The Role of Inflammation In Post-Operative Cognitive Dysfunction

A thesis prepared in the Department of Anaesthetics, Pain Medicine & Intensive Care, Imperial College London. Submitted to Imperial College London For the degree of Doctor of Philosophy

By

Mario Cibelli

Supervisors: Prof. M. Maze and Dr. D. Ma
Abstract

Post-operative cognitive dysfunction (POCD) is a common complication occurring mainly to elderly patients after surgery. The highest incidence of POCD is observed in orthopaedic and cardiac procedures. The background of this thesis is based on the evidence that hippocampal interleukin 1 beta (IL-1β) plays a major role in inflammation and memory performance. Localised IL-1β dysregulation in the hippocampus can be triggered by elevated systemic cytokines resulting from peripheral infective challenge; the underlying immune-to-CNS communication triggers glial activation, sickness behaviour and memory impairment. The aim of this work was to investigate a possible causative relationship between anaesthesia, aseptic surgical trauma, systemic and hippocampal inflammation and memory function in mice. The first part of the work was dedicated to the development of an appropriate model of orthopaedic surgery, which allowed me to show that surgery under anaesthesia, but not anaesthesia alone, causes inflammatory-mediated, hippocampal-dependent, cognitive dysfunction. Post-operative elevated plasma cytokines, assessed by ELISA, and clone expansion of inflammatory cells, such as monocytes (evidenced by flow cytometry techniques), were associated with memory impairment, increased IL-1β expression and reactive microgliosis (evidenced by immunohistochemistry), but not astrogliosis (assessed with western blotting), in the hippocampus. Results from behavioural tests such as fear conditioning showed that blocking inflammation with minocycline prevents these post-surgical changes. Likewise, antagonism of IL-1β receptors mitigated hippocampal-dependent memory dysfunction. Experiments to rule out a possible infection were also performed, and excluded that the results could
depend upon contamination of the wounds in the animal model. Also, age and gender were explored as possible risk factors. These findings support the existence of a surgery-induced IL-1β-mediated inflammatory mechanism that is followed by reactive microgliosis in the hippocampus and underlies memory impairment. This mechanism appears to be dependent on gender and can be attenuated by some anaesthetics, but it is worse in older age.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Table of contents</td>
<td>3</td>
</tr>
<tr>
<td>Table of figures</td>
<td>4</td>
</tr>
<tr>
<td>List of acronyms</td>
<td>9</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>11</td>
</tr>
<tr>
<td>1. Introduction (including aims and hypothesis)</td>
<td>13</td>
</tr>
<tr>
<td>2. General methods</td>
<td>66</td>
</tr>
<tr>
<td>3. Preliminary experiments leading to the development of a surgical</td>
<td>93</td>
</tr>
<tr>
<td>model for the study of Post-Operative Cognitive Dysfunction</td>
<td></td>
</tr>
<tr>
<td>4. Systemic and hippocampal IL-1β-mediated inflammation underlie</td>
<td>124</td>
</tr>
<tr>
<td>cognitive dysfunction following surgery</td>
<td></td>
</tr>
<tr>
<td>5. Lipopolysaccharide-induced hippocampal inflammation and</td>
<td>164</td>
</tr>
<tr>
<td>cognitive dysfunction are attenuated by isoflurane</td>
<td></td>
</tr>
<tr>
<td>6. Effect of Inflammation, Age, Gender and Estrogens on Post-Operative</td>
<td>187</td>
</tr>
<tr>
<td>Cognitive Dysfunction</td>
<td></td>
</tr>
<tr>
<td>7. General discussion and conclusions</td>
<td>220</td>
</tr>
<tr>
<td>8. References</td>
<td>235</td>
</tr>
<tr>
<td>9. Appendix: Manuscripts, articles and presentations</td>
<td>258</td>
</tr>
</tbody>
</table>
# Table of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Caption</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>The first publication about Post-Operative Cognitive Dysfunction by George H Savage.</td>
<td>17</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Incidence of POCD in middle-aged and elderly patients.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Morphological changes to microglial cells during the transition from a resting to an activated state.</td>
<td>56</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>A putative inflammatory and neuroinflammatory pathogenic mechanism leading to POCD.</td>
<td>61</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Diagram of the role of microglial priming in aged animals in exaggerated neuroinflammation and cognitive impairment.</td>
<td>64</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Insertion site of intra-medullary stainless steel fixation wire.</td>
<td>69</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Conditioning chamber.</td>
<td>71</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Diagramatic representation of the primary brain circuit for contextual fear.</td>
<td>74</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Simplified schematic of the neural circuitry involved in fear conditioning.</td>
<td>75</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>The fear trace conditioning testing protocol.</td>
<td>76</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Schematic diagram illustrating Immunohistochemical labelling using a fluorophore-conjugated secondary antibody.</td>
<td>81</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>Schematic diagram illustrating immunohistochemical labelling using the avidin-biotin peroxidase (ABC) technique with a biotinylated secondary antibody.</td>
<td>83</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>Scoring of glial activation visualised by CD11-b staining with the DAB technique in the hippocampus of</td>
<td>84</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.9</td>
<td>Schematic diagram illustrating the detection of target protein using the sandwich ELISA technique.</td>
<td>89</td>
</tr>
<tr>
<td>3.1</td>
<td>Schematic representation of the training session for the trace fear conditioning protocol.</td>
<td>103</td>
</tr>
<tr>
<td>3.2</td>
<td>Schematic representation of the context test after trace fear conditioning.</td>
<td>104</td>
</tr>
<tr>
<td>3.3</td>
<td>Schematic representation of the acoustic-cued test following trace fear conditioning.</td>
<td>104</td>
</tr>
<tr>
<td>3.4</td>
<td>Western blotting to measure the time course of GFAP expression in the hippocampus of rats undergoing laparotomy.</td>
<td>106</td>
</tr>
<tr>
<td>3.5</td>
<td>Western blotting analysis of GFAP in the hippocampus of rats 24 hours after general anaesthesia (GA) alone, or laparotomy under GA, or orthopaedic surgery under GA.</td>
<td>108</td>
</tr>
<tr>
<td>3.6</td>
<td>Freezing levels in the acquisition phase of trace fear conditioning (during tone-to-tone inter-trial intervals) are impaired at the 6th trial, when trained 1 day after tibial surgery, but not at 3 and 7 days after surgery.</td>
<td>110</td>
</tr>
<tr>
<td>3.7</td>
<td>Surgery of the tibia caused an impairment of memory functions assessed by the contextual fear test in animals trained up to three days after surgery. Treatment of minocycline mitigates the negative effects of surgery on memory function in this test.</td>
<td>112</td>
</tr>
<tr>
<td>3.8</td>
<td>Recall of acoustic-cued memories is not impaired after tibial surgery or anaesthesia with trace fear conditioning.</td>
<td>114</td>
</tr>
<tr>
<td>3.9</td>
<td>GFAP immunoreactivity of astroglia in the hippocampus.</td>
<td>116</td>
</tr>
<tr>
<td>3.10</td>
<td>Pilot experiments: dose response effect of LPS i.p. injection on microglial activation as assessed by</td>
<td>117</td>
</tr>
</tbody>
</table>
grading of CD11b staining in the mouse hippocampus 24 hours after injection (A) and time course of hippocampal microglial activation following surgery (B).

| Figure 3.11 | Activation of microglia detected with immunoreactivity for CD11b in the hippocampus. |
| Figure 4.1 | Schematic representation of the training session for the delay fear conditioning protocol. |
| Figure 4.2 | Schematic representation of the contextual test after delay or trace fear conditioning. |
| Figure 4.3 | Schematic representation of the acoustic-cued test following delay or trace fear conditioning. |
| Figure 4.4 | Surgery-induced systemic inflammation is associated with increased expression of hippocampal IL-1β and this is reduced by minocycline treatment. |
| Figure 4.5 | Immunohistochemistry of microglia with anti-CD11b (A-D). Median (horizontal bar) with 25th to 75th (box) and 10th to 90th (whiskers) percentiles for immunohistochemical grading (0-3) of microglia (E-G). |
| Figure 4.6 | Flow cytometric analysis of monocyte subsets in the lungs of mice undergoing surgery or in naive animals. |
| Figure 4.7 | Time course of the Gr-1 high subset of monocyte proliferation in the blood and their margination to the lungs, as measured by flow cytometry. |
| Figure 4.8 | Flow cytometric data demonstrating the time course of polymorphonuclear cell (PMN) proliferation in the blood and their margination to the lungs after surgery. |
| Figure 4.9 | Time course analysis of cell-mediated systemic inflammation following general anaesthesia (GA) alone or surgery under GA assessed by flow cytometry. |
| Figure 4.10 | Time course of inflammatory-mediated margination of cells to the lung microcirculation in response to general anaesthesia. |
anaesthesia (GA) alone or surgery under GA.

**Figure 4.11** Surgery does not cause proliferation of Iba1-labelled microglia when assessed 24 hours after the operation.

**Figure 4.12** Hippocampal-dependent recall of fear memories is impaired after surgery.

**Figure 4.13** Surgery-induced impairment of contextual fear memories is prevented by pre-emptive administration of an IL-1 receptor antagonist (IL-1ra).

**Figure 4.14** Recall of contextual and auditory-cued memories is not affected if surgery is delayed three days after delay fear conditioning.

**Figure 4.15** Recall of contextual and auditory-cued memories is not affected if surgery is delayed 24 hours after delay fear conditioning.

**Figure 5.1** Isoflurane anaesthesia reduces plasma cytokine levels in mice injected with lipopolysaccharide (LPS).

**Figure 5.2** Isoflurane protects against LPS-induced increases in hippocampal IL-1β levels.

**Figure 5.3** LPS-induced microglial activation in the hippocampus, visualised by CD11-b immunohistochemistry, is not reduced by isoflurane anaesthesia.

**Figure 5.4** LPS-induced impairment of contextual fear memories is prevented by isoflurane anaesthesia.

**Figure 5.5** Freezing in the auditory-cued test after delay fear conditioning.

**Figure 6.1** Pattern of the 4-day oestrus cycle in the female adult mouse.

**Figure 6.2** Photomicrographs of counterstained vaginal smear samples from adult female mice.

**Figure 6.3** Plasma levels of IL-6 were increased in young female mice 6 hours after surgery.
| Figure 6.4 | Plasma levels of IL-1β in young female mice were not changed after surgery. | 199 |
| Figure 6.5 | Plasma concentration of TNF-α in young female mice is not affected by either surgery or anaesthesia. | 200 |
| Figure 6.6 | Concentrations of hippocampal IL-1β remain unchanged in young female mice after surgery. | 201 |
| Figure 6.7 | Immunohistochemistry of CD11b-stained hippocampal microglia in young adult female mice: naive and after surgery. | 201 |
| Figure 6.8 | Contextual fear and auditory-cued fear memories are not disrupted by surgery in young female mice. | 203 |
| Figure 6.9 | Surgery under anaesthesia, but not anaesthesia alone induces increased plasma levels of IL-1β in old female animals. Treatment with 17β-estradiol attenuates the effect of surgery on IL-1β plasma concentrations. | 206 |
| Figure 6.10 | Surgery under anaesthesia, but not anaesthesia alone, triggers increased plasma concentrations of IL-6 in old female animals. Administration of 17β-estradiol attenuates the effect of surgery on plasma IL-6 levels. | 207 |
| Figure 6.11 | Plasma levels of TNF-α are increased above naive animals in old female mice at 6 hours after surgery but not after 17β-estradiol treatment. | 208 |
| Figure 6.12 | Surgery is associated with increased concentrations of hippocampal IL-1β in old female mice. Surgery-induced increases in hippocampal IL-1β are reduced by oestrogens. | 209 |
| Figure 6.13 | Photomicrographs of microglia stained with CD11b in hippocampal tissue of old female mice. | 212 |
| Figure 6.13.1 | Levels of microglial activation in old female mice | 214 |
| Figure 6.14 | Memory for context is not affected after anaesthesia, but is impaired after surgery in old female mice | 216 |
List of acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin-biotin peroxidase</td>
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<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation molecule</td>
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<tr>
<td>CR</td>
<td>Conditional Response</td>
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<td>CS</td>
<td>Conditional Stimulus</td>
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<tr>
<td>CSF</td>
<td>Cerebro-Spinal Fluid</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidinetetrahydrochloride</td>
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<td>DAMP</td>
<td>Danger associated molecular patterns</td>
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<td>dB</td>
<td>Decibel</td>
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<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
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<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<td>FITC</td>
<td>Fluorescein-Isothiocyanate</td>
</tr>
<tr>
<td>GA</td>
<td>General anaesthesia</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GLT-1</td>
<td>Glutamate Transporter-1</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High molecular group box 1</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Iba-1</td>
<td>Ionized calcium-binding adaptor molecule-1</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1beta</td>
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<tr>
<td>IL-1Ra</td>
<td>Interleukin-1 receptor antagonist</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
</tbody>
</table>

conditioned with a delay fear paradigm. Administration of 17β-estradiol does not exert any effect on surgery-induced, hippocampal-dependent memory dysfunction.

Figure 6.15 Acoustic-cued fear memory is not impaired after surgery in old female mice trained with delay fear conditioning.
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRF-1</td>
<td>Interferon regulatory factor-1</td>
</tr>
<tr>
<td>ISPOCD</td>
<td>International Study on Post-Operative Cognitive Dysfunction</td>
</tr>
<tr>
<td>kHz</td>
<td>kilo-Hertz</td>
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<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTP</td>
<td>Long Term Potentiation</td>
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<tr>
<td>mA</td>
<td>milli-Ampere</td>
</tr>
<tr>
<td>MAC</td>
<td>Minimal Alveolar Concentration</td>
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<tr>
<td>Mac-1</td>
<td>Membrane Attack Complex-1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMSE</td>
<td>Mini mental state examination</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>OP-CABG</td>
<td>Off Pump-Coronary Artery Bypass Graft</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear cells</td>
</tr>
<tr>
<td>POCD</td>
<td>Post-Operative Cognitive Dysfunction</td>
</tr>
<tr>
<td>POD</td>
<td>Post-Operative Delirium</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TFC</td>
<td>Trace Fear Conditioning</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue-type plasminogen activator</td>
</tr>
<tr>
<td>US</td>
<td>Unconditional Stimulus</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WAIS</td>
<td>Wechsler Adult Intelligence Scale.</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blotting</td>
</tr>
</tbody>
</table>
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A mia moglie Isobel,
a cui devo ogni istante di gioia nella mia vita.
È a nostro figlio John.
Chapter One

Introduction

Contents

1.1 Overview 14
1.2 Historical background 16
1.3 Classification and diagnosis 23
  1.3.1 Post-operative delirium 23
  1.3.2 Post-operative cognitive dysfunction 25
1.4 Prevention 34
  1.4.1 Interventions and long-term studies 36
1.5 Epidemiology 34
1.6 Risk factors 37
1.7 Pathogenesis 40
  1.7.1 Anaesthetics 40
  1.7.2 Inflammation 46
    1.7.2.1 The immune system 48
    1.7.2.2 Neuroinflammation 49
    1.7.2.3 Immune system to brain communication and the effect of neuroinflammation on behaviour 53
    1.7.2.4 Neuroinflammatory consequences of ageing 57
1.8 Aims and hypothesis 60
1.1 OVERVIEW
Ageing governs the processes of life. Nothing in our body, from organs to cells and from vision to thought is ever spared from its ruthless progression.

