yang et al. 2003; Clark, Ma et al. 2005), and post-cardiac arrest (Schmidt, Marx et al. 2005). One mechanism by which xenon exerts such effects is by inducing hypoxia inducible factor one alpha (HIF-1 α) (Limatola, Ward et al. 2010) which can modify apoptotic signalling pathways (Ma, Williamson et al. 2007; Cattano, Williamson et al. 2008). There appear to be no data in the literature that describe the effects of xenon on stress proteins, including Hsp72, or its role in

the prevention of postoperative memory decline. Given the findings in the previous study it would be reasonable to explore the effects of xenon on Hsp72. It is therefore the intention in this study to determine whether pretreatment with xenon induces Hsp72 and therefore prevents the development of POCD.

8.1 Methods

To characterise the role of xenon in preventing POCD, in vivo experiments were conducted using the assessment of memory after tibial fracture surgery under general anaesthesia to demonstrate any evidence of POCD. At the same time an assessment of the systemic inflammatory response was made by measuring the level of the proinflammatory cytokine, IL-1 β at different time points after surgery and anaesthesia.

The tibial fracture surgery, anaesthesia, behavioural studies and laboratory techniques performed in this chapter were described in details in chapter 3, section: 3.1, 3.2.1.a, 3.4.1, 3.4.2.a, 3.5 and 3.6.

8.1.1 Characterisation of the role of pre-treatment with xenon in POCD

The characterisation of the role of xenon pre-treatment in POCD used the assessment of memory impairment by fear conditioning after tibial fracture under general anaesthesia methodology.

Adult wild type C57BL/6 mice were allocated into one of the following groups (n=12 mice/group):

Group 1: Control – naïve mice (Control).

Group 2: 1.8% isoflurane anaesthesia for 20m (1.8% Iso).

Group 3: 70% xenon anaesthesia balanced for 20m (70% Xe).

Group 4: 1.8% isoflurane anaesthesia with surgery of the right hind leg tibia that was pinned and fractured (1.8% Iso-S).

Group 5: Pre-treatment with 70% xenon for 20m followed immediately by 1.8% isoflurane anaesthesia with surgery (Xe-pretreat-Iso-S).

All groups were trained 24h before surgery using a fear-conditioning protocol consisting of paired non-painful (tone/sound) and painful (foot-shock) stimuli. Mice were then assessed in their fear conditioning training environment and in a novel context after corresponding treatments on day 1, and 7 to assess the development of POCD. Different cohorts of mice were used for assessment of memory on day 1 and 7 after surgery and anaesthesia.

8.1.2 Measurement of the level of Phosphoinosite kinase-3 (PI3-kinase) and Hsp72 in the hippocampus after tibial fracture under general anaesthesia

The level of PI3-kinase and Hsp72 in the hippocampus was measured by western blotting on day 1 after corresponding treatments.

Hippocampi from additional mice cohorts (n=6/group) treated as described above, but without behavioural testing, were harvested after administration of

terminal anaesthesia on day 1 after treatment and prepared for the western blotting analysis.

8.1.3 Assessment of the systemic inflammatory response by cytokine value

The inflammatory response after surgery was ascertained by measuring the level of IL-1 β in plasma after corresponding treatments on day 1 and 7 in a different cohort of mice. Blood samples taken from treatment free animals served as controls.

8.2 Results

8.2.1 Physiological variables

No statistical differences were demonstrated in the monitored physiological variables between groups. Mice ages were 3 to 4 months. Mice weighed from 19.8 to 36.2g with a median of 25.2g. Rectal temperature range during surgery and anaesthesia was between 35.8 and 37.8°C with a median of 36.6 $\pm$ 0.4°C (normal range: 35.5-37.1°C). Respiratory rate was between 20 and 30 breath per min during maintenance of anaesthesia with 1.8 $\pm$ 0.3 % MAC of isoflurane. No major bleeding was observed in these sets of experiments and the physiological scores presented as mean $\pm$ SD (range) on day 1 after treatment were 1.04 $\pm$ 1.08 (0-4) for the 1.8% Isoflurane anaesthesia with surgery and 1.45 $\pm$ 1.38 (0-5) for the pre-treatment with 70% xenon followed immediately by 1.8% isoflurane anaesthesia with surgery group. A total of 150 mice were used for these experiments (120 mice for behavioural studies and 30 mice for western blotting and ELISA analysis). There were neither fatalities nor termination by schedule 1 in this set of experiments.

8.2.2 Characterization of the effect of pre-treatment with xenon in POCD

8.2.2.a Learning abilities (acquisition)

All animals were trained 24h before surgery. Memory acquisition by association was achieved by the 4th trial and consolidated by the 6th trial of paired conditional - unconditional stimuli (CS-US) during a single training session. Maximal performance was achieved by the end of the 6th training session (Figure 8.1).

The mean % freezing time during the acclimatisation period was $10.2 \pm 20\%$ increasing to $65.3 \pm 30\%$ by the 6th training session indicating that memory was acquired and established after learning by association.

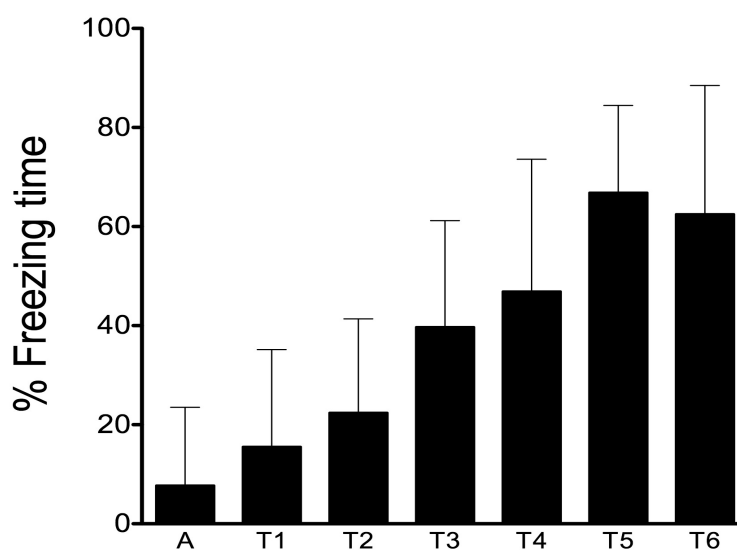


Figure 8.1: This bar histogram illustrates the acquisition of memory during the training phase of the fear-conditioning protocol in C57BL/6-Wt mice. The mice achieved maximal performance by the 6th training session. Values are mean ± SD (n=24 mice).

A= Acclimatisation. T1, T2, T3, T4, T5 and T6= Trial 1, 2, 3, 4, 5 and 6.

8.2.2.b Hippocampal dependent memory

The contextual conditional response a form of hippocampal dependent memory was affected by tibial fracture surgery under general anaesthesia on day 1 and 3 after corresponding intervention.

The surgery under isoflurane anaesthesia group showed reduced memory on day 1 when compare with control, $P=0.001$ (Table 8.1). Mice pretreated with xenon before surgery did not show significant memory impairment on day 1. The memory retention in the group pre-treated with xenon was significantly better than the surgical group that were not pretreated with xenon, $P=0.044$ (Figure 8.2 and Table 8.1).

By day 7, there was no difference in retrieval of hippocampal dependent memory between the two surgical groups, $P=0.69$. However, the surgical group that were not pretreated with xenon exhibited reduced memory retention as compared to control, $P=0.018$ (Table 8.1 and Figure 8.2).

Table 8.1: This table illustrates the effect of different treatments on the hippocampal dependent memory: Contextual conditional response was assessed by % freezing time. The experimental groups were: 1.8% Iso = 1.8% isoflurane anaesthesia treatment, 70% Xe = 70 % xenon treatment, 1.8% Iso-S = 1.8% isoflurane surgery treatment, Xe-Pretreat-Iso-S = xenon pre-treatment +1.8% isoflurane anaesthesia for surgery group. There were 12 mice per group in each cohort (day 1 and day 7) groups.

Data is presented as mean $\pm$ SD. A P value ≤ 0.05 was considered significant. Day 1: § P=0.001 compared to control, §§P=0.044 compared to Iso-S. Day 7: ¶P=0.018 compared to control.

	Control	1.8% Iso	70% Xe	1.8% Iso-S	Xe-Pretreat-Iso-S
Day 1	42.0 $\pm$ 15%	22.1 $\pm$ 16%	33.3 $\pm$ 18%	§19.6 $\pm$ 6%	§§32.1 $\pm$ 9%
Day 7	37.2 $\pm$ 15 %	25.9 $\pm$ 12%	37.3 $\pm$ 13.5%	¶22.0 $\pm$ 6.8%	28.1 $\pm$ 8.5%

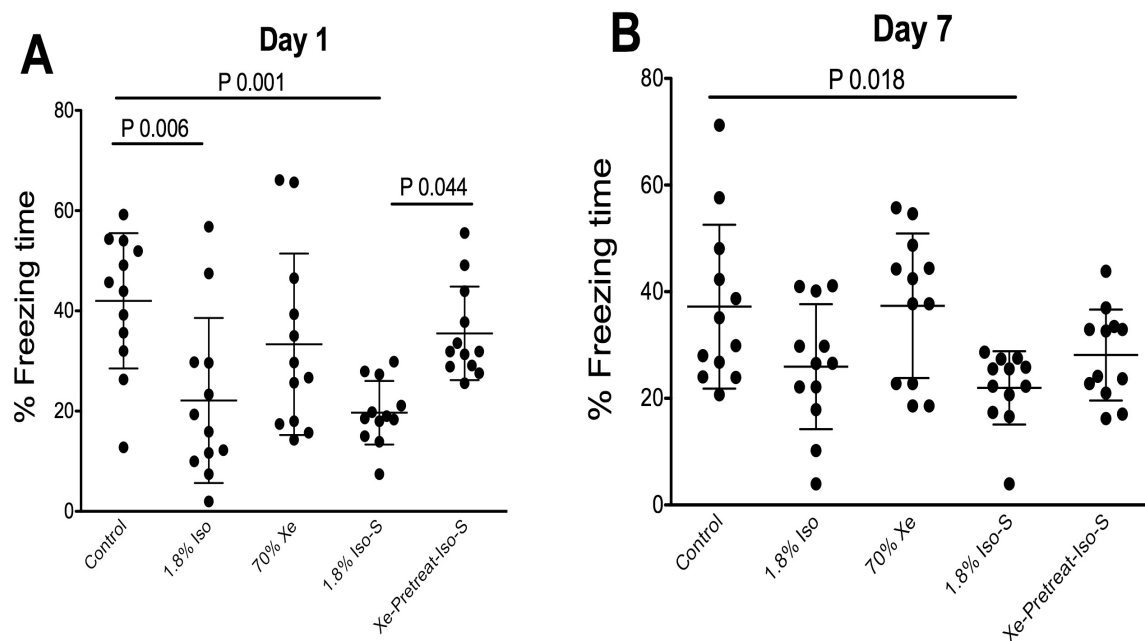


Figure 8.2: Hippocampal dependent memory was impaired following treatment. A contextual conditional response, which is a form of hippocampal dependent memory, was assessed as the percentage freezing time on A) day 1 (n=12/group) and B) Day 7 (n=12/group) after treatment. The experimental groups were: 1.8% Iso = 1.8% isoflurane anaesthesia treatment, 70% Xe = 70 % xenon treatment, 1.8% Iso-S = 1.8% isoflurane surgery treatment, Xe-Pretreat-Iso-S = xenon pre-treatment +1.8% isoflurane anaesthesia for surgery group. Values are the mean $\pm$ SD. A P value ≤ 0.05 was considered significant.

8.2.2.c Hippocampal independent memory

Freezing behaviour during the conditional stimulus assesses hippocampal independent memory, e.g. prefrontal cortex and amygdala. The dependence of the non-conditional response (Tone) to treatments on day 1 and 7 was assessed. It was found that surgery under isoflurane anaesthesia caused memory impairment on day 1 as compared to control, $P=0.034$. By day 7, the surgery under isoflurane anaesthesia group still showed memory impairment when compared to control: 1.8% Iso ($P=0.012$), 1.8% Iso-S ($P=0.052$) and Xe-Iso-S ($P=0.017$).

The variability in hippocampal-dependent memory was minimal compared to that of hippocampal-independent memory, where more inter-animal variability was seen within the groups (Table 8.2).

Table 8.2: This table illustrates the effect of tibial fracture surgery under general anaesthesia on memory. A conditional response, which is a form of hippocampal independent memory, was assessed as the percentage freezing time on day 1 (n=12/group) and on day 7 (n=12/group) after treatment. The experimental groups were: Control, 1.8% Iso = 1.8% isoflurane anaesthesia treatment, 70% Xe = 70 % xenon treatment, 1.8% Iso-S = 1.8% isoflurane surgery treatment and Xe-Pretreat-Iso-S = xenon pre-treatment +1.8% isoflurane anaesthesia for surgery group. Values are the mean±SD. A P value ≤0.05 was considered significant.

Day 1: §P = 0.57, §§P = 0.029, §§§P = 0.034, Day 7: ¶P = 0.012, ¶¶P = 0.052, ¶¶¶P = 0.017, all compared to control.