The ageing process is very variable amongst different species, individuals, organs and cell types. Such variability depends on many factors, including the many diseases one experiences in life. The brain does not escape this rule. In fact, the vulnerability of the brain to noxious external stimuli increases during ageing; a process that can negatively affect both length and quality of life (Iadecola et al., 2009; Sparkman et al., 2008).

Nowadays, an increasing number of ageing individuals are referred for surgery when symptoms of their illness become so disabling as to interfere with everyday functioning. Continuing improvements in surgical technology and anaesthetic care have increased the availability of an expanding variety of surgical solutions to patients at the extremes of age and disability (Shugarman et al., 2009). Unfortunately, this group of patients has the greatest risk of developing post-operative neurological impairment (Moller et al., 1998). Sadly, these patients have exchanged one circumstance (i.e., pain and immobility) for another (the potential inability to cope independently) leaving many with an overall decrease in quality of life and longevity.

Cognition describes the mental activity involved in attention, perception and memory. ‘Post-operative cognitive dysfunction’ (POCD) describes an impairment of these fundamental functions, which manifests itself after a surgical procedure. POCD is a complex syndrome characterized by variable disturbances of attention, perception, memory, thought, information processing, and the sleep-wake cycle that frequently
develops in elderly patients after a surgical procedure (Rasmussen, 1999). It has long been recognised that many patients suffer a cognitive impairment after surgery (Savage, 1887; Bedford, 1955). In the last few years, clinicians, such as anaesthetists, geriatrists and intensive care physicians, have increasingly been interested and involved in the study of POCD (Moller et al., 1998). In the surgical cardiac and orthopaedic settings, POCD is amongst the most prevalent and challenging of clinical problems (Marcantonio et al., 2001; Newman and Stygall, 2000). Unfortunately, systematic enquiries into the nature and cause of this disease have been undertaken only in recent times, prompted by the increasing number of surgical procedures being performed on the elderly, who are the population most at risk of this disease (van Dijk et al., 2000; Newman et al., 2007; Newman and Stygall, 2000).

The most significant risk factor for the development of POCD is age. Eternal life has always been a utopia for human beings. Although it seems to be unobtainable, technological development and innovations in health care have made healthy ageing a reality for many. Alzheimer’s disease and neurological syndromes such as POCD are undermining our endless quest for successful ageing. As part of this endeavour, we need to understand the mechanisms underlying neurological deterioration and cognitive dysfunction.

The primary aims of this study are to explore the links between orthopaedic surgical procedures and inflammation of brain regions leading to cognitive disability with view to gaining an insight into the condition of POCD.
1.2 HISTORICAL BACKGROUND

Delirium is an ancient disease. The word is derived from the Latin term meaning ‘off the track’. Descriptions of similar mental disorders had been mentioned by Hippocrates (400 B.C., Aphorisms, section VII, n. XVIII). However, the first description of delirium had been made by Celsus in the first century A.D.

Hippocrates considered the development of delirium as a consequence of a disease, often following fever, dyspnoea or meningitis. He pointed out how psychomotor hyperactivity, impaired sleep-wake pattern, inability to recognize known people and difficulty in completing easy tasks, were the most common characteristics of people affected and could be often considered as fatal signs (Aphorisms, section IV, n. L).

A pioneer in the study of cognitive dysfunction following surgery was George H Savage, medical superintendent, resident physician and lecturer in mental diseases at Guy’s Hospital, London. In his work (Figure 1.1), entitled ‘Insanity Following the Use of Anaesthetics in Operations’ published by The British Medical Journal in 1887, he described ‘a series of cases of insanity in which the use of anaesthetics, in predisposed subjects, has been followed by insanity’. His remarkable description of the patients, their history and particularly, their symptoms, is a milestone in the field and represents a source of information to understand the involvement at different degrees of the anaesthetics and surgery (Savage, 1887).
Figure 1.1

The first publication about Post-Operative Cognitive Dysfunction by George H Savage published in the British Medical Journal, 3 December 1887 (p1190).

George H Savage wrote 'in the above paper I have thrown together the experience of many years; and, though the time at my disposal will not allow of my placing on
record all the cases in their order, I trust that enough evidence has been brought forward to induce others to give their experience, and thus establish a relation or destroy a fallacy’.

Savage described the possible consequences, on cognition, of interacting variables such as medications and several predisposing factors (Savage, 1887). He argued that, similarly to the symptoms of alcoholic encephalopathy, the insanity that follows an operation could be iatrogenic in origin. The administration of chloroform, ether or belladonna, which were commonly used alone or combined to induce anaesthesia, resulted in a state of mania, or delirium, or forgetfulness, especially in people with a past medical history of risk factors that he indicated as possibly related to the condition. Among these, a previous mental condition, or a disease (such as rheumatic fever and TB), or, more generally, an infection, had a preeminent role in facilitating the appearance of post-operative delirium after administration of several xenobiotics, particularly, anaesthetics. He also pointed out that many of these cases were related to elderly patients, and that the risk factors would be important, as it was the case of young subjects. The form of delirium that the author described in the article seems to be often preceded by the development of other forms of cognitive instability, such as mania, and that, overall, it was self-limiting and short lasting (several weeks was the average duration).

The following description of some of the proposed cases is fascinating.
One of these patients just recovered from a maniac attack, when delirium immediately broke in after chloroform was administered as the anaesthetic for the surgical treatment of a very painful swelling of one hand.

Another was the case of a young woman with anamnesis for previous neurosis. This patient developed depression and serious mania after being exposed to chloroform, when she was supposed to undergo a lower limb operation. The case is made more interesting by the fact that surgery was deferred. As a consequence, the patient was exposed to the anaesthetic, but did not receive any surgery. Further, in an attempt to give a complete description of the case, Savage reported on the impressions of the patient’s friends. This presented the possible scenario that depression, at least, could be related to the chagrin consequence of an impairing illness, which was not possible to treat.

Age as a risk factor was presented in the case of an old man undergoing surgery for treatment of colon cancer under ether anaesthesia. Immediately after surgery, the patient was noticed to be like half-drunk or in a state resembling an incomplete recovery from the anaesthetics. Subsequently, he worsened to a condition of weakness in his body and his mind. He was restless, incoherent, repeating meaningless expressions. His memory seemed very defective. At discharge, the patient was defined as a ‘harmless dement’. Several weeks later, when he returned for further assessment, Savage admitted that he ‘failed at first to recognize in the bright, intelligent clergyman, the dull dement who had so recently left this hospital’ due to such sudden and unexpected recovery.
Other anaesthetics were found to be harmful, as in the case of a woman who became seriously delirious after she was given nitrous oxide, for a tooth extraction.

Many cases are also noted due to an unexplainable relationship between ‘ovariotomy’, as defined in 1887, and delirium. In cases like these, the author noted how nobody suffered an immediate development of the symptoms after exposure to the anaesthetics. He argued that, in cases like ovariectomies, more than a single factor concurred to the causation of the disorder.

A common feature in his patients was the often \textit{restitutio ad integrum} after not long delirium became apparent. However, the author also referred to several other cases in which, after the operation, ‘the progressive weakness of mind has been associated with progressive weakness of body, so that the patients had in the end died of a disease not to be distinguished from general paralysis of the insane’.

A reported example, which serves as a prototype is that of a single girl, aged 30, with a past medical history of fainting fits lasting several months who developed memory loss and behavioural changes after an operation for the removal of a tumour from the breast. Following surgery, the patient suffered a neurological condition, which further worsened with tremors, hesitation in speech, unequal pupils. Five months later the woman died in the hospital.

In conclusion, Savage proposed to further investigate, first, possible risk factors, such as neurosis or previous attacks of insanity and, second, but not less importantly, the
balancing of risk-benefit, for operations that are not essential for either prolonging or saving a life.

In 1955, Bedford opened a debate on the pathogenesis of the disease, which had theretofore been assumed to be a complication of the anaesthesia employed in surgery, rather than the surgery itself or any other factor (Bedford, 1955).

His remarkable research effort consisted in scrutinising the records of 4250 patients over the age of 65 seen in the Oxford Geriatric Unit at Cowley Road Hospital in the five years ending in June, 1954. Of these patients, 1193 had undergone some operation under general anaesthesia within the previous fifteen years (meaning they could have been 50 years old or older at the time of the operation).

He found that, in more than one third (410) of these cases, the near relations or friends alleged that the patient ‘had never been the same since operation’. A subsequent further elaboration of the data in his possession suggested that the above statement was likely to be unjustified in 290 cases (70%), and that there were nevertheless 120 records left containing elements of robustness to justify the enterprise of further study.

Bedford restricted his interest to a group of 29 patients with a very serious impairment of cognition (defined as ‘extreme dementia’) and, of these, he considered those 18 who underwent surgery under his supervision.

From 1955 to 1967 Bedford, Simpson and Blundell debated as to whether anaesthesia was the removable cause of post-operative impairment of cognition in the elderly.
Despite the great interest these studies had aroused in the scientific community, they lacked control groups. Moreover, the testing protocols and the data provided in these studies were insufficient (Bedford, 1955; Simpson et al., 1961; Blundell, 1967).

Further studies have sought to identify pathogenic mechanisms due to perioperative factors such as general anaesthesia, hyperventilation, hypotension, hypoxia, benzodiazepine use and/or alcohol withdrawal, as well as patient-related factors, including polymorphisms and co-morbidities such as underlying neurovascular/neurodegenerative disease and cancer (Heyer et al., 2005; Newman et al., 2007). However, these studies also lacked appropriate controls and did not use a similar battery of tests for clinical assessment. It was thus not possible to prioritise, or even to establish the contribution of these factors for the development of POCD.

The International Study of Postoperative Cognitive Dysfunction (ISPOCD) involved more than 1200 elderly patients (aged 65 or more) undergoing major non-cardiac surgery with general anaesthesia. It included a control group and investigated the role of many of the putative risk factors identified from the other studies. The most significant risk factors associated with POCD were advanced age, duration of surgery, post-operative respiratory and/or infectious complications and a second operation (Moller et al., 1998).

Although a long series of studies has so far been performed to improve our knowledge about post-operative cognitive impairment (the flow chart in table 1.1 shows the appearance of the landmark papers in chronological order), the study of POCD is still
in its infancy, with much research still to be undertaken. Many more investigations will be required to further clarify the multiple aspects involved in this condition.

Table 1.1 Historical flow chart of the milestones in POCD research from 1887.
1.3 CLASSIFICATION AND DIAGNOSIS

Although a real consensus on classification criteria is not clear in the literature, the syndrome characterised by cognitive dysfunction developing after surgery has often been divided into two different entities, termed post-operative delirium and POCD.

1.3.1 Post-Operative Delirium

Post-operative delirium (POD or more simply ‘delirium’) arises in the first two days after an operation, often after an interval of apparent cognitive function integrity (Lipowski, 1990); and usually fades within one week. It can be divided into two main subtypes, ‘hyperalert-hyperactive’ and ‘hypoalert-hypoactive’, depending upon the level of alertness.

In the ‘hyperalert-hyperactive’ subtype, psychomotor and sympathetic nervous system over-activity with mood and cognition disturbances are prominent. Although the patients are often described as simply ‘confused’, the consequences of their changeable behaviour could be serious. Disorientation, increased psychomotor activity, anxiety, fear, nervousness and increased responsiveness to stimuli may often cause the patient to self harm or to take out intravenous catheters, central lines and monitoring equipment. Such actions make it difficult for the nursing staff to attend, post-operatively treat and follow up these patients. Episodes of aggression to the doctors and the nurses are not unusual. If the patient is intubated and connected to the ventilator, as in cardiac or non cardiac intensive care, the consequences of self-extubation may be catastrophic.
Although several tests can be useful to make an accurate diagnosis, very often the patients are not co-operative enough to accomplish the task. Nevertheless, the unexpected change in the patient’s behaviour makes the hyperalert-hyperactive delirium easy to diagnose from its early manifestations.

In the ‘hypoalert-hypoactive’ or ‘quiet’ subtype, decreased responsiveness to stimuli and lethargy (often associated with impaired cognition and memory disturbances) are the most common features. These patients are described as detached from the reality and uninterested in events happening around them. In an interesting subjective report on an experience of general anaesthesia, the patient reported a compromise in alertness and responsiveness. Nothing seemed to matter to her: nursing care requiring co-operation were perceived as important and yet neglected; even pain evoked by an injection was not recognised as aversive (Pockett, 1999).

The diagnosis of quiet delirium is far more difficult. The patient seems to be dormant and, in the absence of an accurate assessment by the doctor, this condition could easily be missed. The incidence and prevalence of hypoalert-hypoactive delirium are, therefore, largely underestimated, despite being the cause of more severe impairment and longer hospitalisation than the hyperalert-hyperactive form (Lipitzin and Levkoff, 1992).

Sometimes delirium may present as a mixed, unspecified form, with unexpected swings from one subtype to the other (Lipitzin and Levkoff, 1992).
Another form of delirium, termed ‘emergence delirium’, has been proposed. It is described when the onset of delirium occurs within the first 24 hours of a surgical procedure (Lipowski, 1990).

Many other terms have been used in the past to describe delirium, such as ‘acute confusional state’, ‘acute brain failure’, ‘organic brain syndrome’ and so forth (Lishman, 1987). Furthermore, a large variety of protocols and guidelines in the literature has made the recognition and classification of delirium rather difficult. However, the most recent text-revised version of the Diagnostic and Statistical Manual of Mental Disorders, DSM IV-TR (American Psychiatric Association, 2000) and the latest International Classification of Diseases, ICD-10, include delirium. They suggest several broadly similar criteria for its recognition. These include: disturbance of consciousness and attention; change in cognition; psychomotor and emotional disturbances; sleep/wake cycle impairment; and early onset. The Royal College of Physicians and the British Geriatric Society have recently published an update of comprehensive evidence-based guidelines 
(www.rcplondon.ac.uk/publications/Pages/Reports-and-guidelines.aspx;  
www.bgs.org.uk/Publications/Clinical Guidelines/clinical_1-2_full-delirium.htm)

In general, a diagnosis of delirium is the result of an accurate clinical assessment obtained mainly by using cognitive questionnaires and blood tests.

1.3.2 Post-operative cognitive dysfunction

Post-operative cognitive dysfunction, or ‘mild neurocognitive disorder’, or more simply POCD, is often diagnosable only weeks or even several months after the operation (early or late POCD, respectively). POCD may be progressive and long
lasting; is associated with longer hospital stay, higher mortality and may even be precursor of subsequent development of dementia (Rockwood et al., 1999).