	Control	1.8% Iso	70% Xe	1.8% Iso-S	Xe-Pretreat-Iso-S
Day 1	56.8±21 %	§30.8±27 %	§§28.3±24%	§§§28.9±20 %	34.5±13 %
Day 7	43.1±16%	¶19.6±15%	32.1±22 %	¶¶13.8±8 %	¶¶¶15.5±9 %

8.2.3 Measurement of level of PI3-kinase and Hsp72 in the hippocampus after tibial fracture under general anaesthesia

Hippocampi of 4 mice out of 6 per group were analysed for PI3-kinase on day 1 after treatment. Tissues from 2 animals per group were not analysed due to technical problems with the western blotting membrane. The level of PI-3-kinase was (mean±SD) 0.96±0.45 in the 1.8% isoflurane anaesthesia, 1.02±0.42 in the 70% xenon anaesthesia group, 0.85±0.28 in the 1.8% Iso-Surgery group and 1.32±0.23 in the Xe-pretreat-Iso-S group. The expression of PI3-kinase in the hippocampi of Xe-pretreat-Iso-S group showed a trend towards a positive modulation compared to that in the control group but it did not reach statistical

significance ($P=0.07$). There appeared to be a difference between Xe-pretreat-Iso-S and 1.8% Iso-S but this did not reach statistical significance ($P=0.54$) (Figure 8.3).

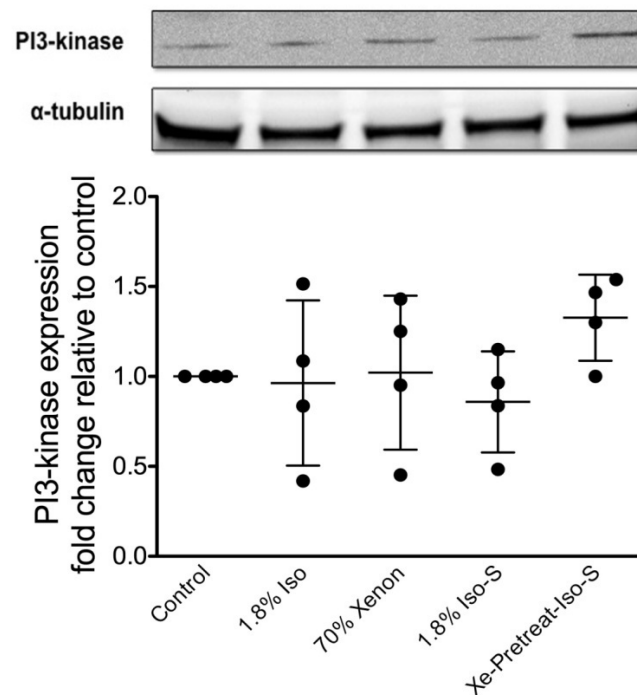


Figure 8.3: This figure illustrates the western blotting analysis for PI3-kinase in mouse hippocampal tissue on day 1 after treatment. The experimental animal groups were: 1.8% Iso = 1.8% isoflurane anaesthesia treatment, 70% Xe = 70 % xenon treatment, 1.8% Iso-S = 1.8% isoflurane surgery treatment, Xe-Pretreat-Iso-S = xenon pre-treatment +1.8% isoflurane anaesthesia for surgery group ($n=4$ /group). The top panel shows a representative immunoblot for PI3-kinase that was reprobbed for α -tubulin as a loading control. The histogram in the bottom panel shows the densitometric measurements of the PI3-kinase signals on the immunoblot, relative to control. Values are the mean $\pm$ SD.

Hippocampi from 4 mice/group were assessed for Hsp72 by western blotting

analysis. The Hsp72 expression was (mean $\pm$ SD) 1.26 $\pm$ 0.18 in the 1.8% isoflurane anaesthesia, 1.36 $\pm$ 0.17 in the 70% xenon anaesthesia group, 1.09 $\pm$ 0.14 in the 1.8% Iso-Surgery group and 1.43 $\pm$ 0.3 in the Xe-pretreat-Iso-S. The expression of Hsp72 in the hippocampi of Xe-pretreat-Iso-S group showed a trend towards a positive modulation compared to that in the control group but it did not reach statistical significance ($P=0.05$). There appeared to be a difference between Xe-pretreat-Iso-S and 1.8% Iso-S on day 1 after treatment but it did not reach statistical significance ($P=0.06$) (Figure 8.4). The apparent difference that is not significant may indicate a signal that needs further exploration or may be a type 1 error.

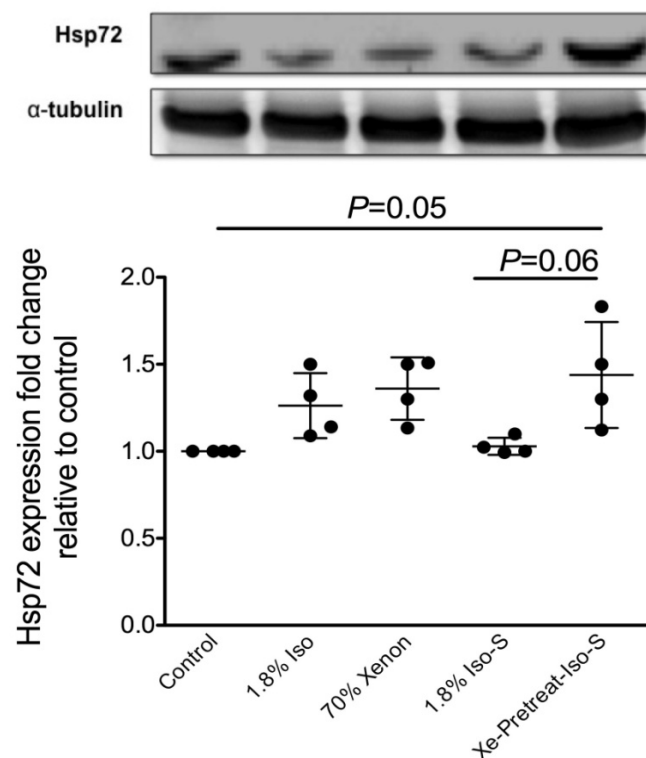


Figure 8.4: This figure illustrates a scatter histogram of the western blotting analysis of Hsp72 in mouse hippocampal tissue on day 1 after treatment. . The experimental mice groups were: Control, 1.8% Iso = 1.8% isoflurane anaesthesia treatment, 70% Xe = 70 % xenon treatment, 1.8% Iso-S = 1.8% isoflurane surgery

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treatment and Xe-Pretreat-Iso-S = xenon pre-treatment + 1.8% isoflurane anaesthesia for surgery group (n=4/group).

The top panel shows a representative immunoblot for Hsp72 that was reprobbed for α -tubulin as a loading control. The scatter histogram in the bottom panel shows the densitometric measurements of the Hsp72 signals on the immunoblot, relative to control. Values are the mean $\pm$ SD. A P value ≤ 0.05 was considered significant.

8.2.3 Assessment of the systemic inflammatory response by cytokine value

The plasma level of IL-1 β was measured on day 1 after treatment. The plasma level of IL-1 β was 8.43 $\pm$ 1.2pg/ml in the control, 7.9 $\pm$ 1.8pg/ml in the 70% Xe anaesthesia, 39.0 $\pm$ 9.4pg/ml in the Xe-pretreat-Iso-S, and 77.3 $\pm$ 29.3pg/ml in the 1.8% Iso-S groups. There was a significant difference between the Xe-pretreat-Iso-S and 1.8% Iso-S groups ($P=0.028$). There was also a significant difference in plasma level of IL-1 β when the 1.8% Iso-S anaesthesia group were compared to the 70% Xe anaesthesia and control groups, on day 1 after treatment ($P=0.0005$), and 1.8% Iso ($P=0.026$) (Figure 8.5).

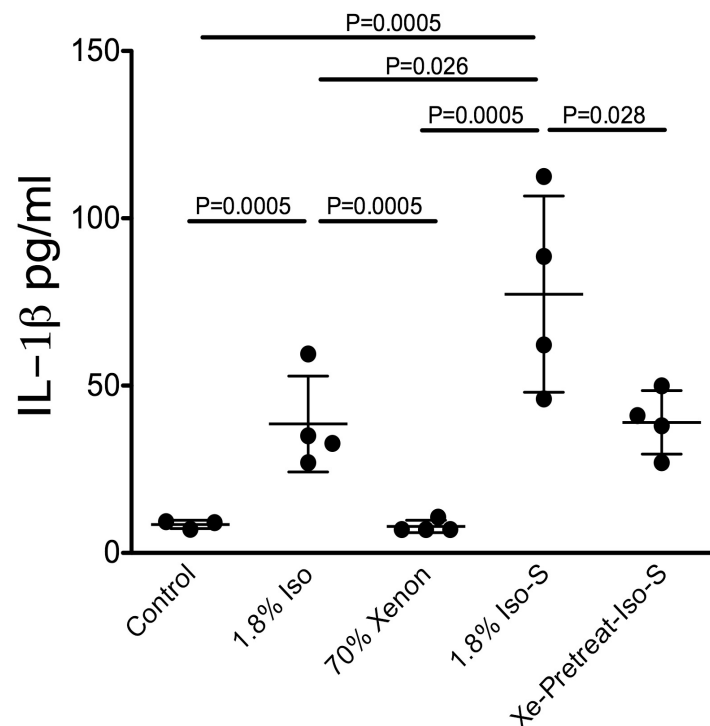


Figure 8.5: This figure illustrates a scatter histogram of the ELISA results on the plasma level of IL-1 β on day 1 after treatment. The experimental mice groups were: Control, 1.8% Iso = 1.8% isoflurane anaesthesia treatment, 70% Xe = 70 % xenon treatment, 1.8% Iso-S = 1.8% isoflurane surgery treatment and Xe-pretreat-Iso-S = xenon pre-treatment +1.8% isoflurane anaesthesia for surgery group (n=4/group). Values are the mean $\pm$ SD. A P value ≤ 0.05 was considered significant.

8.3 Discussion

It appears that POCD after surgery under isoflurane anaesthesia was ameliorated by xenon exposure immediately prior to surgical intervention. This was associated with an increase in Hsp72 and PI3-kinase in the hippocampus and a decrease in the plasma levels of IL-1 β . The experimental data suggested that a detrimental effect of anaesthesia and surgery on memory was demonstrated as

was a systemic and CNS inflammatory response. Both were reduced in effect by pre-treatment with xenon. This was associated with an increase in Hsp72 and PI3-kinase in the hippocampus, where the memory is formed.

In chapter 7 no memory decline was seen after tibial fracture surgery under general isoflurane anaesthesia in Hsp72-overexpressing transgenic mice. Interestingly the set of experiments conducted in this chapter suggest that the increase in levels of Hsp72 in the hippocampus 24h after exposure to xenon may be associated with the molecular mechanism by which xenon potentially could prevent the development of POCD in this mouse model of tibial fracture surgery under general anaesthesia. It is also plausible that the attenuation of memory decline following surgery by xenon pre-treatment might be associated with activation of the PI3-kinase pathway.

The measured inflammatory response was also modified. The xenon pre-treatment reduced IL-1 β level in plasma on day 1 after surgery. Previously, in a porcine model of myocardial damage, right ventricular infarction, the anti-inflammatory effects of xenon included reducing the plasma levels of TNF- α and IL-6 (Hein, Roehl et al. 2008).

The attenuating effect of xenon on early postoperative memory decline may also be modulated by isoflurane anaesthesia. Similar to xenon, isoflurane may also have some anti-inflammatory effects. Previous studies showed that pre-treatment with isoflurane reduced microglia activation at 24 hours after LPS plus interferon gamma exposure in C8-B4 microglia (Xu, Kim et al. 2008).

The main limitations of this set of experiments was mice age and the small number of samples per group for western blotting and ELISA studies which prevents any categorical conclusion about the effect of xenon in Hsp72 expression and activation of PI3-kinase pathways in areas of the brain where memory is formed. A further limitation of these experiments is the short time exposure to xenon before surgery which may not have been adequate to have had an impact on modulation of the synthesis of cytoprotective proteins such as Hsp72.

In summary, it was demonstrated that xenon exposure immediately before surgery prevented early POCD, as assessed by fear conditioning after tibial fracture surgery under general anaesthesia in a mouse model. This was associated with a positive modulation of Hsp72 expression in the hippocampus and a reduction in the plasma level of IL-1 β . These present results thus raise the possibility that xenon anaesthesia may reduce the risk of early POCD. It also reintroduces the possibility that Hsp72 has a role.

Chapter 9:

The role of atorvastatin in POCD

There are two aims in this chapter. Firstly to ascertain the reproducibility of the effect of anaesthesia and tibial fracture surgery under general anaesthesia, on memory shown in the previous chapters in a new surgical model of POCD, unilateral nephrectomy. Secondly to elucidate the role of atorvastatin in POCD. The rationale for this approach is that there is evidence suggesting inflammation is a component for the development of POCD and therefore a potential anti-inflammatory role of statins (Cibelli, Fidalgo et al. 2010).

Statins may be beneficial for both inflammatory diseases and acute brain injury (Corsini, Bellosta et al. 1999; Wang, Lynch et al. 2007; Winkler, Angele et al. 2009). The pivotal role of inflammation in atherogenesis led to the suggestion that statins exert an anti-inflammatory effect (Weitz-Schmidt 2002). Gang Chen et al explored the effect of statin on traumatic brain injury in a rat model of cortical contusion and demonstrated a dramatic reduction in the expression of TLR4, NF κ B and the pro-inflammatory cytokines, IL-1 β , IL-6 and TNF- α , and this was associated with preservation of the blood brain barrier integrity (Chen, Zhang et al. 2009).

The proposed mechanism for this effect is that TLR4 in macrophages is upregulated by oxidized low-density lipoprotein (oxLDL) (Xu, Shah et al. 2001). This leads to activation of the transcription factor NF κ B, which then leads to the synthesis of inflammatory cytokines. It has been suggested that statin, potent inhibitor of the cholesterol biosynthesis affects the production of pro-inflammatory cytokines by inhibiting the activation NF κ B *via* the TLR4 signalling pathways.

This concept is supported by the study that demonstrated that atorvastatin reduced NF κ B activation in a concentration-dependent manner after cells were stimulated with 1 μ g/ml LPS for 24 h and then treated with different concentrations of atorvastatin (0.1, 1.0, and 10.0 μ M) (Reinsfelt, Ricksten et al. 2012). A set of experiments was conducted to investigate whether Atorvastatin could prevent the development of POCD following nephrectomy in a mouse model.

9.1 Methods

To characterise the role of Atorvastatin in POCD, *in vivo* experiments were performed for assessment of memory after unilateral nephrectomy under general anaesthesia and also assessment of the systemic and central inflammatory response, specifically assessment of the inflammatory response in areas in areas of the brain where memory is formed.

The unilateral nephrectomy surgery, anaesthesia, behavioural studies and laboratory techniques performed in this chapter were described in details in chapter 3, section: 3.1, 3.2.1.b, 3.4.1, 3.4.2.a, 3.5, 3.6 and 3.7.

9.1.1 Characterisation of the role of atorvastatin in the development of POCD

The characterization of the role of atorvastatin in the development of POCD assessed by using both fear conditioning and Morris water maze (MWM) (Rosczyk, Sparkman et al. 2008) memory studies following surgery.

Adult wild type C57BL/6 mice were randomly allocated into one of the following groups:

Group 1: Control plus placebo (C-P)

Group 2: Control plus atorvastatin (C-S)

Group 3: Surgery plus placebo (S-P)

Group 4: Surgery plus atorvastatin (S-S)

Group 1 (C-P) and group 3 (S-P) received a placebo (0.4 ml normal saline) administered by gavage once a day for five days. Group 2 (C-S) and group 4 (S-S) received 250µg of atorvastatin (Pfizer, Sandwich, UK) in 0.4ml normal saline administered by gavage once a day for five days. The atorvastatin dose was chosen on the basis of the manufacturer's guidelines of 10mg/kg.

On day 5 of the atorvastatin treatment Group 3 (S-P) and group 4 (S-S) underwent unilateral nephrectomy (Figure 9.1).

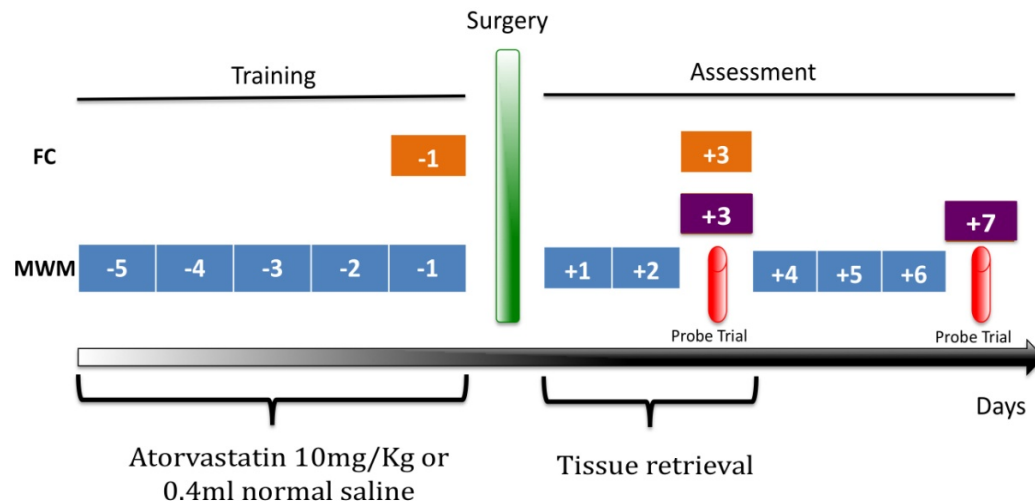


Figure 9.1: This is a schematic representation of the timeline for the experiments conducted to ascertain the effect of atorvastatin on POCD. Training refers to fear conditioning training (FC) (in orange) and Morris water maze (MWM) training (in blue). The orange squares illustrate the days fear conditioning trials took place. (-1) indicates training 1 day before surgery, (+1 and +3) indicate assessment of memory on day 3 after surgery. The blue squares illustrate the days the MWM trials took place. (-5, -4, -3, -2, -1) indicate MWM training days before surgery. (+1, +2) indicate MWM trial days after surgery. (+4, +5 and +6) indicate MWM reversal training for reinforcement of hippocampal dependent memory. (+3 and +7 in purple) illustrate the probe trial during MWM trials to identify the development of POCD after nephrectomies. The green bar illustrates the day of the surgery. The horizontal gray bar illustrates the time line. The first black bracket encompasses the days the mice received treatment and the second black bracket encompasses the days tissues were retrieved for the assessment of the systemic and central inflammatory response.

All groups were trained 24h before surgery using a fear-conditioning protocol consisting of paired non-painful (tone/sound) and painful (foot-shock) stimuli. Mice were then assessed in a novel context on post-treatment day 3. Different cohorts of mice were used for assessment of memory by fear conditioning on day 1 and 3 Figure 9.1.

Another cohort of mice was trained for 5 days before surgery on the Morris water maze and then the assessment was performed on day 3 and 7 after corresponding interventions. For details of the protocol see Figure 9.1.

9.1.2 Measurement of the level of Hsp72 and active NFκB in areas of the brain where memory is formed (hippocampus) after unilateral nephrectomy

The level of Hsp72 and NFκB in the hippocampus was measured by western blotting on day 1 after corresponding treatments.

9.1.3 Assessment of the systemic and CNS inflammatory response by cytokine value

The inflammatory response after surgery was ascertained by measuring the level of IL-1β in plasma and hippocampal tissue after corresponding treatments on day 1, 2 and 3. Blood samples taken from treatment free mice served as controls.

9.1.4 Assessment of microglia activation in areas of the brain where memory is formed (hippocampus) after unilateral nephrectomy

Microglia activation was assessed in the hippocampus by IHC analysis on day 1, 2 and 3 after the intervention in 3 different cohorts of mice that were not trained by fear conditioning. Hippocampal tissue taken from treatment free mice served as controls.

9.1.5 Assessment of the endothelium activation in areas of the brain where memory is formed

Endothelial cell activation was assessed in the hippocampus by FACS analysis. The inducible cyclo-oxygenase-2 (COX-2) was used as a marker of endothelial activation and was measured to assess the effect of statin on its activation.

9.2 Results

9.2.1 Physiological variables

No statistical differences in the monitored physiological variables were detected between groups. Mice ages were 3 ± 0.2 months. Mice weighed from 18.8 to 21.8g with a median of 21.06 ± 1.1 g. The range of rectal temperature during surgery and anaesthesia was between 35.9 and 38°C with a median of 36.5 ± 0.4 °C (normal range: 35.5-37.1°C). Respiratory rate was between 20 and 30 breath per min during maintenance of anaesthesia with 1.8 ± 0.3 % MAC of isoflurane. No major bleeding was observed in these sets of experiments and the

physiological scores presented as median $\pm$ SD (range) on day 1 after treatment were 3.0 $\pm$ 1.8 (0-9) for the surgery-placebo group and 2.0 $\pm$ 2.8 (0-13) for the surgery-statin group.

As MWM training consisted of daily swimming trials for a 13 days period, the weight loss of the mice was calculated both on the day treatment with atorvastatin and or placebo started and prior to termination (Table 9.1).

Additional physiological data relevant to the type of surgery performed on this chapter are summarised in Table 9.1.

Table 9.1: This data (mean $\pm$ SD) (n=9-10 per group) summarises the demographic and physiological data retrieved from animals during this experiments. Significant weight loss was observed in the surgical groups S-P (-6.58 $\pm$ 4.34) and S-S (-12.21 $\pm$ 7.10) compared to the C-P group (-1.64 $\pm$ 6.88) ($P < 0.05$) indicating that surgery caused a loss in body mass. There was no significant difference in weight loss between surgical groups. The volume of blood retrieved was similar across all groups at termination, indicating that no group was dehydrated or suffering from hypovolaemia. Urea and creatinine measurements indicated that surgery did not impair renal function.

	Control- Placebo	Control- Statin	Surgery- Placebo	Surgery- Statin
Weight change (% body weight)	-1.64 $\pm$ 6.88	0.93 $\pm$ 4.31	-6.58 $\pm$ 4.34	-12.21 $\pm$ 7.10

Urea				
(mmol/L)	6.84 ± 0.21	6.74 ± 0.99	7.35 ± 2.19	8.80 ± 0.14
Creatinine				
(μ mol/L)	19.10 ± 1.56	18.65 ± 0.92	21.35 ± 2.96	22.15 ± 5.59
Total				
cholesterol	18.76 ± 1.56	18.65 ± 0.92	21.35 ± 2.10	22.15 ± 3.81
(mmol/L)				
Volume of				
blood	0.59 ± 0.11	0.59 ± 0.16	0.48 ± 0.14	0.51 ± 0.14
retrieved (ml)				

A total of 184 mice were used for these experiments (108 mice for behavioural studies and 76 mice for western blotting, ELISA, IHC and FACS analysis). There were 3 fatalities and (2 in the S-P group and 1 in the S-S group) 3 terminations by schedule -1(1 in the S-P group and 2 in the S-S) on day 1 after surgery and 2 on day 3 after surgery due to poor physiological scoring (9, 13 and 10).

9.2.2 Characterisation of the role of atorvastatin in the development of POCD

9.2.2.1 Fear-conditioning

9.2.2.1.a Learning abilities (acquisition)

All animals were trained before surgery. Memory acquisition by association was achieved and consolidated by trial 6th of paired conditional - unconditional stimuli (CS - US) during a single training session. By the end of the 6th trial for training the percentage freezing times observed in all groups were similar; C-P (84 ± 24), C-S (88 ± 10), S-P (88 ± 12) and S-S (84 ± 24). This suggests that all treatment groups learnt (acquired) the fear association of the conditional and

unconditional stimulus to a similar extent. It also justifies the use of percentage freezing time for comparison of the memory retrieval between different groups (Figure 9.2).

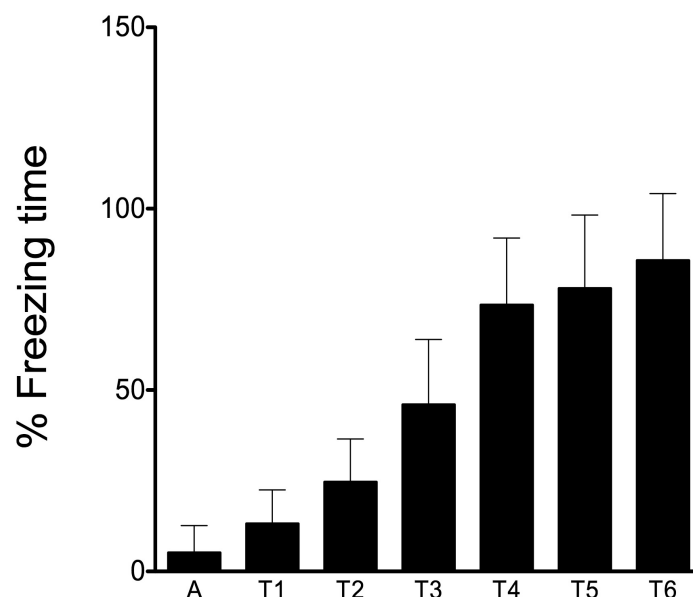


Figure 9.2: This bar histogram illustrates the acquisition of memory during the training phase of the fear-conditioning protocol in C57BL/6-Wt mice. The mice achieved maximal performance by the 6th training session. Values are mean±SD for $n=38$ mice per group. The scale in the y-axis is up to 150% to illustrate the top end of the error bars as there were few mice that had 100% freezing time.

A= Acclimatisation. T1, T2, T3, T4, T5 and T6= Trial 1, 2, 3, 4, 5 and 6.

9.2.2.1.b Hippocampal dependent memory

The contextual conditional response, a form of hippocampal dependent memory, was affected by unilateral nephrectomy surgery under general anaesthesia.

Memory was assessed on day 3 after unilateral nephrectomy surgery. For hippocampal dependent memory assessment there was a decline in memory

retrieval seen in the S-P group (% freezing time: 38 ± 15) compared to the C-P group (70 ± 19) ($P < 0.001$). There was no significant difference between the C-P group (70 ± 19) and the C-S group (79 ± 14). The memory retrieval seen in the S-S group (59 ± 16) was significantly higher than the S-P group (38 ± 15) ($P < 0.05$). Perhaps most remarkably there was no significant difference between the memory retrieval seen in the C-P group (70 ± 19) and the S-S group (59 ± 16), implying that the administration of atorvastatin prior to surgery had prevented the postoperative memory decline seen in the S-P group (Figure 9.3).