The North American Diagnostic and Statistic Manual of Mental Disorders, Fourth Edition (DSM-IV) does not reference POCD directly, yet in several cases, prolonged POCD may resemble the more severe impairment, termed ‘dementia’.

Rasmussen has regarded late POCD as a mild neurocognitive disorder, the definition given by the DSM-IV, he describes it as follows: ‘mild neurocognitive disorder is one of the cognitive disorders not otherwise specified and for which research criteria have been suggested. This presentation is characterized by cognitive dysfunction presumed to be caused by either a general medical condition or substance use that does not meet the criteria for any of the other disorders. An essential feature is that the impairment in cognitive functioning is evidenced by neuropsychological testing’ (Rasmussen, 1999).

In common with the hypoalert-hypoactive subtype of delirium, the detection of prolonged POCD is strictly dependent on neuropsychological testing. It may be extremely difficult to diagnose POCD according to widely accepted testing criteria, as these have yet to be well defined by the scientific community. Amongst the published literature, there is considerable variation in the nature of neurological tests, the timing of testing, and the definition of the commonest psychological deficits in POCD. The inadequacy of several tests makes the standardisation of the results even more difficult. The mini mental state examination (MMSE), for example, was designed by Folstein in 1975. It is commonly used by clinicians to detect dementia by examining
orientation, learning skills, attention, language and visual construction. According to
the reported guidelines included in the article, the MMSE takes approximately 10
minutes to administer, which is the reason for the popularity of the test. The adopted
system of scoring becomes predictive of intact cognitive functions when the overall
performance of the patient ranges between 28 and 30. Unfortunately, the minimal
level of cognitive dysfunction recognized by the MMSE is inadequate for POCD, due
to low sensitivity of a test basically designed for recognition of full scale dementia,
which is, in many cases, far more disabling than POCD (Folstein et al., 1975). This
makes the MMSE, as generally accepted, not appropriate for the diagnosis of POCD.

The ‘clock drawing test’ is used for the assessment of some cognitive functions such
as orientation, conceptualization of time, visual spatial organization (Lam et al., 1998)
memory and executive function (Estaban-Santillan et al., 1998), auditory
comprehension, visual memory, motor programming, numerical knowledge, semantic
instruction, inhibition of distracting stimuli, concentration and frustration tolerance
(Shulman, 2000). The patient is asked to draw the figure of a clock, including all the
numbers, before setting the hands to 11:10 hours. Many other clock-times have been
used including, 3:00, 8:40, 2:45 and so on. The patient needs first to conceptualize the
various features of a clock. Importantly, the patient has to overcome the possible
confusion generated by the number 10 (minutes past 11) while drawing the hand on
the number 2. The usefulness of this test, as many others, resides in the possibility to
assess the patient both before and after surgery, allowing for a diagnosis of POCD, on
the basis of a clear scoring system (Libon et al., 1996). Generally, there is no time
limit to the test, which usually takes only one to two minutes. Amongst the many
different ways to score this test, a simple version is reported in table 1.2.
Paragraph or story memory tests involve reading a paragraph from an article or a story, requiring the patient to repeat the story immediately. After some set time delay, the patient is asked to report the story again and possibly once again (Wechsler, 1997).

Another group of tests, named list-learning tests, involves learning and retaining a list of words. This test has the advantage to be possibly graded in the level of difficulties, to be flexibly tailored to the needs of each single case, for example, depending on the age of the patient (Brandt, 1991).

The subtleness of the symptoms, especially in the initial phase of the development of POCD, makes any of the aforementioned tests insufficient for an appropriate diagnosis. For this reason, a battery of some sensitive tests, rather than a single test, should be considered when assessment for POCD is carried out. A list of the most widely used tests, including the useful ones described above are presented in table 1.3.

The onset of POCD is generally very subtle, in fact. An initial impairment of short-term memory and a certain degree of limitation in the completion of simple tasks tends to go generally unnoticed. Mostly, the patient’s closest relatives are the first to notice a change in the memory, skills and behaviour of the patient. The patient may subsequently be frustrated by a ‘senior moment’ that manifests as a momentary loss of memory as can happen when one has begun an action having forgotten why it was started. Simple tasks easily carried out before the surgery, such as crosswords, may be perceived as impossible, thereafter. Along with the social isolation that accompanies
cognitive dysfunction, the patient may have to give up work. The line ‘Granny has never been the same since her operation’ is becoming increasingly common, especially after a major intervention.

1.3.3. POD vs POCD

The general syndrome that features cognitive deterioration after a surgical intervention can be classified into two main categories: delirium, which may be short-lived, often lasting from days to a few weeks, and POCD, which may last for an indefinite period of time, often detectable only days to weeks after surgery. Delirium is an acute confusional state with alterations in attention and consciousness. In contrast, POCD is characterised by changes in one or more neuropsychological domains and is often more subtle than delirium. Unlike delirium, POCD is not associated with changes in level of consciousness and it does not fluctuate over the course of the day. Due to the subtle nature of POCD, neuropsychological testing is necessary for its detection. While delirium can assume the facet of a clear clinical syndrome, which may be easily detectable by the clinician as in the hyperalert-hyperactive form, the diagnosis of POCD is not based on the subjective symptoms alone, and no standalone suitable questionnaire has yet been developed for this condition. The neuropsychological battery of tests, according to research, must be administered both before and after surgery to maximize sensitivity in the assessment of cognitive function. Pre-operative medical condition, cognitive status, psychological factors, and age may predispose to delirium. The precipitating factors may include polypharmacological treatments, metabolic disorders, cerebral hypoxia and cardiopulmonary bypass. Primary prevention is more effective than intervention after delirium develops. Multicomponent interventions that target well-documented risk
factors for neurocognitive complications can help in preventing the development of post-operative delirium, whereas no effective preventative measures have been documented to be helpful in POCD.

The evidence that will be shown in this thesis and some coming from previous research suggest that surgery and perioperative interventions can cause POCD. The incidence of POCD seems to be independent of the anaesthetic technique, is higher after cardiac than non-cardiac surgery, and increases with age. Other risk factors implicated in the development of the POCD include low base-line cognitive functioning, post-operative infection, microemboli and hypoperfusion during cardiac surgery. The pathophysiology of POCD is poorly understood and there is no clear strategy for preventing this complication at the present time.

Likewise, resolution of symptoms of postoperative delirium may take longer in patients with poor preoperative cognitive function. For some patients, the cognitive effects of delirium may resolve slowly or not at all, but most of the patients with postoperative delirium have good outcomes, with only a slight delay in some segments of cognition, except when the syndrome evolves to POCD in which case prognosis may worsen. Research aimed at unveiling the pathogenesis of these two entities needs to be warranted to ultimately find the mechanisms that could be targeted by either preventative or pharmacological treatments.
<table>
<thead>
<tr>
<th>Score</th>
<th>Description of Clock</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Correct time with normal spacing.</td>
</tr>
<tr>
<td>1</td>
<td>Slight impairment in spacing of lines or numbers.</td>
</tr>
<tr>
<td>2</td>
<td>Noticeable impairment in line spacing.</td>
</tr>
<tr>
<td>3</td>
<td>Incorrect spacing between numbers with subsequent inappropriate denotation of time.</td>
</tr>
<tr>
<td>4</td>
<td>Obvious errors in time denotation (arms misplaced, numbers in wrong place)</td>
</tr>
<tr>
<td>5</td>
<td>Abnormal clock-face drawing with inaccurate time denotation (eg. reversal of numbers, perseveration beyond twelve, misplaced numbers, drawing only to one side, omitting most numbers)</td>
</tr>
<tr>
<td>6</td>
<td>Abnormal clock face drawing with inaccurate time denotation (eg reversal of numbers, perseveration beyond twelve, misplaced numbers and drawing to one side and omitting most numbers).</td>
</tr>
<tr>
<td>7</td>
<td>A recognizable attempt to draw a clock face but no clear denotation of time.</td>
</tr>
<tr>
<td>8</td>
<td>Some evidence that a clock face is drawn.</td>
</tr>
<tr>
<td>9</td>
<td>Minimal evidence that a clock face is drawn.</td>
</tr>
<tr>
<td>10</td>
<td>No reasonable attempt to drawing a clock face (exclude gross visual disturbance, hemiplegia and severe psychotics state).</td>
</tr>
</tbody>
</table>

Table 1.2. Scoring criteria for the ‘clock drawing test’. Source from Lam et al, 1998.
<table>
<thead>
<tr>
<th>Tests used in cognitive battery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Addition</td>
</tr>
<tr>
<td>2. Benton visual recognition</td>
</tr>
<tr>
<td>5. Card sorting</td>
</tr>
<tr>
<td>6. Clock drawing test</td>
</tr>
<tr>
<td>7. Controlled oral word association test</td>
</tr>
<tr>
<td>8. Delayed free recall</td>
</tr>
<tr>
<td>9. Delayed recognition</td>
</tr>
<tr>
<td>10. Digit copying test (DCT)</td>
</tr>
<tr>
<td>11. Digit span backwards</td>
</tr>
<tr>
<td>12. Digit span forwards</td>
</tr>
<tr>
<td>13. Digit symbol from WAIS</td>
</tr>
<tr>
<td>14. Finger oscillation test</td>
</tr>
<tr>
<td>15. Fluid object memory test</td>
</tr>
<tr>
<td>17. Immediate free recall</td>
</tr>
<tr>
<td>20. Mattis Kovner verbal recognition</td>
</tr>
<tr>
<td>21. Object learning test</td>
</tr>
</tbody>
</table>

*Table 1.3.* List of batteries of tests broadly used to diagnose POCD. WAIS, Wechsler Adult Intelligence Scale.
1.4 PREVENTION

It is possible in some cases, to prevent the development of POD. Many studies have considered early recognition of the risk factors, effectiveness of various therapies and special training of the nursing staff, in an attempt to prevent the disorder from occurring.

Marcantonio and colleagues have managed to reduce the overall incidence of post-operative delirium occurring after hip surgery by over one third, by simply improving the pre-operative assessment of the patients with a better recognition of the risk factors (Marcantonio et al., 2001). Rockwood and co-workers have achieved good results by adopting an early recognition strategy of the risk factors through a dedicated training of the nursing staff (Rockwood et al., 1994). In a randomised, two cohort, prospective study, Inouye and Charpentier found a lower incidence of delirium in the treatment group (9.9%), compared with the control group (15%). Hospital stay and the number of episodes of delirium were also reduced (Inouye and Charpentier, 1996). This was achieved by the adoption of a standardized protocol for the management of six risk factors (previous cognitive impairment, sleep deprivation, immobility, visual impairment, hearing impairment and dehydration).

The management of delirium should be systematic. Marcantonio has demonstrated that delirium is independently associated with poor functional recovery one month after hip fracture, even after adjusting for prefracture frailty (Marcantonio et al., 2000). He then proposed to characterize the underlying causes, establish behavioural control, prevent complications and provide adequate functional support (Marcantonio, 2002).
It is generally accepted that the best approach to avoid the development of delirium, is by prevention. This can be achieved by reducing the impact of the risk factors contributing to the condition, including dehydration, immobility, infections, hyponatremia, hypoxemia and pain, which are often easily correctable factors. Any drug that is known to precipitate delirium, such as anticholinergic drugs or psychotropic drugs with anticholinergic side effects, should be avoided.

Antipsychotic medications, such as haloperidol, risperidone or olanzapine represent the treatment of choice. Dexmedetomidine has also been successfully used (Levän et al., 1995). Intramuscular haloperidol may be effective especially in the hyperalert-hyperactive subtype of delirium. In an interesting randomized, placebo-controlled, double-blind, clinical trial, Kalisvaart and colleagues have evaluated the efficacy of haloperidol 0.5 mg three times daily versus placebo on the primary and secondary prevention of post-operative delirium in elderly patients after hip surgery. They found that haloperidol at the administered dose was not effective in preventing the occurrence of post-operative delirium (9.4% vs 12.8% in the treatment and control group, respectively). However, it markedly reduced both severity and duration of this complication (Kalisvaart et al., 2005).

Dementia and depression are risk factors for the development of delirium. They also should be carefully considered in the differential diagnosis. Recognizing these comorbidities permits a precise understanding of the severity of delirium. Dementia and delirium have much in common, and obtaining a differential diagnosis is likely to be difficult. An accurate anamnesis is essential.
1.4.1 Interventions and long-term studies

The possible onset of POCD can be predicted by the recognition of those patients who are exposed to a greater risk of developing the syndrome. One of the subsequent possible actions to undertake within this group of patients involves neuroprotection, which can be achieved in different ways.

The property of some anaesthetics, such as thiopental and propofol, to protect against ischemia by reducing cerebral metabolic demand (Hogue et al., 2006) was tested to prevent POCD, but without success (Zaidan et al., 1991; Roach et al., 1999). On the other hand, pharmacological agents, such as remacemide (a glutamate neurotransmitter antagonist) and lidocaine yielded more interesting results as neuroprotectants (Wang et al., 2002; Arrowsmith et al., 1998; Mitchell et al., 1999).

In cardiac surgery POCD may result from the association of different pathophysiological mechanisms, whereby predictors may be difficult to define. A longitudinal assessment of POCD after cardiac surgery explored the incidence and predicting factors of POCD over a period of 5 years. The investigators found that the incidence of cognitive decline was 53 percent at discharge, 36 percent at six weeks, 24 percent at six months, and 42 percent at five years. After considering all possible predictors of cognitive decline at five years, they showed that cognitive function at discharge was a significant predictor of long-term function (Newman et al., 2001).

Microembolization of particulate debris, which is often observed in both cardiac and orthopaedic surgery, is an important pathophysiological mechanism of brain injury (Moody et al., 1995). The direct evidence of microemboli formation in the cerebral
circulation, coming from studying the retina (Blauth et al, 1988), is supported by indirect evidence provided by the demonstration that cardiopulmonary bypass arterial line filtration reduces cognitive decline (Pughsley et al, 1994). More recently, a correlation between number of microemboli and increasing duration of bypass has also been presented (Brown et al, 2000).

Cardiotomy suction, the technique by which the blood is rescued from the operative field, has been recognized as an important mechanism to produce gaseous and particulate debris, leading to microembolization. Cardiotomy suction scavenges spilt blood from the pericardium, open pleural cavities and mediastinum back to the cardiopulmonary bypass machine. This blood is rich in cytokines and bioproducts which are produced following activation of inflammatory cells by the interaction with foreign surfaces, including air. Filtration of this blood before restitution to the patient does not seem to prevent formation of emboli (Brooker et al, 1998) mostly derived from the adipocytes of the mediastinal fat. These emboli damage the small capillary and arterioles of the blood brain barrier to cause its increased permeability. Recent studies show that when this blood is not returned to the patient neurocognitive dysfunction is observed with a lower incidence, concomitantly with a reduction of the marker S100B.