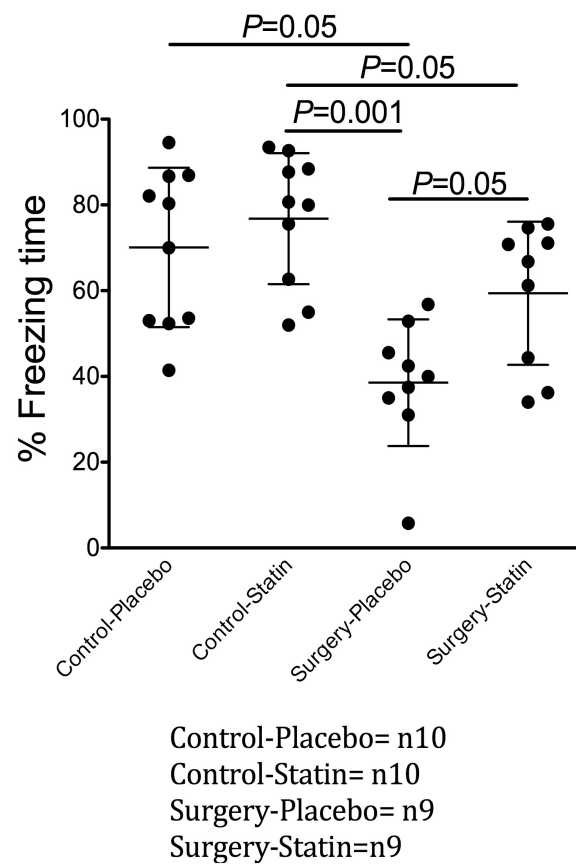


Figure 9.3: This scatter histogram illustrates the result of the assessment of the hippocampal dependent memory on day 3 after unilateral nephrectomy surgery. The histogram shows impairment of hippocampal dependent memory on day 3. A

contextual conditional response, which is a form of hippocampal dependent memory, was assessed as the percentage freezing time on day 3 after treatment. Values are the mean $\pm$ SD from n=9-10 mice per group. A P value ≤ 0.05 was considered significant.

9.2.2.1.c Hippocampal independent memory

Freezing behaviour during the conditional stimulus assesses hippocampal independent memory, e.g. prefrontal cortex and amygdala. The dependence of the non-conditional response (Tone) to treatments on day 3 was assessed.

Memory retrieval during delivering of the conditional stimulus (Tone) in the novel environment was similar across treatment groups with no statistically significant difference between S-P (35 $\pm$ 20) and S-S (50 $\pm$ 30). C-P (59 $\pm$ 26) and C-S (52 $\pm$ 17) produced statistically similar values both in relation to each other and to the surgical groups (Figure 9.4).

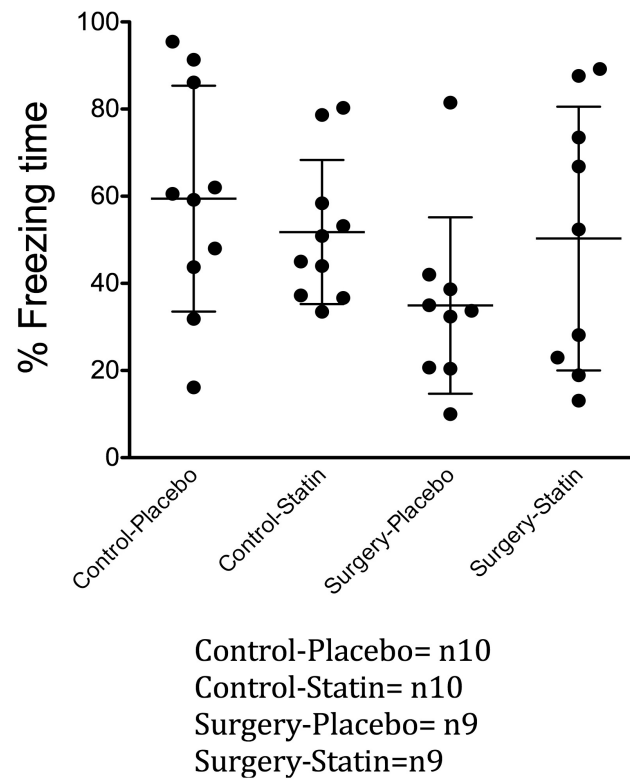


Figure 9.4: This scatter histogram illustrates the result of the assessment of the hippocampal independent memory on day 3 after unilateral nephrectomy. The histogram shows no treatment effect on hippocampal independent memory on day 3. Freezing behaviour during the conditional stimulus (Tone) assesses the hippocampal independent memory and it is reported as % freezing time on day 3 after treatment. Values are the mean $\pm$ SD from n=9-10 mice per group. No statistical difference between any groups.

9.2.2.2 Morris water maze

The MWM was the second behavioural test used for characterization of the effect of atorvastatin in POCD.

The Morris water maze memory assessment data is presented as escape latency or fraction of total time spent in the correct quadrant (mean $\pm$ SD) in the case of the probe trials at day 3 and day 7 postoperatively. The mice from the atorvastatin treated group (S-S) performed significantly better in the memory assessment on day 7 after surgery ($P<0.05$). This was after the reversal-learning task and implies a superior level of behavioural performance at one week post-surgery. The probe trial at day 3 post-surgery revealed a better performance in the surgery-statin group compared to the surgery-placebo group although this failed to reach statistical significance ($P=0.23$). For a more detailed depiction of the results from the postoperative probe trials see Figure 9.5.

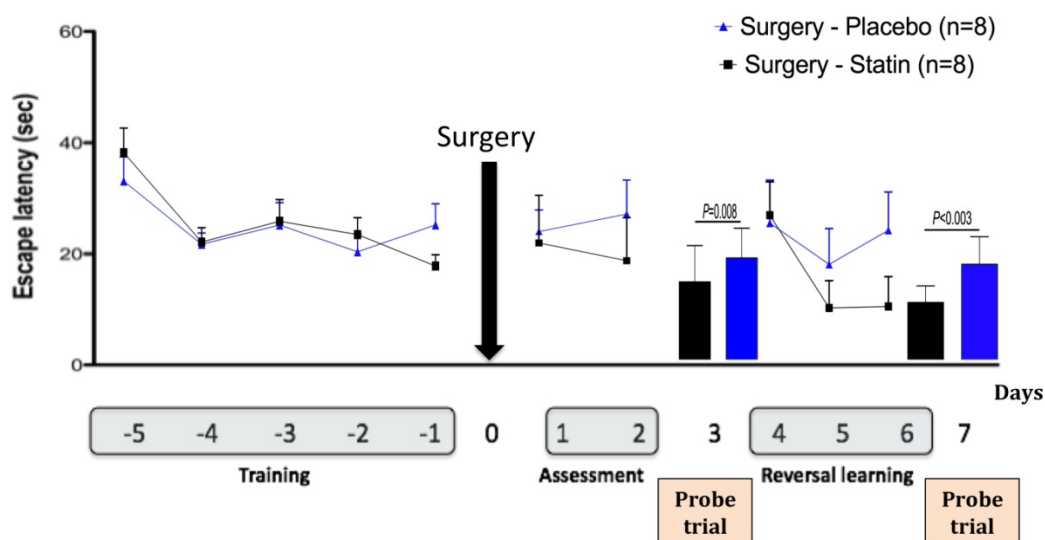


Figure 9.5: This image summarises the MWM results over time. Memory impairment induced by nephrectomy surgery is prevented by atorvastatin. The Morris water maze was used to train animals for their memory formation for 5 days before surgery (Training= -5, -4, -3, -2 and -1) and then to assess their memory (Assessment= 1 and 2) on day 1 and 2 after surgery following by the probe trial to assess the development of POCD on day 3 (3). Reversal learning (4, 5 and 6) on days 4, 5, and 6 was performed to reinforced spatial memory which is also

hippocampal dependent memory. The reversal learning was followed by the probe trial on day 7 (7). The figure illustrates the time the animals spent trying to find the platform during training and initial assessment after surgery (escape latency in sec). The probe trial performed on day 3 and 7 illustrates the time the animals remains in the quadrant where the hidden platform was. Values are the mean $\pm$ SD from n=8 mice per group. A P value ≤ 0.05 was considered significant.

9.2.2.3 Measurement of the level of Hsp72 and active NF κ B in areas of the brain where memory is formed (hippocampus) after unilateral nephrectomy under general anaesthesia

The homogenised hippocampal tissue (non-cell specific) from 4 mice per group were analysed for Hsp72 on day 1 after treatment. The level of Hsp72 was (mean $\pm$ SD) 1.13 $\pm$ 0.21 for the C-S group, 1.14 $\pm$ 0.10 for the S-P group and 1.28 $\pm$ 0.18 for the S-S group. The level of Hsp72 was high in the S-S group when compared to the C-P group ($P=0.04$) (Figure 9.6).

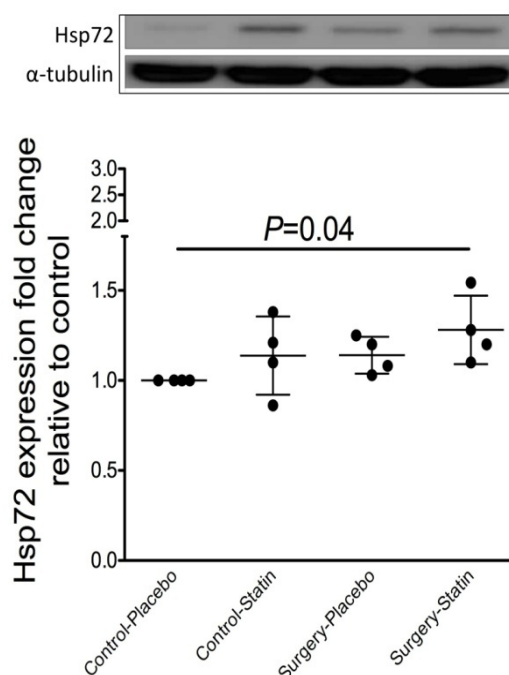


Figure 9.6: This figure illustrates a scatter histogram of the western blotting analysis of Hsp72 in mouse hippocampal tissue on day 1 after treatment. The experimental mice groups were: Control-Placebo, Control-Statin, Surgery-Placebo and Surgery-Statin (n=4/group).

The top panel shows a representative immunoblot for Hsp72 that was reprobbed for α -tubulin as a loading control. The scatter histogram in the bottom panel shows the densitometric measurements of the Hsp72 signals on the immunoblot, relative to control. Values are the mean $\pm$ SD. A P value ≤ 0.05 was considered significant. The level of Hsp72 was higher in the S-S group when compared to the C-P group ($P=0.04$).

The homogenised hippocampal tissue (non-cell specific) from 4 mice per group were also analysed for NF κ B_{p65} on day 1 after treatment. The level of NF κ B_{p65} was (mean $\pm$ SD) 0.69 $\pm$ 0.11 for the C-S group, 2.05 $\pm$ 0.47 for the S-P group and 1.24 $\pm$ 0.34 for the S-S group. NF κ B_{p65} was significantly higher in the S-P

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group than S-S group ($P=0.05$), C-P group ($P=0.001$) and C-S group ($P=0.001$) while there were statistical difference when compared S-S to C-P and C-S. This gross elevation of NF κ B_{p65} in the surgical placebo group might suggest that preoperative administration of atorvastatin had protected against activation of this pro-inflammatory pathway (Figure 9.7).

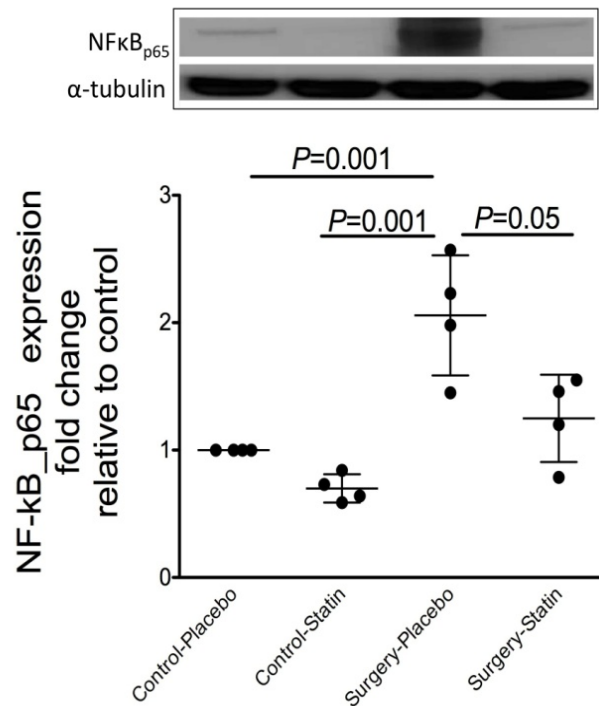


Figure 9.7: This figure is a scatter histogram of the western blotting analysis of NF κ B_{p65} in mouse hippocampal tissue on day 1 after treatment. The experimental mice groups were: Control-Placebo, Control-Statin, Surgery-Placebo and Surgery-Statin ($n=4$ /group).

The top panel shows a representative immunoblot for NF κ B_{p65} that was reprobbed for α -tubulin as a loading control. The scatter histogram in the bottom panel shows the densitometric measurements of the Hsp72 signals on the immunoblot, relative to control. Values are the mean $\pm$ SD. A P value ≤ 0.05 was considered significant.

9.2.2.4 Assessment of the systemic and CNS inflammatory response by cytokine value

The plasma level of IL-1 β was measured on day 1, 2 and 3 after treatment in different cohorts of mice.

On day 1 after surgery, the plasma level of IL-1 β was 7.3 ± 1.6 in the C-P, 7.2 ± 1.0 in the C-S, compared with 59.7 ± 11.7 in the S-P and 59.6 ± 6.0 in the S-S groups ($P < 0.001$). There was a marked increased in plasma level of IL-1 β in the surgical groups (S-P and S-S) when compared to the C-P group ($P < 0.001$). There was no statistical difference between the S-P and the S-S groups ($P = 0.84$). This elevation in IL-1 β for surgical groups was still present on day 2 with C-P (7.3 ± 1.3) and C-S (6.2 ± 2.3) compared to both S-P (89.4 ± 28.2) and S-S (59.8 ± 16.4) ($P < 0.001$). By day 3 there was no difference to the values for IL-1 β across treatment groups: C-P (7.8 ± 1.7), C-S (6.8 ± 0.8), S-P (19.4 ± 7.1) and S-S (13.5 ± 7.1) (Figure 9.8).

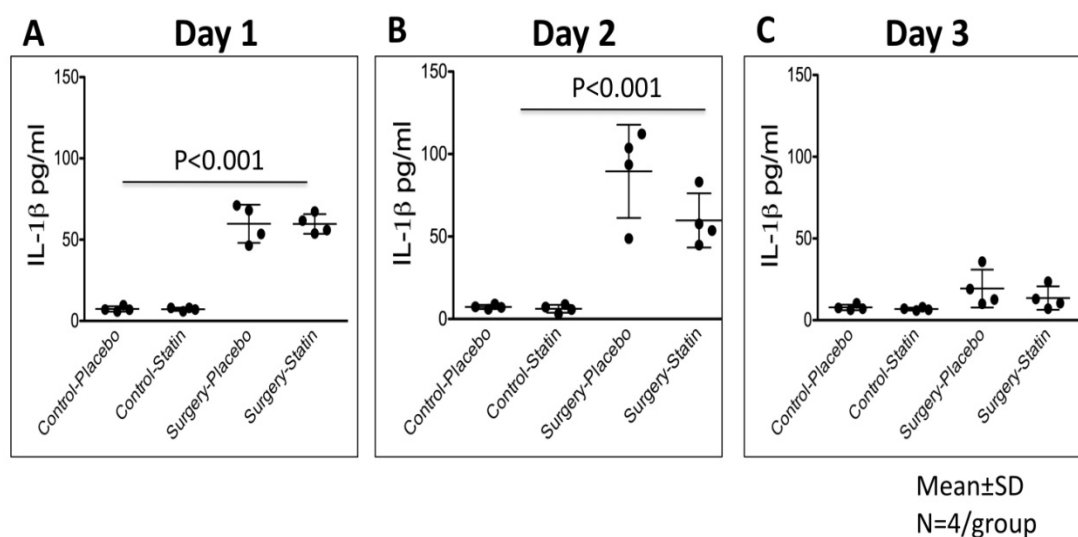


Figure 9.8: This figure is a scatter histogram of the ELISA results for the plasma level of IL-1 β on day 1 (A), 2 (B) and 3 (C) after treatment. The experimental mice Imperial College London

groups were Control-Placebo, Control-Statin, Surgery-Placebo and Surgery-Statin ($n=4/\text{group}$). Values are the mean $\pm$ SD. A P value ≤ 0.05 was considered significant.

The hippocampal level of IL-1 β was also measured on day 1, 2 and 3 after treatment in different cohorts of mice.

On day 1 after surgery the hippocampal level of IL-1 β was 8.1 ± 1.7 in the C-P, 7.4 ± 1.7 in the C-S, 19.8 ± 1.5 in the S-P and 12.3 ± 3.9 in the S-S groups ($P<0.001$) (Figure 9.9 A). On day 1 post-surgery the S-P group had significantly higher levels of IL-1 β than the S-S group ($P<0.05$) and both surgery groups had significantly higher levels of IL-1 β when compared to their respective controls ($P<0.001$).

Day 2; there were no differences between groups. The hippocampal level of IL-1 β was 7.3 ± 2.4 in the C-P, 7.9 ± 1.9 in the C-S, 12.2 ± 4.7 in the S-P and 8.9 ± 0.2 in the S-S groups ($P=0.12$) (Figure 9.9 B).

On day 3 after surgery, when the memory assessment was performed, there were no differences in hippocampal levels of IL-1 β between any of the treatment groups: C-P (5.0 ± 0.7), C-S (5.4 ± 1.0), S-P (5.5 ± 0.9) and S-S (5.4 ± 0.7), $P=0.85$ (Figure 9.9 C).

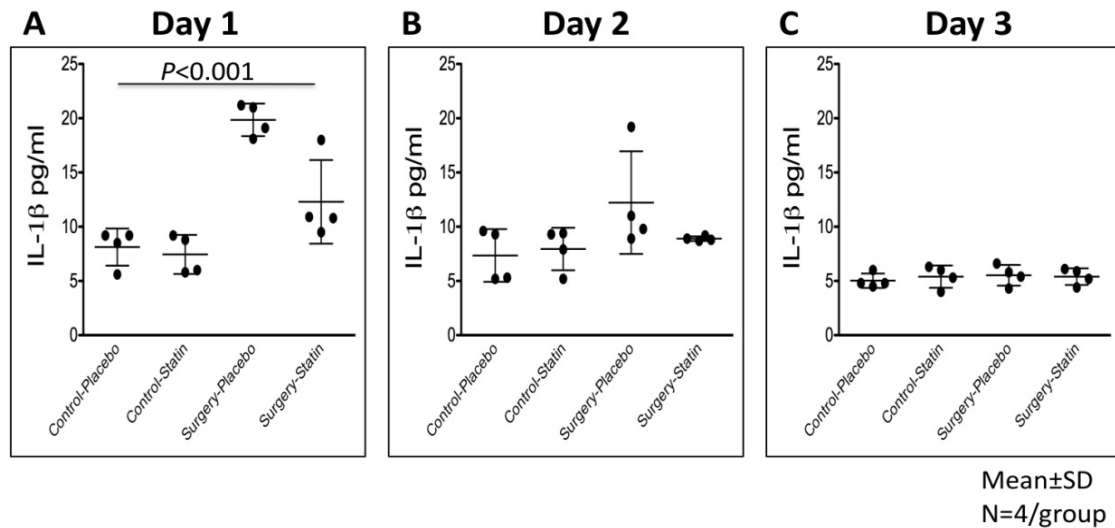


Figure 9.9: This figure illustrates a scatter histogram of the ELISA results on the hippocampal tissue of the IL-1 β level on day 1 (A), 2 (B) and 3 (C) after treatment. The experimental mice groups were Control-Placebo, Control-Statin, Surgery-Placebo and Surgery-Statin ($n=4/\text{group}$). Values are the mean $\pm$ SD. A P value ≤ 0.05 was considered significant.

9.2.2.5 Assessment of microglia activation in areas of the brain where memory is formed (hippocampus) after unilateral nephrectomy

The assessment of microglia was performed in a different cohort of mice for day 1, 2 and 3. All the brains studied ($n=4/\text{group}$) were immunostained with CD11b, which is a cellular marker of microglia activation.

On day 1 after surgery the staining for CD11b revealed active cells in the hippocampus of the treatment groups. There was a mild response (+) in both surgical groups, demonstrating a degree of microglia reactivity. Microglia

activation was more pronounced on the S-P group. This difference was significant when compare to C-P, C-S and S-S group ($P<0.05$) (Figure 9.10).

On day 2 after surgery the S-P showed a moderate response (++) whereas the S-S group showed no response, appearing the same as the controls. Results are summarised in Table 9.2 and Figure 9.10.

On day 3 after surgery the staining for CD11b revealed no active cells in the hippocampus in any of the treatment groups; all slides appeared clear and free of reactive microglia. No microglia could be graded on the scale of morphological activity. The conclusion was that there was no microglia activation in the hippocampus on day 3 after surgery, insomuch as all treatment groups (surgery or no surgery) appeared the same (Table 9.2).

Table 9.2: This table summarises the morphological responses of microglia in the CA1 region of hippocampi from all treatment groups on day 1, 2 and 3 after surgery. The experimental mice groups were Control-Placebo, Control-Statin, Surgery-Placebo and Surgery-Statin ($n=4/\text{group}$). For detailed key see text; 0 = no response/activation, + = mild, ++ = moderate and +++ = intense. S-P showed microglia activation at days 1 (mild) and 2 (moderate) whereas S-S showed only mild activation at day 1 post-surgery.

Microglia activation after unilateral nephrectomy (CD11b)				
	Control- Placebo	Control- Statin	Surgery- Placebo	Surgery- Statin
Day 1	0	0	+	+
Day 2	0	0	++	0
Day 3	0	0	0	0

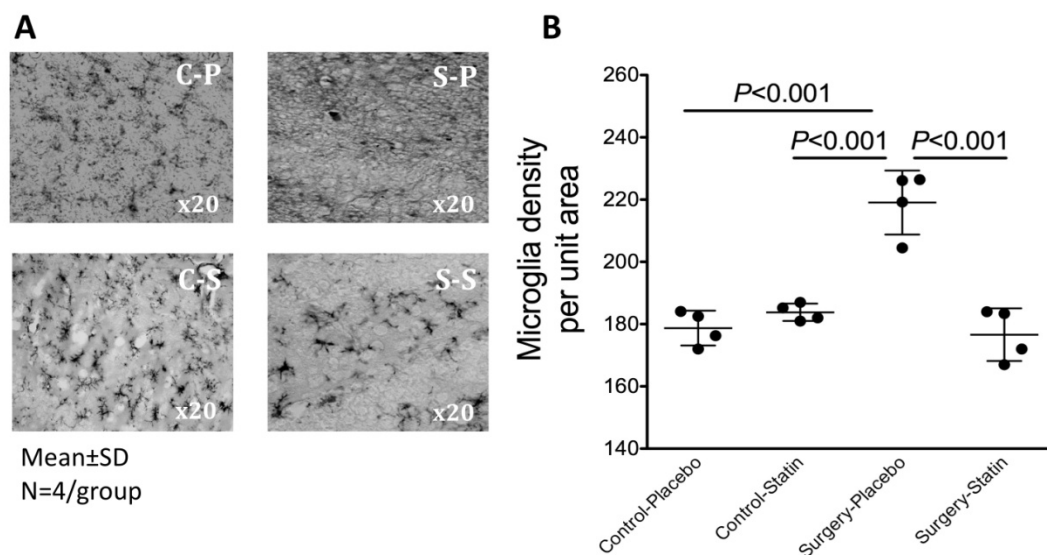


Figure 9.10: Microglia activation was assessed by immunoreactivity for CD11b and microglia density per unit area. A) Representative photomicrographs of the CA1

region of the hippocampus immunostained for CD11b on day one are shown for each of the conditions; control-placebo (C-P), control-atorvastatin (C-S), surgery-placebo (S-P) and surgery-atorvastatin (S-S). Scale bar, x20 magnification. B) The histogram illustrates microglia density per unit area in the CA1 region on day one after mentioned treatment. Values are the mean $\pm$ SD. A P value ≤ 0.05 was considered significant.

9.2.2.6 Assessment of the endothelium activation in areas of the brain where memory is formed

Endothelial cell activation was assessed in the hippocampus by FACS analysis. Analysis was performed in all groups (n=4–6) on day 1 after treatment in a different cohort of mice to quantify the magnitude of the atorvastatin effect on the endothelial cells. Cell suspension was gated for 7AAD and CD31 positive endothelium cells from hippocampi were identified (Figure 9.11A). 2000 endothelium cells were captured per condition. Histograms were plotted to identify COX-2 expression by using mean fluorescent intensity (MFI) difference between groups. The MFI was high in the S-P group (67.48 ± 12.7) when compared to C-P (29.9 ± 7.7 , $P < 0.05$), and S-S group (36.8 ± 2.7 , $P < 0.05$) (Figure 9.11B).

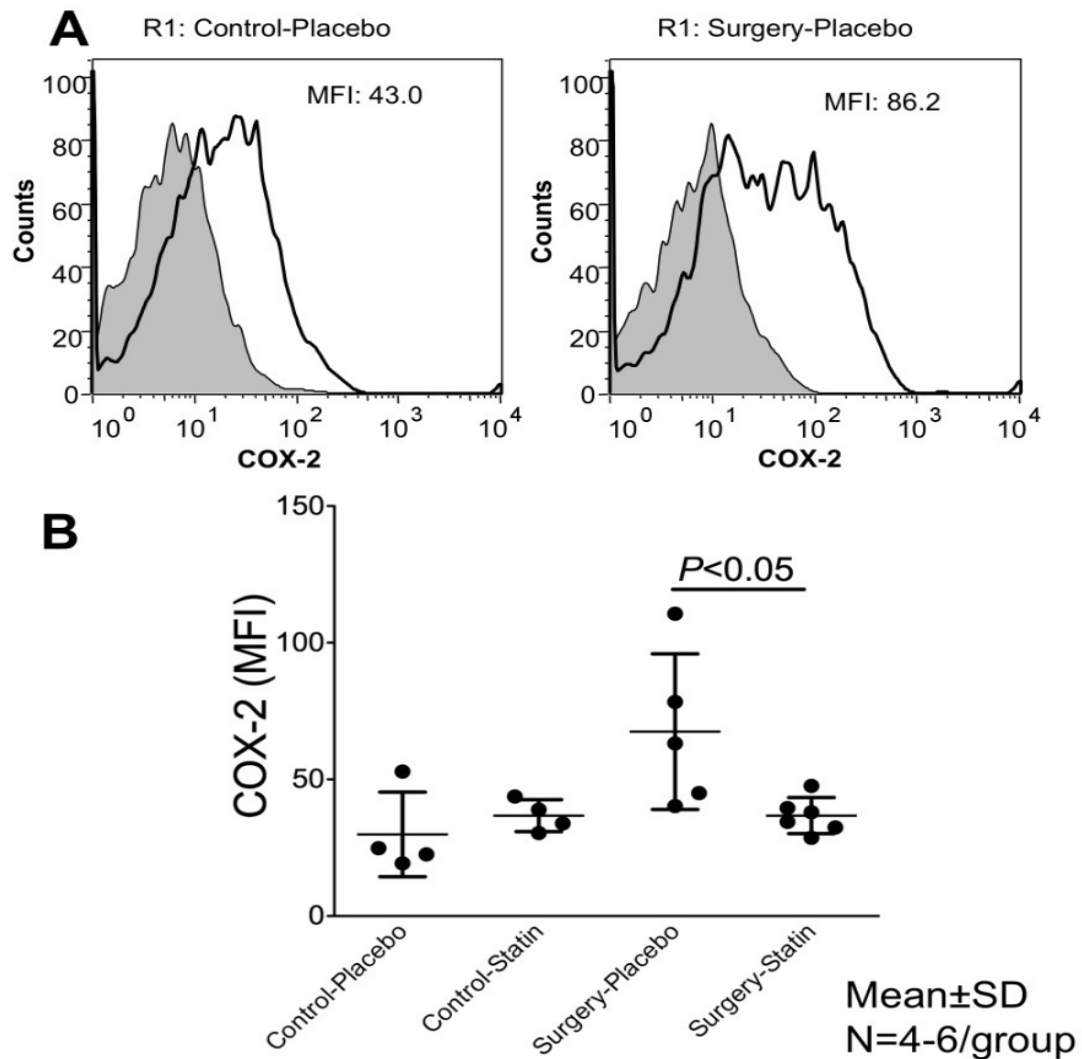


Figure 9.11: Atorvastatin suppresses the expression of COX-2 on endothelial cells. A) Illustrative representation of flow cytometry analysis of COX-2 expression on hippocampi endothelial cells in the control-placebo and surgery-placebo groups on day 1 after treatment. B) Quantification of COX-2 in the hippocampi endothelial cells in the control-placebo, control-statin, surgery-placebo and surgery-statin group. The expression of COX-2 is presented as difference of mean fluorescent intensity (MFI) changes between groups. Values are the mean $\pm$ SD. A P value ≤ 0.05 was considered significant.