Evidence exists that hypoperfusion may lead to cognitive dysfunction. Particularly sensitive are the watershed areas of the brain, whereby protection may be achieved by maintaining an intra-operative higher mean arterial pressure (Selnes et al, 1999).
As explained in this thesis, the inflammatory response is an important contributing intra-operative factor to neurological lesions possibly leading to POCD. Any surgery produces a local inflammatory milieu that can spread anywhere in the body. In areas affected by hypoperfusion, the coagulation cascade is activated together with the fibrinolytic and complement systems, with the consequent release of free radicals, in a spiral of further damage of preexistent lesions.

Interventions aimed at preventing or treating POCD could be tailored to target specific cognitive domains by adoption of psychological support or pharmacological therapy. In a long-term study, Price and co-workers showed that postsurgical cognitive presentation varies with time of testing. At 3 months after surgery, an increased number of older adults experienced memory decline, but only those with executive or combined cognitive decline had functional limitations (Price et al, 2008). These important findings certainly have relevance for patients and caregivers. In another long-term prospective longitudinal study the authors confirmed and association between mortality and POCD, thereby the importance of early detection and treatment to prevent its occurrence (Monk et al, 2008).

All the above cited examples confirm that clarification of the various factors involved in surgery and the adoption of appropriate treatments targeting the inflammatory response may be strategically useful to reduce incidence and impact of POCD in the post-operative period.
1.5 EPIDEMIOLOGY

The frequency of POCD is variable and related to the type of surgery, as well as to several pre-, intra-, and post-operative factors.

Marcantonio and colleagues developed and validated a categorized scoring system to predict the possible onset of delirium prior to an operation. They also found that the incidence of delirium in elective non-cardiac patients is 9% within the first 5 post-operative days (Marcantonio et al., 1994a). The incidence increases in orthopaedic surgery. It is 41% in bilateral knee surgery (Williams-Russo et al., 1992) and varies between 35 and 65% after hip-fracture repair (Gustafson et al., 1988). Elderly patients undergoing emergency surgery have also shown a high incidence of POCD: 42.3% after bypass surgery for lower limb ischaemia (Sasajima et al., 2000) and 73% after lung transplant (Dyer et al., 1995).

A large international multicentre trial enrolling 1218 patients aged at least 60 years, noted the occurrence of POCD at a rate of 25.8% one week after surgery and in 9.9% of patients 3 months after surgery (Moller et al., 1998). Johnson and co-workers found an incidence of 19.2% one week after surgery in middle-aged surgical patients compared to 4.0% in the non-surgical control group (Johnson et al., 2002; for details, see figure 1-2).
Figure 1.2. Incidence of POCD in middle-aged and elderly patients. Although clinical features of cognitive deterioration are evident even in middle-aged patients, this impairment is longer lasting in older subjects. Source data: (Moller et al., 1998; Johnson et al., 2002).

These results have recently been confirmed by another study of 417 patients undergoing major surgery, reporting, at discharge, cognitive decline in 186 (56%) patients, with an equal distribution in type and severity of cognitive deficits (Price et al., 2008). At 3 months after surgery, 231 patients (75.1%) had normal levels of cognitive functions, 42 (13.6%) experienced memory decline alone, 26 (8.4%) showed only executive function impairment, and 9 (2.9%) had a decline in both executive and memory domains. Of those with cognitive decline, 36 (46.8%) had mild, 25 (32.5%) had moderate, and 16 (20.8%) had severe impairment. The combined group had more severe deficits. Executive function or combined (memory and executive) decline involved greater levels of functional impairment (i.e., instrumental activities of daily living).

Little is known about POCD occurring beyond one year. It has nevertheless been reported that some elderly patients (1%) still experience the disabling effects on
cognitive function one year after surgery (Abildstrom et al., 2000), other than an increased risk of death within a year for those patients whose symptoms were still evident 3 months after surgery (Monk et al., 2008).

Yet, given the nature and timing in the onset of late POCD, the prevalence may be largely underestimated in common practice.
1.6 RISK FACTORS

The risk factors for delirium have been recognized and categorized in two different groups: predisposing and precipitating factors. Predisposing factors, such as old age, hip fracture, alcoholism, are already present in the patient at the time of hospital admission. Precipitating factors are usually hospital-related insults, such as orthopaedic or cardiac surgery, physical restraint and hyponatremia.

They have also been divided in pre- intra- and post-operative factors. Age, alcoholism, severity of the illness leading to surgery, previous cognitive dysfunction and renal impairment have been considered as pre-operative risk factors (Francis et al., 1990; Levkoff et al., 1992; Schor et al., 1992; Inouye et al., 1993; Pompei et al., 1994). Sharon K. Inouye and co-workers have noted that an association of these factors contributes to increase the risk of developing early POCD (Inouye et al., 1999). Intra-operative factors include: use of anticholinergic compounds, psychoactive medications and anaesthetics, as well as the type of surgical procedure (Marcantonio et al., 1994b).

In the ISPOCD study, the identified risk factors for the development of POCD were increasing age, duration of anaesthesia, little education, a second operation. Amongst the post-operative factors, they found respiratory complications and post-operative infections. However, age was the only risk factor for late post-operative cognitive dysfunction (Moller et al., 1998).

Apart from age, time of formal education has been consistently reported as an important risk factor related to POCD (Moller et al., 1998; Monk et al., 2008). Low
educational attainment has been shown to predict cognitive impairment, whereas a protective effect seems to be related to high educational levels as described in POCD following procedures requiring cardiopulmonary by-pass (Newman et al., 1995).

The concept of cerebral cognitive reserve has been advocated as the rationale underlying the proposed relationship between POCD and level of education (Maze et al., 2008). Stern refers to passive and active models of cognitive reserve; in the passive model, reserve is represented by increased brain size and/or number of synapses available. Within this model, any cognitive deterioration consequent to a brain injury can be compensated for by means of neuronal pathway substitution. The active model features an improved processing of the available information by exploitation of pre-existent redundant neuronal networks (Stern, 2002). This theory of synaptic enrichment provides a convenient explanation for the observation that high levels of intelligence and educational attainment are good predictors of brain resistance to injuries before cognitive dysfunction is manifest (Elkins et al., 2006). Higher educational levels and occupational attainment have been associated with an improved ability to recruit and re-arrange redundant neuronal structures and pathways in replacement of those damaged by an insult (Satz, 1993). The cognitive reserve theory also explains the role of a previous stroke (even though this may occur without any apparent neurological deficit), as an exacerbating factor for POCD.

Although research on POCD and related risk factors is still at a relatively early stage and extensive interest in the predictive value of general co-morbidities would be expected, these have largely been ignored, thus far.
Several risk factors, known to strongly affect post-operative morbidity and mortality, such as hypertension, anaemia, atherosclerosis, diabetes, chronic pulmonary disease, congestive heart failure, severely impaired left ventricular function and unstable angina, have little been taken into account when researching about predisposing factors for POCD. Surprisingly, many brain-related co-morbidities, such as depression and pre-operative dementia, are often excluded from the studies as possible confounding factors.

The degree of increased risk related to the presence of mental co-morbidities in the past medical history was alluded to in a retrospective study of 975 patients, 57 of which had post-operative complications (Bernstein and Offenbartl, 1991). Importantly, those patients who displayed symptoms of dementia pre-operatively, were the same who had a fatal complication later on (25 of 32 who died had dementia), in accordance with more recent findings relating POCD and mortality (Monk et al., 2008).
1.7 PATHOGENESIS

The pathogenesis of POCD is still surrounded by a degree of uncertainty. Several authors support the possible involvement of anaesthetics (Culley et al., 2003; Crosby et al., 2005; Xie et al., 2008). Although it is commonly believed that a patient’s unresponsiveness after a general anaesthetic is transient and completely reversible, this has yet to be scientifically proven. Several investigators believe that other mechanisms underlie the development of POCD. Speculation about impairment of adrenergic or cholinergic transmission, or about the role of some genetic markers from dementia studies based upon their predictive value as risk factors or causative elements, have opened new interesting research avenues (Hshieh et al., 2008; Leung et al., 2007). Even if ongoing research fails to demonstrate direct association between anaesthetics and POCD, there is reason to speculate that post-operative changes in cognition could result from the physiological effects of the anaesthetics, thus implicating their indirect role. Hyperventilation, hypotension and hypoxia constitute only some of the possibilities (Bekker and Weeks, 2003).

The putative main mechanisms suggested to underlie POCD are explained here, with a special emphasis on the pathogenetic mechanism representing the centre of the investigation of this dissertation, which is surgery-induced inflammation.

1.7.1 Anaesthetics

General anaesthetics (especially the inhalational type) are largely used in clinical practice. Since their discovery, in 1799 by Humphrey Davy, the surgical treatment of a wide array of diseases became possible (Evers and Maze, 2004).
General anaesthesia is the dynamic speciality. New compounds are discovered at a fast rate, resulting in significant advances in terms of effectiveness and safety profiles, and making even complicated surgical procedures safe, which is important at the extremes of age. Nonetheless, the general anaesthetics are an exceptionally potent class of drugs, and exert their effect at any level in the nervous system (Franks and Lieb 1994). Cerebral blood flow, metabolism, neurotransmission, protein expression, ion channels, and neuronal membranes are known to undergo significant perturbations by the anaesthetics (Franks and Lieb 1994). Modulation of \( \gamma \)-amino-butyric acid (GABA) receptors or N-methyl-D-aspartic acid (NMDA) glutamate receptors contributes to the desirable hypnotic effects of the anaesthetics, but could also be the cause of interference to neurotransmission, as this occurs with developmental neurotoxins (Bittigau et al. 2002). Furthermore, as senescence progresses, the brain undergoes regressive changes, which critically affect function and responsiveness to toxic insults (Iadecola et al., 2009; Sparkman et al., 2008). Aging, one of the aforementioned risk factors for POCD, causes several structural and morphological changes to brain tissue, which are likely to be correlated with a reduction in cognitive reserve. These include reduced brain weight and volume (Schwartz et al., 1985) as well as loss of cellular bodies and myelinated fibers in several brain regions (Pakkenberg and Gundersen 1997) including the hippocampus (Simic et al., 1997); an area of the brain that is critical for memory. Subcellular changes are documented as a reduction in synaptic density (Morrison and Hof, 1997), rarefaction of cerebral microvasculature (Riddle et al., 2003), and alterations to DNA repair systems including the mechanisms for removal of neurons with damaged nuclear DNA (Rutten et al. 2003). Oxidative stress has been cited as a likely cause of age-related neurodegeneration (Coyle et al., 1993).
Thus, it is possible to imagine how such processes cause the ageing brain to have reduced functional reserve and limited ability to compensate for the deleterious effects of the anaesthetics, making it more vulnerable to the possible development of cognitive dysfunction.

Recent evidence suggests that anaesthetic-induced swelling of mitochondria and endoplasmic reticulum leads to a transient vacuolization of cerebral neurons in rodents (Jevtovic-Todorovic et al., 2000), and this process prominently occurs in aged rodents (Jevtovic-Todorovic and Carter, 2005), following administration of ketamine, or nitrous oxide, or a combination of both. Studies of Alzheimer’s disease and anaesthetics have suggested a causative relationship between increased production of Aβ-protein (one of the recognized molecules involved in the pathogenesis of Alzheimer’s disease) and apoptosis following exposure to isoflurane (Xie et al., 2006). This effect is blocked by an inhibitor of apoptosis, while isoflurane-induced apoptosis itself can be prevented by inhibitors of Aβ aggregation, implying the existence of a self-perpetuating cycle of apoptosis and Aβ protein generation (Xie et al., 2007).

The premise that isoflurane anaesthesia increases deposition of Aβ protein, however, has been challenged by a study in which the investigators were still able to demonstrate post-anaesthetic cognitive dysfunction, but were unable to detect any change in Aβ protein deposition (Bianchi et al., 2007).
Further evidence in support of a causative relationship between POCD and anaesthetics is provided by a number of studies all converging towards the same conclusion. They all demonstrated an association between enduring learning deficits and/or persistent changes in hippocampal gene expression following general anaesthesia with desflurane, isoflurane or nitrous oxide, occurring in aged but not young rats (Culley et al., 2004; Futterer et al., 2004; Crosby et al., 2005; Culley et al., 2006; Culley et al., 2007). However, some degree of post-anaesthetic memory impairment alone (with no analysis of possible gene expression change) was reported to develop in young animals as well (Culley et al., 2003).

This literature supporting the hypothesis of anaesthetic-induced POCD is counterbalanced, to some degree, by the growing evidence of anaesthetic-induced neuroprotection.

Isoflurane appears to reduce the extent of neurological damage following focal cerebral ischemia in rats, independent of ischemia duration, 2 and 8 weeks after the insult (Sakai et al., 2007). Also, when applied on hippocampal and cerebellar slices, isoflurane reduced or prevented neuronal degeneration and cell death in rat models of hypoxia or oxygen-glucose deprivation or when it was concurrently administered with α-amino-3-hydroxyl-5-methyl-4-isoxazol propionic acid (AMPA) measured up to 14 post-experimental days (Gray et al, 2005; Li et al., 2002; Popovic et al., 2000; Sullivan et al., 2002). Furthermore, isoflurane is well known to exert preconditioning effects. Preconditioning with isoflurane prior to permanent or transient focal cerebral ischemia significantly improved brain injury in rodents (Kapinya et al., 2002; Liu et al., 2001; Zheng and Zuo, 2005).
Although sevoflurane and desflurane have been studied less extensively than isoflurane, available evidence strongly suggests that neuroprotection from focal or global cerebral ischemia occurs with both agents (Pape et al., 2006; Warner et al., 1993; Werner et al., 1995; Haelewyn et al., 2003; Tsai et al., 2004; Kurth et al., 2001; Engelhard et al., 1999). Furthermore, in contrast to the lack of studies investigating the possible preconditioning effects of desflurane, recent investigations have been conducted in support of the protective effects of sevoflurane. Sevoflurane has been shown to reduce neuronal damage when administered 24 hours prior to global cerebral ischemia in rats (Payne et al., 2005) and to protect in an in vitro model of hypoxia (Kehl et al., 2004).

The noble gas xenon is an anaesthetic with demonstrated neuroprotective properties starting from concentrations that are below those required for anaesthesia. It has been shown to decrease neuronal injury resulting from exogenous administration of excitotoxins or after oxygen and glucose deprivation, in neuronal-glia cell culture. In vivo, xenon is protective in a model of hypoxia/ischemia in rats (Dingley et al., 2006) and prevents the deleterious consequences of excitotoxins (Wilhelm et al., 2002), acute neuronal injury provoked by middle cerebral artery occlusion (ischaemia) in mice (Homi et al., 2003) and by cardiopulmonary bypass in rats (Ma et al., 2003).