9.3 Discussion

In this chapter the POCD following unilateral nephrectomy was significantly prevented after treatment with a preoperative regime of atorvastatin. This was shown using a fear conditioning paradigm. There was no statistical difference between the surgical group given atorvastatin and the non-surgical placebo group implying pronounced beneficial effects. This could imply that postoperative memory decline had been prevented through administration of atorvastatin. The amelioration of postoperative memory deterioration was reinforced by the results from the water maze which found that at one week after surgery the atorvastatin treated group demonstrated superior spatial memory performance to the placebo treated group. After surgery there was a reduction in COX-2 in endothelium cells from the hippocampi and also lower levels of hippocampal IL-1 β in mice treated with atorvastatin on day 1. There was, however, an increase in the plasma levels of IL-1 β in both the placebo treated and the atorvastatin treated groups for up to 2 days. These results imply that as the plasma values indicate inflammation that is unaffected by atorvastatin the cellular effects in the endothelium cells in the hippocampus may be a direct effect of atorvastatin.

Lower levels of IL-1 β seen in the CNS of the atorvastatin treated surgical group were associated with a significant improvement in hippocampal dependent memory retrieval. The presence of IL-1 β in the hippocampus has previously been seen to affect the synaptic plasticity with a reduction in long-term potentiation (LTP). LTP is generally accepted as the biological basis of memory (Lynch and Lynch 2002). Sources of IL-1 β in the hippocampus include activated

microglia and migrated blood-borne monocytes (Giulian, Woodward et al. 1988; Maher, Clarke et al. 2006). Potentially the migration of leucocytes from the periphery can thus set up an IL-1 β rich environment. Statins have been shown to interfere with the migration of leucocytes from the periphery to the CNS (Stüve, Youssef et al. 2003). Hence this could provide a potential mechanism by which the hippocampi of the atorvastatin treated group were 'sheltered' from the elevated IL-1 β and associated inflammatory milieu of the peripheral stress response to surgery.

From a different perspective it could be argued there may be a residual effect or a low level of Hsp72 that results in continued impairment of neural function. The observation that the level of Hsp72 was not significantly different between groups mitigates against this possibility. The limitation to these postulates is that the number of mice included for the analysis of Hsp72 expression in the hippocampus was too small to draw conclusions.

Another potential explanation is that that microglia activation was directly affected by atorvastatin. Previous data in the field of traumatic brain injury found that atorvastatin significantly decreased microglia response after injury for up to 7 days (Wang, Lynch et al. 2007). Activated microglia have been identified as a source of not only inflammatory mediators but also the excitatory neurotransmitter glutamate (Wible and Laskowitz 2010). This can potentially lead to secondary neuronal injury via the mechanism of excitotoxicity. Interestingly, there is also evidence that glutamate mediated excitotoxicity may be significantly attenuated by statin (Zacco, Togo et al. 2003).

However, on day 3 after surgery, when memory differences between groups were observed, there was no elevation of IL-1 β in the hippocampi of any of the treatment groups whilst, high levels of IL-1 β in plasma were still seen in the surgical groups. The acute hippocampal inflammatory situation seen in POCD may be akin to an extremely localised brain injury initiated by endothelial cells. This set of experiments demonstrated that the hippocampus endothelium was activated soon after surgery and its degree of activation was measured by the level of expression of COX-2. This was reduced by the administration of atorvastatin. These findings suggest that the mechanisms of persistent memory decline or in fact memory decline beyond day one may be linked to other mechanisms so there is a need to explore further the endothelial cells within the CNS and their activation after acute non-neurosurgical surgery.

To take this forward the study should be repeated with a group of animals treated with both atorvastatin and mevalonate prior to surgery. Mevalonate is the downstream product of the HMG-CoA reductase catalyzed reaction prevented by statin. Addition of this molecule would also demonstrate whether or not the memory benefits seen are due to inhibition of the mevalonate pathway (Weitz-Schmidt 2002). Adding surgical groups treated with an interleukin-1 receptor antagonist (IL-1ra), would allow examination of whether the difference in cognitive function observed in these experiments was causally associated with differences in IL-1 β levels. This is relevant as it has already been shown that the hippocampal dependent cognitive impairment associated with LPS injection can be prevented by IL-1ra administration (Pugh, Kumagawa et al. 1998; Terrando,

Rei Fidalgo et al. 2010). It would also be shrewd to investigate the optimal dose and duration of preoperative administration of atorvastatin to confer the greatest degree of perioperative protection.

In summary, nephrectomy in a murine model leads to POCD which was prevented by preoperative administration of atorvastatin. This surgical model produced a systemic and central inflammatory response, with the central inflammation being attenuated by administration of atorvastatin.

Chapter 10:

Main Discussion

The hypothesis; the mechanism underlying POCD is inflammation mediated in part by low levels of inducible Hsp72 and activation of P2X7 receptors. Hence POCD is ameliorated by xenon and statins.

Suppressing the activity of the receptor P2X7 has been shown to alter the inflammatory response after major insults. Both xenon and atorvastatin, an anti-lipid drug have been shown to have anti-inflammatory properties. It was therefore suggested that either pre-treatment with xenon or with atorvastatin might, increase the expression of Hsp72. This in turn might result in obtunding of the neuroinflammatory response presumed accountable for the development of POCD.

The aims of this PhD thesis were to use existing animal models that have been validated for both inflammatory response after surgery and the assessment of memory changes to firstly attempt to identify techniques to enable study the systemic and central nervous system inflammatory response to surgery and anaesthesia and secondly to develop a model to predict the development of POCD based on the inflammatory response. Then to use these methods to elucidate the role of P2X7 receptors and Hsp72 in POCD. Finally to assess if the action of the anti inflammatory agents xenon and atorvastatin on up regulation of Hsp72 might assist in the clearer identification of a preventative mechanisms for POCD.

The summary of the findings of this thesis and their limitations are presented in the order the aims were proposed at the beginning of this thesis following by an

explanation about how the aims were met. Finally it will conclude with whether the hypothesis was proven.

In this thesis it has been demonstrated that the SIMCA analysis, to assess cytokine profile after surgery, could be used to identify those cytokines associated with inflammation. SIMCA showed a range of cytokines that were involved in the initial phase of response to surgery and anaesthesia. There were inadequate numbers in the study for definitive outcomes but it is a technique that shows potential promise.

The FACS analysis was refined to demonstrate different cell lines at different time points and the technique was shown to work. The P2X7 receptors were investigated and were seen on microglia and astrocytes. They appear to affect sickness behaviour but in these studies, not POCD. Over expression of the Hsp72 did reduce the development of POCD. Pretreatment with xenon seems to be anti-inflammatory and mediated *via* Hsp72 and both prevent POCD.

10.1 Assessment of the inflammatory response by SIMCA and FACS

To pursue the relationship between inflammatory effects and POCD, two techniques for evaluating these effects were assessed. The assessment of memory changes and inflammatory response after surgery by using the statistical isolinear multicomponent analysis, SIMCA showed a good separation

between the surgery group and its respective controls on day 1 and 7 after tibial fracture surgery under general anaesthesia. The plasma level of cytokines identified on day 1 and 7 after surgery also allowed good class separation within the models but was not adequate to allow the creation of a SUS-plot to predict the inflammatory response in plasma on these days after tibial fracture surgery under general anaesthesia. The hippocampus level of cytokines on day 1 and 7 after tibial fracture surgery under general anaesthesia had a clear signal and discriminative ability between groups in each model and they had good Q2 allowing the creation of SUS-plot for assessment of reproducibility and predictability of the neuro-inflammatory response on days 1 and 7 after surgery. IFN γ , GM-CSF and MCP-3 were identified as the key discriminative cytokines in hippocampi on day 1 after surgery whilst the IFN γ , GM-CSF and IL-5 were identified as the key discriminative cytokines in hippocampi on day 7 after surgery.

The SIMCA is a new tool that has not previously been used for the identification of a physiological signature for the development of POCD, and several limitations with this approach were identified. The mice were young and therefore not representative of older mice. The sample size was adequate to assess if the method has potential use but too small to develop a reliable model. The cytokine samples were stored at very low temperature then brought back to room temperature for processing and therefore it is difficult to ascertain if some proteins were potentially denatured.

Given these limitations this method is potentially an exciting new technique for looking at complex mechanisms. The conclusions that can be drawn are limited but it does suggest that there is a systemic and CNS inflammatory response to surgery but that it is difficult to pin point a single cytokine as the key in POCD. The study demonstrates that there is potential usefulness with this method and that it warrants further investigation.

The FACS analysis was used to distinguish different cells types in the hippocampus in sufficient numbers and accuracy to quantify key markers of activation. This would complement IHC findings by identifying individual cells response to different insults. By implementing a method of simultaneous fixation and homogenization during the performance of FACS the microglia were clearly identified in sufficient numbers to perform quantification of the different antigens with minimal alterations in their levels which may result from the traditional tissue disaggregation methods. In this set of experiments microglia activation was identified after surgery by FACS with a higher yield of cells. This was in line with previous immunohistochemical findings reported in the literature (Cibelli 2011). The FACS analysis permits the assessment of different cell lineages, at different time points, from specific areas of the brain. This could, therefore, facilitate more complex experiments aiming to identify cellular communication during ongoing inflammatory processes. The method is limited by the size of the mouse hippocampus and the contamination of sample with myelin debris. The use of DNA dye (7AAD) was successful in reducing myelin debris from analysis and hence noise from data acquisition. Therefore, a simple and economic method was identified which enables the performance of direct

flow cytometric analysis of multiple samples obtained from small CNS tissue samples.

10.2 P2X7 receptors

The presence and activity of P2X7 receptor in microglia and astrocyte cells has been demonstrated. P2X7 receptor does appear to influence sickness behaviour, but blocking the P2X7 receptor after surgery did not influence POCD.

10.3 Heat shock protein 72

Over expressing the Hsp72 seems to prevent the development of POCD in Hsp72-Tg mice up to day 7 after surgery, although with a reduction in sensitivity. For example reduction in freezing time with time following training. These findings suggest that Hsp72 is important either to memory retrieval or protection from a process that degrades memory retrieval following anaesthesia or surgery. Limitations include the possibility that transgenic mice may have developed chronic mechanisms to adapt to the high level of Hsp72. Also the wide age range may prevent seeing effects which may be occurring in older mice. Although the high expression of Hsp72 appears protective, it needs further investigation. Therefore the following set of experiments were conducted aimed at exploring strategies to enhance the expression of Hsp72 and thereby prevent the development of POCD.

10.4 Xenon

The effect of xenon on POCD was explored. POCD after surgery under isoflurane anaesthesia was ameliorated by xenon exposure and was associated with an increase in Hsp72 and PI3-kinase in the hippocampus and a decrease in the plasma levels of IL-1 β . Limitations of this set of experiments were the mice age, the short time exposure to xenon before surgery the costing and cumbersome system to deliver the noble gas, Xenon.

10.5 Atorvastatin

POCD following unilateral nephrectomy was significantly prevented after treatment with a preoperative regime of atorvastatin. The amelioration of postoperative memory deterioration was reinforced by the results from the water maze which found that at one week after surgery the atorvastatin treated group demonstrated superior spatial memory performance to the placebo treated group. After surgery there was a reduction in COX-2 in endothelium cells from the hippocampi and also lower levels of hippocampal IL-1 β in mice treated with atorvastatin on day 1.

10.6 What is already known in this area?

Postoperative cognitive decline, as originally described in cardiac surgery, has been attributed to specific intraoperative pathophysiological events. These include air and other embolism, hypotension and periods of hypoxaemia (Newman, Grocott et al. 2001). It has been clearly shown that POCD also occurs

in nearly 10% of non-cardiac surgical patients not exposed to these risk factors

and can be demonstrated three months after surgery (Moller, Cluitmans et al. 1998; Bekker and Weeks 2003). Theoretical advances have considered the potential for a peripheral stressor such as surgical trauma itself to increase levels of pro-inflammatory cytokines both peripherally and in the CNS (O'Connor, Johnson et al. 2003). There is evidence that activation of the systemic innate immune system leads to alterations in the chemical environment and function of the CNS (Linthorst, Flachskamm et al. 1995). Also that this inflammatory milieu could be associated with impaired memory retrieval, the putative mechanism for POCD (Wan, Xu et al. 2007; Rosczyk, Sparkman et al. 2008).

Infection with gram-negative bacteria is a powerful stimulant to activate the peripheral immune system and this has been shown to exert a detrimental effect on the memory of mice. This effect was reversed by inhibition of the pro-inflammatory cytokine IL-1 β (Gibertini, Newton et al. 1995; Terrando, Rei Fidalgo et al. 2010). Further support for this concept came when Pugh *et al* demonstrated that peripheral administration of LPS created an IL-1 β mediated deficit in hippocampus dependent but not hippocampus independent memory (Pugh, Kumagawa et al. 1998). The role of IL-1 β is increasingly established and the presence of IL-1 β in the hippocampus has been shown to inhibit synaptic plasticity and thus cause a reduction in long-term potentiation, which is generally accepted as the biological basis of memory (Lynch and Lynch 2002).

An additional observation has been that after non-neurosurgical procedures microglia activation seems to be a common finding (Wan, Xu et al. 2007; Cibelli, Fidalgo et al. 2010) and it has been implicated to memory loss after surgery.

10.7 How these findings are linked to the literature?

10.7.1 Assessment of the inflammatory response by SIMCA and FACS

It is very likely that the inflammatory response is complex and may involve a spectrum of cytokine activity (Rosczyk, 2008 4028). If that is the case then assessment of inflammatory response using the principal component analysis would, potentially, seem potentially a reasonable method for identification of a physiological signature consisting of multiple discriminatory cytokines. If it can be shown to work it would be potentially useful for modelling the CNS and peripheral inflammatory response. SIMCA, as a method has not been used before for assessment of the inflammatory response and is still in an embryonic phase in biological science generally. The results shown here suggest it is, at least, feasible to apply multivariable analysis approach to identify proteins that may interfere with memory formation (consolidation) or retrieval after major injuries. The numbers in these studies reported here were inadequate to draw conclusions but provide some evidence that the method can work and should be explored further in the future.

While the preliminary data with FACS proved to be an alternative to ascertain individual cell activity within the brain after different noxious insults it also complements the IHC analysis as it has also shown its usefulness previously in models of chronic pain and inflammation (Blackbeard, et al. 2007).

10.7.2 P2X7 receptors

The role of P2X7 in inflammation is well established in the literature. P2X7

receptors are present in microglia and neurons and expression of these receptors is increased during stress. Activation of P2X7 receptors leads to synthesis of pro-inflammatory cytokines such as IL-1 β , which is present in the hippocampus and is important for the formation of spatial memory (Labrousse, Costes et al. 2009). Increased levels of IL-1 β in the hippocampus have been implicated in memory loss induced with severe infection (Barrientos, Higgins et al. 2006), following splenectomy (Wan, Xu et al. 2007) and traumatic brain injury (Clausen, Hanell et al. 2009).

In the studies reported in this thesis, postoperative memory decline after tibial fracture under general anaesthesia was not prevented in P2X7-KO mice. It might have been expected that were P2X7 receptors pivotal then there would be a reduction in memory decline. An explanation might be that memory may be governed by a baseline level of IL-1 β within the hippocampus and in the absence of P2X7 receptors, chronic compensatory mechanisms may be involved, such as an over expression of P2X4 receptor (Hracsko, Baranyi et al. 2011). Further studies using highly selective P2X7 antagonists may help us to further characterise the role of this receptor on memory decline after surgery. Other explanations include that it could simply be that the fear-conditioning paradigm used for assessment of memory was not sensitive enough to identify differences between groups.

10.7.3 Heat shock protein 72

Hsp72 has been implicated in the development of POCD. It was previously shown by Yenari et al that Hsp72 ameliorates the inflammatory response by

inhibiting NF κ B in astroglia after ischaemic injury (Yenari, Liu et al. 2005). Hsp72 is known to inhibit iNOS gene transcription and synthesis via the inhibition of uptake and activation of NF κ B (de Vera, Kim et al. 1996; Feinstein, Galea et al. 1997; Zheng, Kim et al. 2008). Hsp72 also stabilises the mitochondrial membrane potential and reduces ROS production (Ouyang, Xu et al. 2006).

In these experiments over expression of Hsp72 reduced the activation of microglia as assessed by cell count and morphology and prevented memory loss after non-neurosurgical procedures. Mice over expressing Hsp72 showed no memory impairment following surgery. However, the C57BL6-Wt surgical group still showed significant memory deficit on day 3 after surgery. Whilst the effects of anaesthesia such as those on blood pressure or blood gases, cannot be excluded as potential causes of this effect, the anaesthetic was brief, and it was unlikely that any haemodynamic effect was different between groups of genotypes. It is also relevant that neither reduced blood pressure, oxygenation, nor depth of anaesthesia were associated with POCD in human trials (Steinmetz, Funder et al. ; Moller, Cluitmans et al. 1998).

Hsc70, a precursor of Hsp72, has been shown to be lower in the hippocampus of mice after 3 days of training on the Morris water maze, which suggests that Hsc70 may be implicated in the learning process of spatial memory (Pizarro, Haro et al. 2003; Ambrosini, Mariucci et al. 2005). The exact relationship between learning and the expression of Hsp72 is not yet clear. In these experiments there was no difference in *learning (anterograde acquisition)*

between C57BL/6-Wt and Hsp72 over-expressing mice. However, *memory (retrograde retrieval)* was fully protected in Hsp72 over-expressing mice after surgery and anaesthesia before day 7. The sensitivity of the fear-conditioning test appears to be reduced at day 7 as all mice showed reduction in memory at this time point. These findings suggest that Hsp72 is important either in memory retrieval or protects from a process that degrades memory following anaesthesia or surgery. Whether the observed protection of memory involves anti-inflammatory mechanisms based on inhibition of iNOS and NFκB or reduced oxidative stress, or both, remains to be evaluated.

These findings taken together with the current literature suggest that the neuroinflammatory response may be responsible for the impaired memory after non-neurosurgical procedures and the antiinflammatory effect of Hsp72 can prevent it. While long lasting over expression of Hsp72 has been demonstrated to prevent POCD further investigations are required to identify if the over expression of Hsp72 for brief period before surgery has the same impact on the development of POCD.

Further research would also be necessary and appropriate to distinguish whether this protective Hsp72 up-regulation takes place in the endothelium, microglia and/or astrocytes. This may have an influence on potential protective pharmacological interventions.

10.7.4 Isoflurane

The role of isoflurane needs to be discussed as a potential confounder in these

studies. Isoflurane anaesthesia has been shown to be a risk factor for cellular toxicity *in vitro* (Xie, Dong et al. 2006), and pro-inflammatory cytokine release and memory decline *in vivo* (Culley, Baxter et al. 2004; Bianchi, Tran et al. 2008; Valentim, Alves et al. 2008; Wu, Lu et al. 2010).

Isoflurane has been reported to suppress learning in a dose-dependent fashion (Dutton, Maurer et al. 2001). It has been demonstrated that isoflurane interferes with anterograde but not retrograde memory for Pavlovian fear conditioning (Dutton, Maurer et al. 2002; Culley, Baxter et al. 2003). In these studies, fear-conditioning training took place during delivery of anaesthesia. However, the animals were trained before surgery and anaesthesia. This allowed the influence of the acquisition phase on assessment of memory postoperatively to be excluded. Previous studies have demonstrated that ketamine, xylazine and acepromazine anaesthesia interfere with retrograde memory after fear-conditioning training (Gerlai and McNamara 2000). This is the first report showing impaired memory (retrieval of information) after isoflurane anaesthesia.

Isoflurane has been reported to have some anti-inflammatory effects. Xu *et al* showed that pre-treatment with isoflurane reduced activation of C8-B4 microglia 24 h after LPS plus IFN γ exposure (Xu, Kim et al. 2008). Sevoflurane, another inhalational anaesthetic, induced an increase in expression of Hsp72 in cardiac muscle (Kitahata, Nozaki et al. 2008). However, in the studies reported in this thesis, isoflurane alone did not increase in Hsp72 in the hippocampus on day one after surgery and did not reduce the plasma level of IL-1 β .

10.7.5 Xenon

Xenon exposure immediately prior to a surgical intervention ameliorates early memory decline after surgery under isoflurane anaesthesia. This was associated with an increase in Hsp72 and PI3-kinase levels in the hippocampus and a decrease in plasma levels of IL-1 β . Once again the major limitations of the Xenon studies were the age of the mice used, the cumbersome system to deliver this noble gas and the sample size. The major limitation in applying this clinically would be the prohibitive costs so even translating the results of this thesis to clinical studies and applications would need to take this into account.

10.7.6 Atorvastatin

A pre-operative regime of atorvastatin improved hippocampus dependent memory recall as assessed by the fear conditioning paradigm after unilateral nephrectomy. This beneficial effect was pronounced to the degree there was no statistical difference in recall between the surgical group given atorvastatin and the non-surgical placebo group. An improvement in the atorvastatin treated group was also demonstrated by superior spatial memory performance in the water maze test one week after surgery.

The degree of activation of endothelium in the hippocampus soon after surgery was assessed by measuring the level of expression of COX-2. Atorvastatin treatment was associated with a reduced COX-2 in the endothelium. Lower levels of IL-1 β by ELISA in the hippocampus of animals treated with atorvastatin on day one after surgery were also found but plasma levels of IL-1 β , were increased in both the placebo and the atorvastatin group for 2 days post-

operatively. On day 3 after surgery, when significant differences in memory between groups were observed, there was no elevation in IL-1 β levels either in plasma or in the hippocampi of the atorvastatin treatment group whilst high levels of IL-1 β in plasma were found in the placebo surgical group.

There was no significant difference between the atorvastatin and placebo groups in the level of Hsp72 in the hippocampus even though there was a trend to higher levels with atorvastatin. This lack of statistical difference between the surgery groups may be due to the small sample size utilised to perform western blotting of the hippocampus. These findings are highly promising and if confirmed, easily translatable to clinical settings.

The surgical model used in the xenon experiments is a very specific iatrogenic injury generated under general anaesthesia where other variables were tightly controlled. This may not mimic more complex human clinical situations. Moreover, the tibial fracture model may not trigger more behavioural changes than those seen after exposure to isoflurane alone. A group of animals pretreated with isoflurane followed by surgery and a group treated with xenon followed by isoflurane alone would contribute to a better understanding of the memory findings.

To interpret these findings in the context of other existing research in this area it is important to bear in mind other limiting factors such as interspecies variability (e.g. different immune response in mice and rats), different surgical models (e.g. splenectomy, bone fracture, nephrectomy), different memory tests,

different time points of memory testing and the difference between learning and memory.

10.8 In conclusion

The first part of the hypothesis was that the mechanism underlying POCD is inflammation. This was investigated by SIMCA and FACS analysis. Both methods seem to be potentially useful techniques for analysis of the inflammatory response and they could be complementary to current available methods once the limitations are further explored and amended.

The involvement of P2X7 receptors in the development of POCD could not be demonstrated even though its presence was identified in the changes in microglia and astrocytes and the data suggest the involvement of P2X7 in sickness behaviour.

However the role of Hsp72 in POCD was clearly shown. The prevention of the development of POCD by using xenon was also demonstrated with the implication that this mechanism might be involved. Atorvastatin also reduced POCD by suppressing the inflammatory response.

Therefore inflammation is involved in POCD and the chaperone Hsp72 is part of the mechanism. Xenon reduces POCD partly by effects on Hsp72 while atorvastatin has an anti inflammatory effect which also reduces POCD,

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12 Appendices

12.1 Appendix 1

Table 3.1: Physiological scoring system used for assessment of animals activity in the perioperative period.

Animal Number:

Date & Time	Score
Weight	
> Pre-operative weight	0
95-100% pre-operative weight	1
90-95% pre-operative weight	2
<90% pre-operative weight	4
Activity	
Normal	0
Moves round cage spontaneously, but reduced	1
Moves to stimulus, but not spontaneously	2
Huddled, not moving to stimulation	5
General appearance	
Normal	0
Evidence of poor grooming	2
Behaviour	
Traumatisation of wound	1
Muscle twitching	3
Back-arching	5
TOTAL (max=16)	

Judgment:

0: No evidence of symptoms

1-4: Continue to monitor closely, on at least a daily basis. Give analgesics if pain is considered possible and reassess.

5-6: Consider killing by Schedule 1 method. Give analgesics if pain is considered possible and reassess. If necessary, seek second opinion of veterinary.

7 or above: kill by schedule 1 method.

Table 3.5: Baseline assessment of C57BL6-Wt, P2X7-KO and Hsp72-Tg mice. * Tests were not performed. Assessment of 2 or more physiological abilities were sufficient to characterise the mice baseline physiology before allocating them to corresponding experiments.

	C57BL/6-Wt mean±SEM (95% CI) (n)	P2X7-KO mean±SEM (95% CI) (n)	Hsp72-Tg mean±SEM (95% CI) (n)
Acoustic Reflex at 110 dB	46.0±3.0 (38.29–53.88) (n=6)	*	50.1±5.9 (36.02–64.36) (n=8)
Latency to startle (sec)			
Pain Threshold	18.8±1.6 (15.39–22.31) (n=17)	16.01±1.9 (11.3–20.8) (n=7)	19.09±1.8 (15.11–23.07) (n=17)
Latency to pain (sec)			
Motor Activity	457.5±39.8 (355.0–560.1) (n=6)	*	416.2±99.2 (160.9–671.4) (n=6)
Travel Distance (cm) – Smart cage			
Motor Activity	41.3±18.1 (-3.1–85.8) (n=7)	35.7±13.1 (3.5–67.8) (n=7)	*
Travel Distance (cm)- Rotarod			
Long-Term memory	75.6±3.3 (69.0–82.2) (n=34)	71.2±3.5 (63.9–78.6) (n=30)	82.1±2.9 (76.23–88.10) (n=34)
Freezing Time after Training (%)			

12.2 Appendix 2

Table 3.7: Solutions and corresponding components used for IHC studies.