The association between anaesthesia and POCD is still under debate, with some researchers being convinced of its involvement and others who are not. The contrasting effects of the anaesthetics, both detrimental and protective, make it even more difficult to draw convincing conclusions regarding their possible role in POCD.
On the clinical side, in an attempt to clarify the putative role of the general anaesthetics in the development of POCD because of their central effects, numerous studies have been conducted to compare general versus loco-regional anaesthesia.

An important investigation enrolling 438 patients found no significant difference in the incidence of cognitive dysfunction 3 months after either general or regional anaesthesia in elderly patients. The authors concluded that there seems to be no causative relationship between general anaesthesia and late POCD (Rasmussen et al., 2003). Two structured, evidence-based, clinical updates of the available literature have also failed to find any difference. Both the studies reached the common conclusion that other risk factors, like inflammation, may be involved (Bryson and Wyand, 2006; Newman et al., 2008).
1.7.2 Inflammation

Backache, muscular pain and knee arthritis often share the same pathogenetic mechanism. This mechanism is also shared by other conditions, such as allergies, asthma and atopy. Symptoms arising from life-threatening conditions like heart disease, infections and Alzheimer’s disease also depend upon the same, basic, mechanism (Silverman, 1991; Akiyama et al., 2000; Leonard, 2007).

This mechanism is inflammation.

Inflammation has been known since the ancient Egyptians, and was described by the Romans (Celsus) as the underlying cause of rubor, calor, tumor, dolor, functio laesa (redness, warmth, swelling, pain, loss of function) which are the typical symptoms that develop after the immune system has been activated by an insult (Porth, 2007).

Acute inflammation is initiated by immune cells that are already resident in tissues, such as macrophages and dendritic cells. Following an infection or tissue insult, these cells are stimulated to release inflammatory mediators that are responsible for initiating the clinical signs of inflammation. Vasodilation results in a local net increase in blood supply (responsible for tissue redness and heat). Increased permeability of vessels allows exudation of blood plasma proteins (causing oedema). Extravasation of immunocompetent leukocytes and recruitment to the site of injury by chemotaxis helps to maintain the inflammatory response by releasing pro-inflammatory mediators, some of which sensitize sensory nerves resulting in pain and loss of function (Doan et al., 2005; Stein et al., 2009).
Inflammation describes the body’s immune response to tissue damage or disease. It involves recruitment of inflammatory cells as well as the generation and release of a wide range of chemical agents. In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism. However, unrestricted inflammation can also lead to a range of chronic conditions, such as allergies, atherosclerosis, and autoimmunity. Under certain circumstances, while serving a vital protective immunological function, the inflammatory response escapes control. As a consequence, an excessive or inappropriate inflammatory response may itself result in a chronic pathological condition (Silverman, 1991). Chronic inflammation is characterised by the dominating presence of macrophages in the injured tissue. These cells release toxins, including reactive oxygen species, as part of a defensive strategy to prevent the growth of microorganisms but they are injurious to the organism's own tissues as well as invading agents (Marques et al., 2008). Consequently, chronic inflammation is almost always accompanied by tissue destruction and disease (Cotran, 1998).

The inflammatory response is part of the immune system. An imbalanced regulation of the inflammatory process follows a complex pathway, starting from dysregulated immunological reactions and culminating in the development of disease.
1.7.2.1 The immune system

The immune system has evolved in vertebrates primarily to prevent an invasion from pathogens. This defence system is made up of cells and tissues from the haematological and lymphatic systems; the lymphatic system enables the body to screen for foreign organisms while the haematological system provides a range of cells capable of orchestrating an immune response. The immune system is highly conserved phylogenetically, and highly effective. It can be divided into two types of immunity: innate and adaptive. The innate immune response is rapid and unspecified involving several effector cells types, including macrophages and neutrophils. The innate response initiates a cascade of events that leads to the adaptive immune response, which is slower but involves a highly focused and potent reaction to a specific pathogen requiring recruitment of T-cell and B-cell leukocytes (Doan et al., 2005; Janeway et al., 2005).

Activation of the immune system often follows the invasion of the body by a foreign substance, an antigen (from the words antibody generator), a molecule expressed on pathogens, such as bacteria or viruses. This invasion primes a particular group of inflammatory cells, antigen presenting cells (APCs), such as macrophages or dendritic cells, which engulf the pathogen. Inside the APCs, pathogens undergo an elaborated sequence of enzymatic reactions culminating in the fragmentation of the foreign substance in smaller pieces, some of which will be recognized as antigens. These antigens will be subsequently moved on the surface of the APC’s in order to facilitate recognition by a specific population of white cells: the T cells. The interaction between the APC’s and the proper subclass of T cells, the T-helper cells (T_H’s), depends on highly specific receptor-ligand binding (for any given antigen), enabling
T_{H}\text{’s} activation and proliferation. The next step is the recruitment of other immune cells, such as the B cells, in response to the mediators secreted by the T_{H}\text{’s} (Maier and Watkins, 1998; Janeway et al., 2005). The defence mechanisms, as such, are particularly effective in recognizing specific antigens against which the immune system should be primed. Also, the proliferating clones of T_{H}\text{’s} and B cells are provided with the perfect machinery, devised to specifically target the recognized presented antigen, that is, the invading pathogen. Although adaptive immunity is a costly and rather slow apparatus of cells and mediators it has the great advantage to be precise and extremely fast when the same antigen is recognized following a subsequent invasion. This is because memory cells, persisting beyond the end of the immune response and bearing receptors for the known antigen, are ultimately formed.

1.7.2.2 Neuroinflammation

Microglia are considered to be the major resident macrophage-type immunocompetent cells of the CNS. These cells make up 5–10% of the adult CNS glia population and are thought to function as sensors for a range of stimuli that may act to compromise brain tissue homeostasis (Kreutzberg, 1996; Stoll and Jander, 1999). Although normally present in a quiescent state, microglia can be primed or activated by the presence of foreign antigens (Sparkman and Johnson, 2008). In their activated state, microglia are capable of mounting macrophage-type innate immunity (van Rossum and Hanisch, 2004; Raivich et al., 1999). Activated microglia secrete IL-1β and other pro-inflammatory cytokines (Aloisi, 2001), reactive oxygen species, excitotoxins, such as glutamate (van Rossum and Hanisch, 2004) and neurotoxins such as β-amyloid precursor protein (Farber et al., 1995). Upon activation, microglia exhibit discernible morphologic changes, including cell body enlargement, thickening of
proximal processes and decreasing ramifications of distal branches (figure 1.3) (Kreutzberg, 1996). Expression of αMβ2 integrin, also referred to as Mac-1, OX-42, or CD11b/CD18 (a binding site for the complement factor 3 component C3bi as well as for ICAM1), increases markedly upon microglial activation (Kloss et al., 1999). Successful immunohistochemical identification of activated and primed microglia by staining with CD11b has been demonstrated previously (Block et al., 2007). Activated microglia release pro-inflammatory cytokines, notably interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNFα) (van Rossum & Hanisch, 2004; Giulian et al., 1996). A subset of microglia also resides in an intermediate state between rest and activation. As with activated microglia, primed cells have shortened processes and express cell surface markers but appear to lack the cytokine secretory properties of activated microglia (Sparkman and Johnson, 2008). In severe pathological states microglia can adopt an amoeboid morphology and assume phagocytic properties in common with the innate immune response carried out by peripheral macrophages (Kreutzberg, 1996).
Figure 1.3
Morphological changes to microglial cells during the transition from a resting state (far left) to activated cells (far right) include enlargement of the cell body, thickening of proximal processes and ramification of distal branches. In response to stimuli, resting microglia retract their processes, increase expression of surface markers, including CD11b and major histocompatibility complex (MHC) class II and release cytokines, such as IL-1β and TNFα. In severe pathological states, activated microglia may also become phagocytic (Adapted from Kreutzberg, 1996; Sparkman and Johnson, 2008).

The time course for microglial activation varies depending on the type of stimulus or pathological insult. For example, the microglial activation period can begin rapidly (within hours) reaching a plateau 2 weeks after an inflammatory insult caused by chronic infusion of lipopolysaccharide (LPS) into the brain (Gao et al., 2002), whereas prion infection induces microglial activation within a few days (Marella and Chabry, 2004). Excessive microglial activation, as may occur in chronic
neuroinflammation, can provoke damage to neurones and glia either by disproportionate release of toxic substances (reactive oxygen species, nitric oxide and glutamate) or cytokines, and/or by the induction of destructive cascades (van Rossum and Hanisch, 2004; Sparkman and Johnson, 2008).

Astrocytes also play an important role in neuroinflammation and regeneration during neurological inflammatory insults (Eddelston et al., 1993). These cells greatly outnumber neurones in the CNS and are crucially involved in antioxidant defence, toxic insult, neurotrophic functions, tissue repair, and extra-cellular volume control. They protect neurones by preventing excitotoxic damage and are able to mount an inflammatory reaction stimulated by cytokines in response to injury (Rothstein et al., 1996). Cytokine-mediated activation of astrocytes develops with a morphological change of the cell and the development of a dense arborisation of the end-feet processes following an up-regulation of glial fibrillary acidic protein (GFAP) (Lapping et al., 1994). After activation, astroglia shows either a promoting or inhibiting action on tissue injury. Its involvement in neuronal tissue repair is carried out with the formation of a scar as a consequence of astrogliosis. This is to limit the extension of the damage. However, under certain conditions, this mechanism could prevent the restoration of axonal regeneration and pre-existent inter-neuronal synapses which in turn leads to neurodegeneration (Silver and Miller, 2004). Furthermore, astroglial activation may cause detrimental effects through sustained release of neurotoxic macromolecules such as reactive oxygen species, nitric oxide, and excitotoxins. Once started, the inflammatory reaction can be effectively sustained by activated astroglia with the release of IL-1β and TNF-α (Lieberman et al., 1989; Hu and Van Eldik, 1996).
1.7.2.3 Immune system to brain communication and the effect of neuroinflammation on behaviour

A function of the immune system, which has received only limited scientific attention, is its ability to act as a diffuse type of sensory organ to inform the brain of infection and injury events. A bi-directional communication is suggested to occur between the immune system and the CNS in order to explain how an infection or injury in the periphery can lead to changes in neurophysiology and behaviour; a psychoneuroimmune interaction (Maier and Watkins, 1998). This kind of peripheral to central communication explains a co-ordinated group of adaptive ‘sickness’ behaviours that occur in response to a peripheral infection or injury and, rather than simply occurring as a symptom of weakness from the injury/infection, these behaviours have evolved as a defensive strategy to aid recovery and survival. Sickness behaviours include responses, such as lethargy, sleepiness, depression, anorexia, reduced grooming and impaired concentration (Hart, 1988). Pro-inflammatory cytokines released into the blood by macrophages and other peripheral immune cells in response to peripheral infection/injury and transmitted to the brain by activation of humoral and nervous system communication pathways are critical to activating sickness behaviour (Maier and Watkins, 1998). This was established because blocking cytokine signals with receptor antagonists or antisera prevented sickness behaviours and these behaviours could be induced by administration of cytokines in the absence of an inflammatory state (Dinarello, 1991; Dinarello & Thompson, 1991).

Evidence suggests that fast peripheral to central transmission of the cytokine signal occurs via the vagus nerve (Goehler et al., 1997; Goehler et al. 1999; Maier et al., 1998), whereas slower transmission is thought to occur either by one of several
mechanisms. Firstly, direct entry of cytokines into the brain from the peripheral blood can occur via passive diffusion in areas where the blood-brain barrier (BBB) is absent (Maier et al., 2003) or by active transport across the BBB (Banks et al., 1991; Banks et al., 1994; Banks et al., 2001; Banks et al., 2006). Alternatively, activation of in situ production of the same cytokine in the brain as in the periphery also occurs (vanDam et al., 1992). Endothelial cells from the BBB are capable of secreting immune-related molecules, including cytokines, in response to humoral peripheral immune signals (Reyes et al., 1999) and induction of cytokine expression occurs in activated microglia (Ban et al, 1992). Peripheral inflammation stimulated experimentally by LPS injection was demonstrated to induce IL-1β mRNA expression in activated brain microglia, which peaked 6–8 hours after LPS injection (Buttini and Boddeke, 1995).

IL-1β is a key pro-inflammatory cytokine implicated in initiating behavioural responses to peripheral injury and infection, including fever and sickness behaviour, as well as acute CNS disorders such as stroke, and chronic neurodegenerative disorders (Alzheimer’s and Parkinson’s disease) (Allan et al., 2005; Maier and Watkins, 1998). IL-1 has two forms α and β that are agonists at the main receptor for this cytokine IL-1R1. IL-1R1 associates with an accessory protein (IL-1RAcP) to trigger a range of effector functions, including production of cytokines, chemokines, prostaglandins and growth factors, via activation of specific intracellular signalling pathways. The IL-1 signal is terminated by a naturally occurring receptor antagonist (IL-1RA) or a non-signalling receptor (IL-1R2), which acts as a soluble or membrane-bound binding protein for IL-1 (Pinteaux et al., 2009).
Research has demonstrated that a number of peripheral inflammatory stimuli activate the transcription and translation of IL-1β protein in the brain and that this cytokine acts on specific brain receptors to induce sickness behaviour that includes cognitive impairment (Kelley et al., 1997; Dantzer et al., 2008). IL-1 receptors are found throughout brain tissue but binding is highest in regions associated with learning and memory, including the hippocampus (Parret et al., 2002). Increasing the levels of IL-1β in the hippocampus either experimentally or by inducing a peripheral inflammation with LPS, have the effect of impairing hippocampal-dependent memory (Rachal Pugh et al., 2001). In fact, a peripheral infection or increasing IL-1β levels blocks long-term potentiation (LTP); the cellular mechanism implicated in memory formation in the hippocampus and also disrupts hippocampal-dependent spatial learning tested with the Morris water maze (Bellinger et al., 1993; Barrientos et al., 2006). Infusion of IL-1RA into the dendate gyrus of the hippocampus attenuates stress-induced impairment of learning and memory.

Just as inflammation is considered to have a central role in the pathogenesis of cardiovascular disease, multiple sclerosis and diabetes, neuroinflammation is a shared pathogenic mechanism common to a number of neurodegenerative diseases (Alzheimer’s disease, dementia, and depression), which feature deterioration in cognitive functions including memory as a prominent clinical symptom (Leonard, 2007). From this perspective, the cognitive impairment seen after surgery (POCD) may be similarly triggered by neuroinflammation. In common with LPS injection, surgery is also a peripheral inflammatory insult that should hypothetically result in elevated plasma cytokine levels that are communicated to the CNS where there is an
induction of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) by activated microglia that produce neurophysiological changes to modify behaviour (Figure 1.4).

**Figure 1.4**
Schematic diagram illustrating a putative inflammatory and neuroinflammatory pathogenic mechanism leading to POCD.
1.7.2.4 Neuroinflammatory consequences of ageing

The homeostatic state that balances pro-inflammatory and anti-inflammatory cytokines in the adult brain is known to slowly deteriorate over time, shifting the balance towards a pro-inflammatory state with increasing age. This makes the aged brain more vulnerable to the effects of pro-inflammatory factors, such as stress, infection, or disease. Several features noted in aged brain tissue have been suggested to increase the susceptibility to an inflammatory insult (Sparkman and Johnson, 2008).

Age changes brain morphology, physiology, and function. There is a decrease of blood flow, loss of dendritic tree and synapses, as well as modification of numerous neurotransmitter systems (Mrak et al., 1997). Evidence from several age-related neurological disorders such as Alzheimer’s disease, dementia and Parkinson’s disease suggest that changes to cholinergic transmission are responsible for the impairments in mood, memory, motor function and behaviour (Eikelenboom et al., 1999; Strong, 1998).

The aged brain also has an increased number of activated and primed microglia (Perry et al., 1993; Sheffield et al., 1998). In a primed state, microglia can achieve a more rapid induction and greater cytokine release in response to a triggering stimulus (Frank et al., 2007). Elevated plasma levels of pro-inflammatory cytokines have been reported in elderly human subjects and have been associated with reduced cognitive function (Weaver et al., 2002). IL-6 is also elevated in the hippocampus of aged mice (Ye and Johnson, 1999). Psychological and peripheral immunological stressors (e.g. LPS) are known to sensitise immune responses to antigenic stimulation and cause
increased cytokine release in the brain (Frank et al., 2007). It has been suggested that the effect of these stressors is amplified in aged brains because the microglia are already in a primed state, leading to exacerbation of the normal neuroinflammatory responses in ways that lead to cognitive impairment (Neupert et al., 2006). The levels of pro-inflammatory cytokines measured in hippocampus, cerebellum and cerebral cortex in response to a peripheral LPS injection is significantly higher in aged mice brains compared with adult mice (Godbout et al., 2005). There is also a reduction in the levels of anti-inflammatory mediators, such as IL-10 (Ye and Johnson, 2001) in aged brain tissue making it less able to counteract pro-inflammatory insults.

All these age-related changes lead to creation of a pro-inflammatory state in which normal stressors, such as a peripheral inflammation due to infection or trauma, have an exacerbated neuroinflammatory effect in the brain leading to more pronounced cognitive impairment. As a demonstration of this effect, the level of cognitive impairment measured by behavioural performance tests in mice after a peripheral infection with LPS is higher in aged mice compared with adult controls (Godbout et al., 2005).
Figure 1.5
Schematic diagram illustrating the role of microglial priming as occurs in the aged brain, which enhances the sensitising effects of psychological or systemic infection stressors. Priming leads to heightened release of pro-inflammatory cytokines in response to a triggering insult, which leads to an exaggerated neuroinflammatory response and cognitive impairment (Adapted from Sparkman and Johnson, 2008).
1.8 AIMS AND HYPOTHESIS

**Aims:** This study is intended to further characterise the association of peripheral and hippocampal inflammation with hippocampal-dependent learning and memory dysfunction in mice following orthopaedic surgery.

**Hypothesis:** Glial activation and release of proinflammatory cytokines in the hippocampus are triggered by the inflammatory response to a surgical intervention in the periphery. It is the resulting neuroinflammatory response in the hippocampus that contributes to the development of POCD-correlated behaviour in mice.
Chapter Two

General methods

Contents

2.1 Introduction 62
2.2 Experimental groups and procedures 62
   2.2.1 Animals 62
   2.2.2 Experimental groups 62
   2.2.3 Tibial fracture surgery 63
   2.2.4 Minocycline pre-treatment 65
   2.2.5 Anaesthesia treatment 65
   2.2.6 Peripheral injection with lipopolysaccharide 65
   2.2.7 Tissue harvesting and blood sampling 66
2.3 Behavioural studies 66
   2.3.1 Fear conditioning 67
   2.3.2 The trace fear conditioning experimental paradigm 70
2.4 Immunohistochemistry 71
   2.4.1 Tissue fixation 72
   2.4.2 Antibody staining 74
   2.4.3 Antibody labelling 75
   2.4.4 Avidin-biotin system 77
   2.4.5 Microscopy and image analysis 79
   2.4.6 Qualitative (semi-quantitative) evaluation of CD11b-stained microglia in hippocampal tissue 79
   2.4.7 Data analysis 81
2.5 Western blotting 81
2.6 Enzyme-Linked Immunosorbent Assay (ELISA) 83
   2.6.1 Introduction 83
   2.6.2 Sandwich assay 84
   2.6.3 ELISA procedure 85
2.7 Flow cytometry 86
2.1 INTRODUCTION

This chapter provides background information and theoretical explanations for the main experimental procedures employed in this thesis. These include experimental design (organisation of experimental groups); a general description of the main animal procedures used (including animal housing, behavioural testing and tissue harvest) and specialist techniques (immunohistochemistry; Western blotting and Enzyme-Linked Immuno-sorbent Assay [ELISA]). Detailed descriptions of the methods used for individual experiments are provided at the beginning of each of the data chapters in this thesis.

2.2 EXPERIMENTAL GROUPS AND PROCEDURES

2.2.1 Animals
All the experiments were conducted under Home Office approved licence and were performed using 12-14 weeks old male C57-BL6 mice (Harlan, Oxon, UK) housed in groups of up to 4 animals/cage, under a 14:10 hours light-dark cycle, in a constant temperature and humidity controlled environment, with free access to food and water. Acclimatization to light/dark cycle for a minimum of 7 days preceded any intervention or assessment. Prior to each procedure, the mice were allowed to a period of 1 week of adaptation to the housing environment in light/dark cycle.

2.2.2 Experimental groups
Mice were randomly assigned to a range of study groups, including a control group of age-matched naive animals that did not undergo any experimental procedure. Examples of typical animal groupings for experiments in this thesis are provided in the following list of interventions:
• Tibial surgery with anaesthesia
• Anaesthesia without surgery
• Tibial surgery with a pre-treatment e.g. minocycline,
• Control group of age-matched naive animals

Duplicate groups of animals were used for harvesting tissue samples, and for behavioural studies. This design was employed to avoid any possible interference caused by the fear conditioning testing (Nguyen et al., 1998).

2.2.3 Tibial fracture Surgery
Following 1 week of adaptation in a light/dark cycle, mice were randomly assigned to experimental groups.

Under general anaesthesia and aseptic conditions, the mice in the surgery group were subjected to an open tibial fracture with intramedullary fixation. Isoflurane 1.5 MAC, which corresponds to a concentration in air of 2.1% (Engelhardt et al., 2006) and buprenorphine 0.1mg/kg subcutaneously (sc), were given to provide approximately 15-20 minutes of deep anaesthesia and post-operative analgesia.
The surgical field was prepared with sterile drapes and the instruments were autoclaved. The mouse skin was shaved and disinfected with chlorexidine gluconate 0.5% and isopropyl alcohol 70%. The opened diaphyseal surgery of the tibia in the left hind paw is a modification of the model previously described by Hiltunen and co-workers (Hiltunen et al., 1993). Briefly, a longitudinal incision was made through the skin and fascia laterally to the tibia to expose the periosteum. A 0.5mm hole was drilled just above the proximal third of the tibia where an intramedullary, stainless steel fixation wire was inserted (Figure 2.1). Subsequently, the fibula and the muscles surrounding the tibia were isolated, and an osteotomy was created with scissors in the middle of the distal third of the tibia. The skin was sutured and intra-operative fluid loss was replaced with a subcutaneous injection of 0.9% NaCl solution. The long-acting analgesic buprenorphine provided post-operative analgesia.

Figure 2.1 Insertion site of an intra-medullary stainless-steel fixation wire.
By the third post-operative day the initial haemorrhage was organised into a haematoma that was infiltrated with neutrophils, lymphocytes and macrophages, which were also present around the necrotic material at the fracture site. The cellular infiltrate became most pronounced in the muscles by the 5th postoperative day. By the 7th postoperative day, a fusiform soft callus formed at the fracture site. The systemic inflammatory response associated with this surgery appeared to be similar to that seen following joint replacement and was unlike a septic response (Harry et al., 2004).

2.2.4 Minocycline pre-treatment
In addition to the same surgical protocol described above, the animals were given an intraperitoneal (i.p.) injection of minocycline or enrofloxacin antibiotic two hours prior surgery and once daily until they were sacrificed. All animals in the groups receiving anaesthetics were allowed free, unrestricted food and water intake and weight bearing in cages following recovery from anaesthesia.

2.2.5 Anaesthesia treatment
The anaesthesia protocol was the same for animals undergoing surgery as well as those receiving anaesthesia without surgery. This consisted of isoflurane 1.5 MAC for 20 minutes and a sc injection of buprenorphine.

2.2.6 Peripheral injection with lipopolysaccharide
Naive animals received an injection of lipopolysaccharide (LPS) ip. LPS is a cell wall fragment from Gram negative bacteria and peripheral administration of this compound is used to produce a robust systemic inflammation. LPS injection can also be used as a positive control for neuroinflammation as it generates measurable increases in pro-inflammatory cytokines (primarily IL-1β, IL-6 and TNFα) in the
hippocampus, cerebellum, and cerebral cortex (Godbout et al., 2005) as well as microglial activation (Qin et al., 2007) and sickness behaviour (Kelley et al., 2003).

2.2.7 Tissue harvesting and blood sampling
Mice in all groups were sacrificed at different time course after intervention. They were either perfusion-fixed for immunohistochemistry (IHC) or sacrificed by a Home Office Schedule 1 procedure. The brain or relevant neural tissues were then excised, snap frozen in liquid nitrogen and stored at -80°C for use in Western blotting analysis (WB). Blood samples were taken from experimental animals immediately before sacrifice (or immediately prior to perfusion) and were centrifuged at 3000 rpm for 4 minutes. The supernatant was then carefully removed and stored at -80°C.

2.3 Behavioural studies
The behavioural studies were conducted using a dedicated conditioning chamber (Figure 2.2).

Figure 2.2 Conditioning Chamber.
The back and the side walls of the chamber are made of aluminium, whereas the front door and the ceiling are made of transparent Plexiglas. The floor of the chamber consists of 36 stainless steel rods (1 mm diameter) spaced 0.5 cm apart (centre-to-centre). The rods are wired to a shock generator and scrambler for the delivery of foot shock. Prior to testing, the chambers were cleaned with a 5% sodium hydroxide solution scented with 0.5% almond extract, and pans containing a thin film of the same solution were placed underneath the grid floors. Background noise (60dB, A scale) was provided by means of a fan positioned on one of the side walls. The infra-red video camera mounted in front of the chamber was the main element of an automated computer-assisted, video-based system used to estimate freezing behaviour (Freezeframe, Actimetrics Software). It also allows the animals’ behaviour to be observed and recorded by an experimenter both on- and off-line.

2.3.1 Fear conditioning

Fear conditioning refers to the mechanism by which organisms learn to fear a new stimulus. It plays a normal and essential role in activating the adaptive behaviours that function to defend the organism against environmental threats (Fanselow, 1984). For this defensive reason, it has evolved to be an extremely rapid and robust form of learning that can occur both by the subject experiencing a traumatic event or by watching others display a fear reaction (Mineka & Cook, 1993). The rapid and robust properties of this type of learning make it readily transferable to the laboratory setting where it has been used successfully in rats and mice. For laboratory use, fear conditioning is achieved by associating a particular neutral stimulus, such as a tone, with the fear stimulus, such as a brief electric shock to the animal’s paw, (Pavlov, 1927; Maren, 2001). Thus, fear conditioning contributes to the behaviour that occurs immediately after the first presentation of an aversive or threatening stimulus.
Fear conditioning experiments use the standard Pavlovian nomenclature, where the ‘foot-shock’ or fear stimulus that reinforces learning is termed the Unconditional Stimulus (US) and the neutral stimulus (the tone) that occurs concurrently with the US is termed the Conditional or dependent stimuli (CS). The CS is the stimulus that is the one capable of evoking a learned defensive or fear response termed the Conditional Response (CR). Of note, only the stimuli that have a dependent relationship with the US become a CS capable of producing a learned response (CR) (Rescorla, 1967).

There are several measures that have been used to assess the acquisition of fear as a learned response in the experimental setting in animals. These include fear-potentiated startle, increased blood pressure, defecation, analgesia and freezing. In rats and mice, freezing is a robust response to fear that is readily observed in nature and is also easy to detect by human observers or automated systems. It has the important advantage of not requiring constraint or administration of probe stimuli that could influence behaviour. Although the CS used in most conditioning experiments is a temporally discrete event, such as a tone or light, the unique features of the specific environment in which the fear ‘shock’ stimulus is applied are also in a dependent or conditional relationship with the fear stimulus. In fact, the environmental stimuli provide a context for the shock (US), being present before, during and after this stimulus. The context can also act as a CS and helps to mediate the learned response to a US. Testing for conditional freezing behaviour immediately after a shock provides a convenient test of short-term memory, whereas either the context or a discrete CS at a presented a remote time point from the training session is a test of long-term memory (Fanselow, 1980a; Fanselow, 1986). The basic neural circuitry responsible for fear
conditioning is well described (Figures 2.3 and 2.4). The hippocampus is important for context but not auditory conditioning (Kim & Fanselow, 1992). Hippocampus-independent (tone fear) memories and hippocampus-dependent (context fear) memories and can be measured using the same freezing response paradigm. This provides a useful analytical tool for implicating the involvement of particular brain regions during investigations of behavioural responses to inflammation and surgery.

Figure 2.3 Diagramatic representation of the primary brain circuit for contextual fear. Adapted from Hunt PS, et al. Dev Psychobiol 2007, 49: 649-663
Figure 2.4 Simplified schematic of the neural circuitry involved in fear conditioning. The diagram highlights the signalling pathways for different classes of input conditioning stimuli (CS) starting at the thalamic level (black), aversive reinforcing unconditional stimuli (US) inputs (grey) and regions where CS-US integration occurs (blue) then response-generation pathways (red). Adapted from a presentation by M.S Fanselow ‘Features and fundamentals of Pavlovian Fear Conditioning’.

2.3.2 The trace fear conditioning experimental paradigm
Trace fear conditioning (TFC) is here described as an example of possible implementation of a pavlovian fear conditioning paradigm, as other paradigms have been used in this thesis. In TFC each behavioural testing session was carried out at time course 1, 3 or 7 days. On the day of testing, the mice were transported to the behavioural room and placed into the conditioning chamber. Training consisted of a 198-second acclimatization period. It was followed by six trace fear conditioning trials, each cycle lasting 214 seconds. Trace fear conditioning consists of playing a tone (16 seconds, 2 kHz), followed by a trace interval lasting 18 seconds and a foot-
shock (2 seconds, 0.9 mA). The inter-trial interval, defined as the time elapsing from the end of each tone to the onset of the next, is of 198 seconds.

Tone ———— 16 sec ———— 198 sec ————
Shock ———— 18 sec ———— 2 sec ————

**Figure 2.5** Trace Fear Conditioning testing procedure. An electric shock is delivered 18 seconds after a tone. The test is repeated six times.