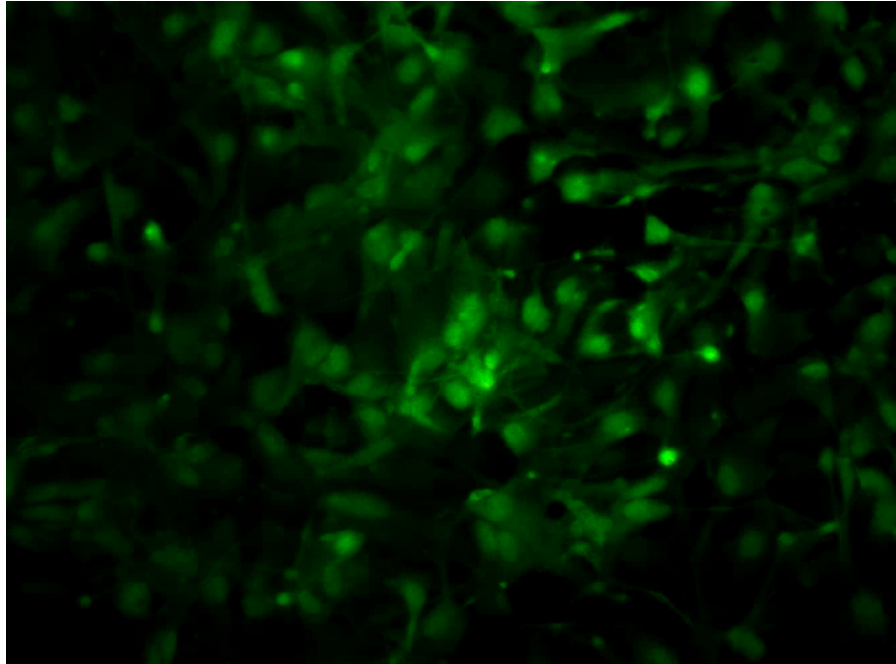
4% Paraformaldehyde (PFA)	40 gr Paraformaldehyde in 1 lt PBS -Heat to 56 °C on a stirrer plate -Clear the solution by adding NaOH -Filter with large paper filters -Adjust pH to 7.4 Store solution at 4 °C
0.9% Saline Prewash	9 gr NaCl in 1lt dH ₂ O (add heparin (c. 10 units per ml) in the final solution) Store at 4 °C
30% Sucrose	150 gr Sucrose in 500ml dH ₂ O (stir to dissolve) add 0.25 gr Sodium Azide (preservative) Store solution at 4 °C
0.1 M K ₂ HPO ₄ buffer (Monohydrogen phosphate buffer)	17.4 gr K ₂ HPO ₄ powder in 1lt dH ₂ O
0.1 M KH ₂ PO ₄ buffer (Dihydrogen phosphate buffer)	13.6 gr KH ₂ PO ₄ powder in 1lt dH ₂ O
0.1 M Phosphate Buffer (PB)	Mix: 4x 0.1 M K ₂ HPO ₄ buffer & 1x 0.1 M KH ₂ PO ₄ buffer (e.g. for 1lt PB mix: 800 ml 0.1 M K ₂ HPO ₄ buffer and 200ml 0.1 M KH ₂ PO ₄ buffer). Store solution at 4 °C
1% Phosphate Buffer Saline (PBS)	Add 100ml 10% PBS (stock buffer) in 900ml dH ₂ O (adjust pH to 7.4). Store solution at 4 °C
Cryoprotectant Solution	-300gr Sucrose -300gr Ethylen Glycol, -1lt 0.1M PB stir to dissolve and adjust pH to 7.4 Store solution at 4 °C
Tris Buffered Saline (Tris) (stock solution)	-48 gr Tris Base -36 gr Sodium Chloride, -1lt dH ₂ O adjust pH to 7.3
0.1 M Tris	Dilute stock solution 1 in 4 with dH ₂ O adjust pH to 7.3
Gelatinised Subbing Solution	-0.5 gr Chrome Alum (Chromium potassium sulphate) -5 gr Gelatin, 1 lt dH ₂ O warm solution on a heat plate and once dissolved add 0.1% Na Azide
0.1% Triton in PBS	-50µl Triton X100 -50 ml 0.1% PBS

Table 3.8: Summary of antibodies used for WB on my studies.

Primary	Hsp72	Pk-I3	NFκBp65	COX-2
Species	Mouse	Mouse	Mouse	Rabbit
Class	Mono-IgG	Polyclonal	Mono-IgG	Mono-IgG
Manufacture	Assay Designs	Cell signalling	Santa Cruz	Santa Cruz
Concentration	1:1000	1:1000	1:1000	1:1000
Cellular location	Cytoplasm	Nuclear	Nuclear	Cytoplasm
Secondary	HRP- conjugated	HRP-conjugated	HRP- conjugated	HRP- conjugated
Manufacture	Assay Designs	Millipore	Biosciences	Abcam
Concentration	1:1000	1:2000	1:1000	1:1000

12.3 Appendix 3

Video 1: This video illustrates the activation of pro-caspase-1 after stimulation of cortical astrocytes with ATP.



12.4 Appendix 4

Publications and meetings generated during my PhD study.

11.4.1 Original publications

1. **Vizcaychipi M**, Watts HR, O'Dea KP, Lloyd DG, Penn JW, Wan Y, Pac-Soo C, Takata M, Ma D. (2012). The therapeutic potential of atorvastatin on post-operative cognitive decline in mice. *Annals of neurology*. Submitted September 2012.
2. Katsouri L, **Vizcaychipi M**, McArthur S, Harrison I, et al. (2012). Prazocin, an alpha 1-adrenoreceptor antagonist, prevents memory deterioration in the APP23 transgenic mouse model of Alzheimer's disease. *Neurobiology of Aging*. Accepted August 2012 (Reference: NBA-12-103R1).
3. **Vizcaychipi MP**, Lloyd LG, Wan Y, Palazzo MG, Maze M, Ma D. (2011). Xenon Pre-treatment May Prevent Early Memory Decline after Isoflurane Anaesthesia and Surgery in Mice. *Plos One*6(11):e26394. Nov 3.
4. **Vizcaychipi MP**, Xu L, Barreto GE, Ma D, Maze M, Giffard RG. (2011). Heat shock protein 72 overexpression prevents early postoperative memory decline after orthopedic surgery under general anesthesia in mice. *Anesthesiology*114(4):891-900.
5. Wan Y, Xu J, Meng F, Bao Y, Ge Y, Lobo N, **Vizcaychipi MP**, Zhang D, Gentleman SM, Maze M, Ma D. (2010). Cognitive decline following major surgery is associated with gliosis, β -amyloid accumulation, and τ phosphorylation in old mice. *Crit Care Med*38(11):2190-2198.
6. Terrando N, Rei Fidalgo A, **Vizcaychipi M**, Cibelli M, Ma D, Monaco C, Feldmann M, Maze M. (2010). The impact of IL-1 modulation on the development of lipopolysaccharide-induced cognitive dysfunction. *Crit Care*14(3):R88. Epub. May 14.
7. Zhang L, Huang H, Cheng J, Liu J, Zhao H, **Vizcaychipi MP**, Ma D. (2011). Pre-treatment with isoflurane ameliorates renal ischemic-reperfusion injury in mice. *Life Scie*88(25-26):1102-1107. Epub. Apr 30.

8. Rizvi M, Jawad N, Li Y, **Vizcaychipi MP**, Maze M, Ma D. (2010). Effect of noble gases on oxygen and glucose deprived injury in human tubular kidney cells. Exp Biol Med**235**(7):886-91. Epub. May 14.

11.4.3 Review articles

1. Lloyd D, Ma D, **Vizcaychipi M**. (2012). Cognitive decline after anaesthesia and critical care. BJA, Continuing education in Anaesthesia, Intensive care and Pain. June.
2. Fung A, **Vizcaychipi M**, Lloyd D, Wan Y, Ma D. (2012). Central nervous system inflammation in disease related conditions: mechanistic prospects. Brain Res**29**;1446:144-155.
3. Pac-Soo C, Lloyd DG, **Vizcaychipi MP**, Ma D. (2011). Statin: The Role in the Treatment and Prevention of Alzheimer's Neurodegeneration. J Alzheimers Dis. July 6.
4. J Bostock J, **Vizcaychipi MP**. (2011). Delirium in the Intensive Care Unit. European Annals of Delirium**6**; July.

11.4.4 Invited speaker

11.4.4.a International meetings

1. Hungary: October 2012 – An anaesthetic approach to memory decline after surgery.
2. The Netherlands: June 2012 – Postoperative memory loss after anaesthesia and orthopedic surgery does not correlate with hippocampal inflammation in mice. (Presented by Professor R. Giffard as I was working on my PhD thesis amendments).
3. Austria: February 2012 – Delirium and POCD; A pragmatic approach.
4. Chicago: October 2011 - Heat shock protein 72 prevents early postoperative memory decline after surgery.
5. Nanjing: September 2011 – Postoperative memory decline; protecting the brain at risk in non-neurological critical illness.
6. Watsonville, California: October 2009 – Role of heat shock protein 72 on prevention of postoperative cognitive dysfunction.

11.4.4.b National meetings

1. Birmingham UK: December 2011 - Perioperative care; the role of anaesthesia. How we can prevent delirium after surgery: A multidisciplinary approach.
2. London UK: November 2011 - Perioperative care: Highlighting the mechanisms of statin on prevention of memory decline.
3. London UK: July 2011 *Royal Society of medicine*: Brain Failure: Protecting the “at risk” brain in non-neurological diseases in critical illness.
4. London UK: July 2010 - 3rd Perioperative medicine Research Training Conference. Heat shock protein 72 prevents early postoperative memory decline after surgery. St Thomas Hospital.

11.4.4.c Regional meetings

1. Magill Department of Anaesthesia at Chelsea & Westminster Hospital, London UK: February 2010 - An introduction to postoperative memory decline after surgery under general anaesthesia..
2. Kennedy Institute of Rheumatology, London UK: February 2009 - Progress on postoperative memory decline. The role of cytokines.
3. Charing Cross Hospital, London UK: August 2009 - Introduction to postoperative cognitive dysfunction; the role of the anaesthetists.

11.5 Posters (Poster presented by starred authors)

11.5.1 International meetings

1. D Lloyd*, S Spurr, J Li, H Watts, **MP Vizcaychipi**, Z Xie and D Ma. Metabolome changes induced by anaesthetic in an *in vitro* Alzheimer model. ICAD conference July 2011, Paris, France.
2. S.W Penn*, **M.P. Vizcaychipi**, A.R. Fidalgo, C. Pac-Soo, D. Ma. The therapeutic potential of atorvastatin in attenuating POCD after

- unilateral nephrectomy in mice. 23rd ESICM Annual Congress.10 – 13 October 2010. Barcelona, Spain
3. S. Grover*,**M.Vizcaychipi**, L. Zhuang, M. Palazzo, M Maze,D. Ma. Xenon preconditioning attenuates cognitive impairment after orthopaedic surgery under general anaesthesia in mice. Euroanaesthesia. 12 – 15 June 2010. Helsinki, Finland.
 4. Fertleman M*, Mani S, Nurse B, Hobson J, Nanchahal J, **Vizcaychipi M**, Harrison J, Ritchie CW, Feldmann M, Maze M. Effective cognitive assessment in the management of post-operative delirium.13th Congress of the European-Federation-of-Neurological-Societies. 16:199-199, Oct 2009. Florence, Italy.
 5. N. Terrando*, M. Cibelli, A Rei Fidalgo, **M.Vizcaychipi**, D Ma, M. Feldman, M Maze. Immune-to-brain signalling mediates cognitive dysfunction following surgery in mice. Association of University Anesthesiologists (AUA), April 2009. Galveston, USA.
 6. **M Vizcaychipi***,L Xu, X Xiong, Y Ouyang, L Volobueva, J Emery, M Maze, R Giffard. Assessment of hippocampus-dependent memory after tibial fracture on C57BL/6-Wt and Hsp72 over expressing mice. Neuroscience retreat, October 2008. Watsonville, California, USA.
 7. M Cibelli*, D Ma, A Rei Fidalgo, **M Vizcaychipi**, M Maze. Microglial activation in the hippocampus is related to postoperative cognitive dysfunction in Mice. Anesthesiology October 2008, Chicago, USA.

11.5.2 National meetings

1. D Lloyd*, H Watts, **M Vizcaychipi**, and D Ma. Alzheimer's: A problem emerging from Anaesthesia. Meeting for clinician Scientists in Training. Royal College of Physicians, London, UK. February 2011. (Presented by D. Lloyd who won second prize for best oral presentation), London UK.
2. S.W Penn*, **M.P. Vizcaychipi**, C. Pac-Soo, D. Ma. The therapeutic potential of atorvastatin in attenuating POCD after unilateral nephrectomy in mice. Winter scientific meeting of the Anaesthetic Research Society. The Royal

College of Anaesthetists. Nov 2010 (First President's Award for Undergraduate Research), London UK.

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