The data were elaborated by a computer-based system (Freezeframe, Actimetrics Software) that was used to retrieve the information in a video format, recorded by the infra-red camera positioned in front of the chamber. Freezing was recognized by the software as a total lack of movement, including absence of fur, vibrissae and skeletal movement (except for breathing), whilst significant muscle tone is shown. The percentage of time spent freezing over the total time spent in the chamber to accomplish the test was used to score the hippocampal-dependent memory and learning abilities. A decrease in the percentage of time spent freezing indicated impairment of these abilities. Figure 2.5 shows a schematic representation of the experimental procedure.

### 2.4 IMMUNOHISTOCHEMISTRY

Immunohistochemistry combines anatomical, immunological and biochemical techniques to enable specific proteins to be identified by means of a specific antigen/antibody reaction tagged with a visible label. This technique enables the distribution and localisation of specific proteins within a tissue to be visualised. It involves staining the cells by means of antibodies against a target antigen expressed in tissue. This first antibody can be labelled directly but, more frequently, a secondary
antibody directed against the first (an antiIgG) is labelled as, unlike the primary antibody, this can be added in excessive concentrations to improve the strength of the labelling signal. This second antibody is conjugated either with fluorochromes or appropriate enzymes that can be used to generate colourimetric reactions so that the location of the primary antibody and, thus, the target antigen, can be recognised. The term immunohistochemistry is the demonstration of a target antigen in tissue by detecting specific antibody-antigen interactions where the antibody has been tagged with a visible label (Stanley et al., 1995).

2.4.1 Tissue fixation

For immunohistochemical analysis, it is essential that the antigenic sites in tissue are preserved and accessible, and that the morphology of the tissues and cells is retained. Fixation of tissue is, therefore, necessary to avoid decomposition of its structure, to prevent diffusion of soluble tissue components, to arrest endogenous enzymatic activity, and to protect the tissue against the deleterious effects involved with the various stages of the immunohistochemical process. Fixatives help tissue preservation, but have the disadvantage of cross-linking tissue components so that it can become difficult for the immunochemical reagents to penetrate the tissue to reach antigenic sites. Four variables must be considered in determining the optimal fixation conditions for immunohistochemical localisation, namely: (i) preservation of tissue morphology; (ii) immobilisation of the antigen; (iii) preservation of antigen immunoreactivity; and (iv) adequate penetrability of tissue to the immunochemical reagents. The optimal fixation conditions with respect to the first two variables usually involves rapid and complete fixation, whereas the optimal conditions for the second two variables are best achieved with the lowest possible degree of fixation. Therefore, the most appropriate fixation conditions are a carefully balanced
compromise between these considerations. Fixation tends to lower immunoreactivity and penetrability of immunoreagents leading to well-defined tissue morphology but low intensity immunolabelling. On the other hand, less than optimal fixation is usually associated with an intense immunohistochemical signal that is diffuse and poorly localised (Berod et al., 1981).

Formaldehyde-based fixatives stabilise tissues by reacting primarily with basic amino acids to form methylene cross-links. The number of methylene bridges formed depends on the concentration of formaldehyde, temperature, pH and time of exposure (Puchtler & Meloan, 1985). The disadvantages of using this type of fixative include shrinkage and distortion of the tissue and, therefore, the antigenic sites. Formaldehyde is the most commonly used fixative in immunohistochemistry because of its high degree of tissue penetration and its good preservation of immunoreactivity (Berod et al., 1981).

The fixation procedure used for immunohistochemical experiments in this thesis was as follows: Under terminal anaesthesia with pentobarbitone, animals were euthanized by transcardial perfusion with cold heparinised 0.1M phosphate buffer solution (PBS) followed by 4% paraformaldehyde in 0.1M PBS at pH 7.4. The brain was harvested and post-fixed in 4% paraformaldehyde in 0.1M PBS at 4°C and subsequently left for 12 hours in 15% sucrose in 0.1M PBS and 30% sucrose in 0.1M PBS for further 48 hours both at 4°C. The sucrose solution provides cryoprotection to prevent contraction and expansion of the tissue during freezing and defrosting procedures that would otherwise disrupt tissue integrity. After freeze-mounting the brains in embedding
medium, 30μm coronal sections of the area comprising the hippocampus were cut sequentially in groups of 4 on a cryostat and thaw-mounted onto slides.

2.4.2 Antibody staining

Antibodies are serum glycoproteins, known as immunoglobulins (IgGs), which are secreted by terminally differentiated B cells (lymphocytes). In the vertebrate immune system, antibodies are produced as a defence against foreign substances. The IgG molecule consists of a pair of light amino acid chains and a pair of heavy chains joined together by disulphide bonds. There are two antigenic binding sites on each IgG molecule, which are formed by the N-terminal regions of the light and heavy chain pairs (the Fab fragment). The specificity of the antibody for an antigen is determined by the amino acid sequence of the variable domains within the Fab fragment (Figure 2.6). The antibody only binds to a specific portion of the antigen, the epitope, which is typically made up of 3–8 amino acids (Koss and Melamed, 2005). Antibodies may, thus, be used as biochemical tools to identify and locate specific amino acid sequences within cell components.

Polyclonal antibodies can be made by injecting the antigen (isolated cell constituent or molecule) into a host animal (e.g. goat, rabbit or mouse) to stimulate production of multiple B-cell clones, each of which produces a single antibody specific to the antigen. The animal plasma is then harvested and the clotting factors are removed to produce a serum containing the antibodies (antiserum). A polyclonal antiserum will contain multiple antibodies each with varying affinities and specificities (recognising different epitopes of the same antigen). Polyclonal antibodies have the advantage over monoclonal antibodies in that they contain a mixture of high and low affinity
antibodies identifying multiple epitopes of the target protein, which encourages antigen-antibody interactions. However, polyclonal antibodies require purification from antisera (absorption with unwanted antigens and affinity purification with the pure antigen) to prevent non-specific labelling of tissue (Koss and Melamed, 2005). By contrast, monoclonal antibodies are produced by fusing lymphocytes from immunized animals with a myeloma cell line. The resultant hybridoma producing the antibody is then grown in tissue culture. Thus, monoclonal antibodies consist of a single molecular species with specificity restricted to a single epitope (Cuello et al., 1983).

2.4.3 Antibody labelling
The location of the antigen-antibody complex in tissue can be labelled using one of two methods. Immunofluorescence involves the conjugation of either the primary but usually a secondary antibody with a fluorescent substance (fluorophore), such as fluorescein isothiocyanate (FITC), enabling the site of the bound antibody to be visualised under light of a particular wavelength required to excite the fluorophore (Figure 2.6). Where a secondary antibody is used, it must be generated against the immunoglobulins of the primary antibody source, e.g., if the primary antibody is raised in rabbit, then the secondary antibody should be raised in another species against rabbit IgG. For example, a monoclonal antibody, CD11b (low endotoxin, clone M1/70.15, labelling mouse CD11b, raised in rat, Serotec, UK) was used to label microglia in hippocampal tissue sections. Visualisation of immunoreactivity for CD11b was achieved a fluorescent secondary goat anti-rat antibody conjugated to the fluorophore FITC. Use of secondary antibody labelling methods requires a blocking step to obstruct the sites responsible for non-specific binding of antibodies to tissues. Normal serum is the most popular blocking agent for immunohistochemical staining.
The principle is that non-immune serum from the host species of the secondary antibody is applied to the tissue at the beginning of the procedure and will adhere to protein-binding sites either by non-specific adsorption or by binding of specific, but unwanted, serum antibodies to antigens in the tissue (Pierce Protein Research Products, 2009a).

Figure 2.6
Schematic diagram illustrating immunohistochemical labelling using a fluorophore-conjugated secondary antibody.

The second antibody labelling method involves the use of secondary antibodies conjugated to enzymes, such as horseradish peroxidase or glucose oxidase. The horseradish peroxidase labelling procedure requires the addition of 3,3’-diaminobenzidine tetrachloride (DAB) as a chromagen substrate for the bound peroxidase enzyme, which produces a coloured ‘brown’ precipitate on reaction with peroxidase (Koss and Melamed, 2005) (Figure 2.7). Final analysis of this staining method is complicated by the presence of endogenous peroxidase activity in the cells.
and tissues resulting in non-specific staining of the tissue or false positives (Ramos-Vara, 2005). Several methods have been devised to inhibit or destroy endogenous peroxidase activity after tissue fixation, which is termed a quenching step. The most common methods are pre-incubation of tissue sections in a dilute solution of hydrogen peroxide (3% H$_2$O$_2$ in PBS, or H$_2$O$_2$ in methanol), which should occur prior to the addition of blocking serum and the primary antibody. This is to ensure that the peroxidase inhibitor does not affect immunoreactivity of the primary antibody (Pierce Protein Research Products, 2009a).

2.4.4 Avidin-biotin system
The avidin-biotin peroxidase (ABC) technique is a method of amplifying the signal from a primary-secondary antibody interaction that employs a variation on the horseradish peroxidase labelling technique. Avidin is a 68,000 molecular weight glycoprotein with an extraordinarily high affinity for the small molecular weight vitamin, biotin. This affinity is several million times higher than that of antibody for most antigens. Hence, the binding of avidin to biotin (unlike antibody-antigen interactions) is essentially irreversible. In addition to this high affinity, the ‘avidin/biotin system’ can be effectively exploited because avidin has four binding sites for biotin and most proteins (including antibodies and enzymes) can be conjugated with several molecules of biotin (Koss and Melamed, 2005). The first step in detecting a primary antibody is the addition of a biotinylated, affinity-purified secondary antibody. The biotinylated secondary antibody can then be detected using addition of preformed macromolecular complex between avidin and biotinylated horseradish peroxidase enzyme, which still retains biotin-binding sites (e.g., the Vectastain ABC system) (Vector Laboratories, 2004) (Figures 2.6 and 2.7).
The ABC system has several advantages. It increases the enzyme label at the tissue antigen site and provides increased detection efficiency. It requires less primary antibody and involves a short assay time. Against this, the ABC complex is large, and may hinder tissue penetration in some applications. If the avidin-biotin complex becomes too large to penetrate the tissue, an alternative method can be used, called the ‘labelled avidin-biotin (LAB)’ method. This employs an avidin-enzyme conjugate (or streptavidin-enzyme conjugate) to detect the bound biotinylated-primary antibody on the tissue section. This smaller complex allows better tissue penetration (Pierce Protein Research Products, 2009a).
2.4.5 Microscopy and image analysis

Micrographs of immunolabelled tissue were obtained using an Olympus BX-60 microscope, with a green fluorescence filter (rhodamine, 530 nm) for the fluorescent stained slides, or normal light for the DAB-labelled slides and captured with a Zeiss KS-300 colour 3CCD camera.

Figure 2.8 Scoring of glial activation visualised by CD11-b staining with the DAB technique in the hippocampus of mice. Magnification 40x. Graded from 0 to 3 as a measure of the degree of microglial stained slides.

2.4.6 Qualitative (semi-quantitative) evaluation of CD11b-stained microglia in hippocampal tissue

The qualitative rating was based on a 4-point categorical scale modified from the scale developed by Colburn and colleagues (Colburn et al., 1999). It depends on a
combined evaluation of the level of glial (microglia and/or astrocytes) activation based on cell morphology and immunoreactivity as explained in table 2.1.

Assessment of the qualitative rating scale was performed in a single blinded fashion by an observer who was not informed about the prior treatment of the tissue. Figure 2.8 contains representative photomicrographs of microglia as an example from each condition tested illustrating the categorical rating scale (images visualised with DAB).

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation state</td>
<td>Resting</td>
<td>Mild</td>
<td>Moderate</td>
<td>Intense</td>
</tr>
<tr>
<td>Criteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ramified cells with fine processes.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very low CD11b immunoreactivity.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells evenly distributed throughout the hippocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ramified, evenly spaced microglia.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slight increase in the number or density of cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertrophy of cell bodies and retraction of processes, apparent amoeboid morphology.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shortened and clumpy processes, densely concentrated in the hippocampus with slight overlap between the cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overlap between cells and increased CD11b immunoreactivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1
Morphological, immunoreactive state and scoring of microglial activation (modified from Colburn et al., 1999).
2.4.7 Data analysis
Data were summarised with descriptive statistics; usually mean ± SEM (unless otherwise stated). Statistical analysis was performed with analysis of variance followed either by the Student-Newman-Keuls test for numerical data or the Dunn’s comparison for categorical data. A $p < 0.05$ was considered to be of statistical significance.

Power calculations were carried out to estimate the minimal requirements, in terms of sample size for each experiment in order to achieve statistical significance for the endpoint tested. For example, immunohistochemical identification of activated microglia and astroglia requires a cohort size of $n=8$ to show a 30% change with 80% power at 5% significance. Likewise, identification of 30% maximal change in the mean, with 80% power at 5% significance required a cohort size of $n=10$ in the Trace Fear Conditioning paradigm.

2.5 WESTERN BLOTTING
Western or immunoblotting coupled with polyacrylamide gel electrophoresis are techniques used to detect a target protein in a tissue sample (containing a complex mixture of proteins). The first step, gel electrophoresis, refers to the technique of using a cross-linked polymer matrix, or gel, to support and separate protein molecules derived from homogenised tissue samples on the basis of size, shape, or isoelectric point under an electric current. The separated proteins are transferred to a nitrocellulose membrane and Western blotting describes the subsequent use of a polyclonal or monoclonal antibody specific to the cytokine or other protein of interest in order to label it. Thus, the combination of these techniques can be used to
determine the presence and/or relative abundance of a protein of interest (O’Neill and Bowie, 2001)

In this thesis, both techniques were used to detect and quantify proteins markers present in activated microglial that were derived from frozen hippocampal tissue samples harvested from mice. Hippocampal samples (and a positive control for the marker being assayed) were thawed to 4°C and homogenised in lysis buffer. The lysates were centrifuged and the supernatants collected. Total protein concentrations were then determined by protein assay and used to normalise protein concentrations to 10µg per sample. Samples were then denatured and negatively charged by exposure to a sodium dodecyl sulfate detergent. Then they were loaded in small wells into the gel and separated by electrophoresis. The separation process involves applying an electric current to the gel and allowing the proteins to migrate though the matrix. Bands separate in each sample lane on the basis of protein component size. A molecular weight marker that produces bands of known size is used to help identify proteins of interest. After separation, the proteins can be transferred onto a nitrocellulose membrane. The transfer process operates on the same principle as separation, except that the electric current is applied at 90° to the gel so that the proteins migrate out sideways onto a nitrocellulose membrane.

After transfer to the membrane, immunoblotting can be carried out on the proteins. As with immunohistochemistry, the first stage in this process is to block non-specific protein interactions between the membrane and the antibody. This is achieved by placing the membrane in a solution of non-fat dry milk and mild detergent. Depending on its specificity and affinity for the target protein, the primary antibody can also be
diluted in the blocking solution to reduce non-specific labelling. Membranes are usually incubated with the primary antibody over night. This is followed by washing steps and then application of the secondary antibody, which should detect and label the bound primary antibody. Secondary antibodies are typically conjugated to horseradish peroxidase enzyme and can be located by applying a suitable enzyme substrate. The peroxidase reaction product is then visualised using a system for detection of chemiluminescence. Images of the labelled bands can then be captured and densitometric analysis performed to quantify the immunoreactivity from each sample band according to relative label intensity. In this way, a measurement of the relative amount of immunoreactive protein in each sample can be compared using statistical analysis. This enables the effect of different experimental treatments on the level of activated microglial protein in hippocampal tissue to be assessed.

2.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

2.6.1 Introduction

Enzyme-linked immunosorbent assay (ELISA) is a plate-based assay employed to detect the presence, and quantify an analyte. It is also a specific and highly sensitive method for quantitative measurement of cytokines. With this technique, the antigen is immobilised onto a solid surface by binding with monoclonal antibody coated onto the wells of a polystyrene microtitre plate. The antigen-antibody binding product is then complexed with an antibody linked to an enzyme. As with immunohistochemical techniques, this is usually horseradish peroxidase (HRP) or alkaline phosphatase (AP) (Crowther, 1995). Enzyme activity is measured by incubation with a substrate to produce a coloured reaction product. The colour intensity is proportional to the amount of analyte bound and the concentration can be determined by comparison to a
standard curve developed in the same plate with known concentrations of the analyte standard. The sensitivity of the ELISA is dependent on the affinity of the antibodies and the efficiency of the amplification technique used (Crowther, 1995; http://www.mabtech.se)

2.6.2. Sandwich assay

The ‘sandwich assay’ is the most effective ELISA assay type. Here, the target antigen is bound between two primary antibodies – the capture antibody and the detection antibody to provide an assay that is both sensitive and robust. The assay is made highly sensitive by having two antibodies each raised against a different and non-overlapping epitope from the target antigen (O’Neill and Bowie, 2001). The basic stages of the sandwich assay procedure are illustrated in figure 2.9.

Figure 2.9
Schematic diagram illustrating the detection of target protein using the sandwich ELISA technique
2.6.3. ELISA procedure

The first stage involves adsorption of the capture primary antibody diluted in an alkaline buffer, such as phosphate buffered saline (pH 7.4), to the assay plate. This is achieved through passive adsorption of the protein to the plastic of the plate as a result of hydrophobic interactions between the plastic and non-polar protein residues of the antibody (O’Neill and Bowie, 2001). The coating can be done by the manufacturer of the ELISA kit to standardise inter-assay variability during the coating procedure. Pre-prepared ELISA kits are available for the detection of specific cytokines and other targets, including TNFα, interferon gamma (IFN gamma) and interleukin 6 (IL-6). Following washing steps to remove unbound antibody, blocking buffer is added to ensure that all remaining available binding surfaces of the plastic plate are covered. The buffer is a solution of irrelevant protein, mixture of proteins, or other compound, that passively adsorbs to binding surfaces on the plate that are not already bound with capture antibody. The failure to provide an effective blocking step will result in excessive background and a reduced signal: noise ratio from the assay (Crowther, 1995; Crocker and Murray, 2003)

Washing stages between the additions of reagents are carried out using a physiological buffer, such as Tris-buffered saline (TBS) or phosphate-buffered saline (PBS). Washes are necessary to remove non-bound reagents and to decrease background, thereby increasing the signal: noise ratio. Samples tested in ELISA include serum, plasma and supernatants from solubilised tissue samples. The samples are loaded in duplicate to improve accuracy. Standard concentrations of the cytokine or protein of interest are prepared in the same diluent buffer as the samples. Blank wells are included on the plate in order to correct the final colourmetric readings for
background. Following washes after incubation of samples in the plate, the second primary antibody is added for an additional incubation period. This is followed by the secondary antibody conjugated to HRP. For development of the final colour reaction, several HRP substrates can be used, including 2,2’-azino diethylbenzothiazoline-sulfonic acid (ABTS), 5-aminosalicylic acid (5AS) and tetramethylbenzidine (TMB). Termination of the reaction is achieved by adding a ‘stop solution’ containing a strong acid or base to denature the HRP enzyme. The optical density of each well is measured using a microtitre plate reader set to the appropriate wavelength for the particular colour reaction product. Concentrations of the cytokine or protein of interest are derived from the standard curve (Crocker and Murray, 2003, Crowther, 1995).

2.7 FLOW CYTOMETRY

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle’s relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. A flow cytometer is made up of three main systems: fluidics, optics, and electronics (Loken, 1990).

The fluidics system transports particles in a stream to the laser beam for analysis. The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors. The electronics system converts the detected light signals into electronic signals that can be processed by a computer. For some instruments equipped with a sorting feature,
the electronics system is also capable of initiating sorting decisions to charge and deflect particles. In the flow cytometer, particles are carried to the laser intercept in a fluid stream. Any suspended particle or cell from 0.2–150 micrometers in size is suitable for analysis. Cells from solid tissue must be disaggregated before analysis. When particles pass through the laser intercept, they scatter laser light. Any fluorescent molecules present on the particle fluoresce. The scattered and fluorescent light is collected by appropriately positioned lenses. 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 particle or event. The characteristics or parameters of each event are based on its light scattering and fluorescent properties. The data are collected and stored in the computer. This data can be analyzed to provide information about subpopulations within the sample.
Chapter Three

Preliminary experiments leading to the development of a surgical model for the study of Post-Operative Cognitive Dysfunction
Contents

3.1. Introduction 90

3.2. Methods 92

3.2.1 Experimental groups 92
3.2.2 GFAP Western blotting 94
3.2.3 CD11b and GFAP immunohistochemistry 95
3.3.4 Behavioural study: Trace fear conditioning 97

3.3. Results 100

3.3.1 GFAP expression in the hippocampus was not increased in rats undergoing laparotomy under general anaesthesia. 100

3.3.2 GFAP expression in the hippocampus was not significantly different between groups of rats undergoing anaesthesia alone, laparotomy under general anaesthesia, or orthopaedic (tibial) surgery. 102

3.3.3 The impairment of consolidation of hippocampal memories after trace fear conditioning is short lived following tibial surgery in mice and the impairment can be mitigated by minocycline treatment. 104

3.3.3.1 Trace fear conditioning tests after tibial surgery 104
3.3.3.2 Contextual fear testing after tibial surgery 106
3.3.3.3 Acoustic cued memory testing after tibial surgery 108

3.3.4 Astroglia are not activated following anaesthesia or surgery of the tibia. 110

3.3.5 Microglia activation occurs after orthopaedic surgery of the tibia. 113

3.4. Discussion 115
3.1 INTRODUCTION

The pathogenesis of POCD continues to be controversial and, as previously explained in this thesis, several theories are currently being proposed in an attempt to describe it, many emphasizing the role of the anaesthetics; yet none of them fully able to explain it.

The complexity of the pathophysiology of POCD, its incidence, impact on the aged population and the heterogeneity of its manifestations, necessitate the development of an animal model to facilitate the study of this condition.

Mice and rats are a good option for an experimental model since they usually develop homogeneous surgical lesions that are easy to replicate. Also, rodents are often used for the study of memory, especially when this needs to be coupled with an impairment of the normal neurological homeostasis (Havekes and Abel, 2009).

Evidence suggests that, whereas the peripheral nervous system relies heavily upon the systemic adaptive immune system for protection against ischaemia, infection, and other types of injury (Medzhitov and Janeway, 2000), the central nervous system (CNS) relies upon resident glia (i.e. microglia, astrocytes, and ependymal cells), neurons, perivascular cells, and choroid plexus cells to provide innate immune responses that are capable of differentiating between self and non-self pathogens (Nguyen et al., 2002; Elward and Gasque, 2003; Griffiths et al., 2007).

My hypothesis for POCD is that a peripheral inflammatory message derived from a surgical insult is translocated into the brain to activate resident glial cells. Activated
glial cells then produce inflammatory mediators, which sustain and perpetuate a vicious cycle of glial activation, neurodegeneration and cognitive dysfunction.

The hippocampus is the most likely target tissue for investigation in an experimental model, since it represents the brain area where the initial memory engram is built up after a connection between sensory processing and memory formation is made, at the early stages of cognition, before the progression towards more complex associative learning constructs (Amadio et al., 2004).

The objective of the present study was to produce a surgical lesion under general anaesthesia in rodents, which would be followed by the appearance of detectable markers of peripheral and then hippocampal inflammation, possibly coupled with an equivalent level of measurable memory impairment.
3.2 METHODS

3.2.1 Experimental groups

Following 1 week of adaptation in a light/dark cycle, Wistar rats were randomly divided into four groups: anaesthesia (A), orthopaedic surgery (S), laparotomy (P), and negative control (C). Also, C57BL6 mice were randomly assigned to five groups: orthopaedic surgery (S), surgery and minocycline (M), anaesthesia (A), positive control (L) and negative control (C).

Group A was given an anaesthesia protocol without surgery: isoflurane 1.5 MAC, which corresponds to a concentration in air of 2.1% and buprenorphine 0.1mg/kg subcutaneously (sc), given to provide approximately 20 minutes of deep anaesthesia and analgesia. The animals in group A did not undergo any surgery and did not receive any other pharmacological treatment.

Under general anaesthesia (as in group A) and aseptic conditions, rodents in group S were subjected to an open tibial fracture with intramedullary fixation.

Opened diaphyseal surgery of the tibia in the left hind leg was performed as previously described in Chapter 2, section 2.2.3. Following surgery, the skin was sutured and intra-operative fluid loss was replaced with a subcutaneous injection of 0.9% NaCl solution. The long-acting analgesic buprenorphine was given at induction and provided intra- and post-operative analgesia.
In group M, in addition to the same surgical protocol described above, the animals were given an intraperitoneal (i.p.) injection of minocycline 40 mg/kg two hours prior to surgery and once daily until they were sacrificed.

In group P, rats underwent laparotomy, consisting of a 3 cm long abdominal skin incision under general anaesthesia (provided as previously described for the S group). After thorough disinfection of the surgical area with chlorhexidine gluconate 0.5% and isopropyl alcohol 70%, the abdominal cavity was exposed and left opened for 10 minutes, after which it was sutured and disinfected again. Intra-operative fluid loss was replaced with a subcutaneous injection of 0.9% NaCl solution. Buprenorphine was injected subcutaneously at induction of general anaesthesia to provide intra- and post-operative analgesia.

Lipopolysaccharide 3mg/kg ip was given to mice in group L. It has been used as a positive control for peripheral and neuroinflammation, since LPS administration causes microglial activation (Qin et al., 2007) and sickness behaviour (Kelley et al., 2003).

Group C was composed of naive animals.

Animals in all groups were allowed free, unrestricted food and water intake and weight bearing in cages, and the same happened following recovery from anaesthesia for the groups exposed to anaesthetics.
Mice in all groups, except C, were sacrificed at day 1, 3 and 7 after intervention. They were perfusion-fixed for immunohistochemistry (IHC), or used for behavioural assessment. The rats, which were used for Western blotting (WB), were sacrificed at time course of 2, 4, 8, 12 and 24 hours after any intervention. A blood sample was taken from all animals immediately prior to decapitation and processed as described in Chapter 2. The brain was harvested and the hippocampus isolated, excised, snap frozen in liquid nitrogen and stored at -80°C.

3.2.2 GFAP Western blotting

Hippocampal tissue from rats was collected as previously described. Frozen tissue samples were thawed to 4°C and homogenized in lysis buffer (pH 7.5, 20mM tris(hydroxymethyl)aminomethane [Tris]-HCl, 150mM NaCl, 1mM Na2DTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na3VO4, 2mM DL-dithiothreitol, 1mM phenylmethanesulfonyl, and 1 μg/ml leupeptin). Lysates were then centrifuged (3,000 G for 10 minutes). Supernatants were collected and the total protein concentration determined by protein assay (Bio-Rad, Herts, United Kingdom). Samples, including positive control tissue (normalized for protein concentration to 10μg per sample) and a biotinylated molecular weight marker (New England Biolabs) were denatured in Laemmli sample loading buffer (Bio-Rad) at 70°C for 10 minutes. Samples were then separated by 10.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrotransferred to a nitrocellulose membrane in transfer buffer (Invitrogen, United Kingdom). Prior to transfer, membranes were pretreated with blocking solution (5% non-fat dry milk in Tween-containing, Tris-buffered saline [10mM Tris, 150mM NaCl, 0.1% Tween, pH 8.0]). The membranes were then incubated with anti-GFAP primary antibody (goat
anti rat, 1:1,000 in blocking solution; Serotec, United Kingdom) overnight at 4°C. The horseradish peroxidase–conjugated mouse antibody to goat IgG (1:2,000; New England Biolab) was then used to detect the primary antibodies. The bands were visualised with an enhanced chemiluminescence system (ECL; Amersham Biosciences, Little Chalfont, United Kingdom), and intensities were quantified by densitometry. The membranes were then stripped and re-probed with antibodies against the housekeeping protein, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:10,000 Sigma). The resulting chemiluminescence signal from GAPDH housekeeping protein was used to normalise the GFAP signal from samples, which and were expressed as fractions of naive tissue controls. Densitometric analysis of gel bands was performed using QWIN image analysis software (Leica Microsystems). Images of the bands were captured under standard light conditions, and the optical densities of pixels sampled from images of each gel band were measured using a standard-sized area selection tool. Average pixel measurements were obtained from a minimum of three Western blots and compared between experimental groups using a one way ANOVA and post-hoc Tukey test.

3.2.3 CD11b and GFAP immunohistochemistry

Under terminal anaesthesia with pentobarbitone, the IHC group was euthanized by transcardial perfusion with cold heparinised 0.1M phosphate buffer solution (PBS) followed by 4% paraformaldehyde in 0.1M PBS at pH 7.4. The brain was harvested and post-fixed in 4% paraformaldehyde in 0.1M PBS at 4°C and subsequently left for 12 hours in 15% sucrose in 0.1M PBS and 30% sucrose in 0.1M PBS for further 48 hours. After freeze-mounting the brains in OCT embedding medium, coronal sections
of the area comprising the hippocampus, 30μm thick, were cut sequentially in groups of 4 and mounted on superfrost plus slides (BDH).

The correct dilutions and incubation periods of each antibody were determined prior to use on experimental tissue.

The monoclonal antibody, anti-CD11b (low endotoxin, clone M1/70.15, labelling CD11b, rat anti-mouse, Serotec, UK) was used at a concentration of 1:200 to label microglia in hippocampal tissue. Visualisation of immunoreactivity for CD11b was achieved with a fluorescent secondary (FITC goat anti-rat, Serotec, UK), at a concentration of 1:200. The polyclonal antibody, rabbit anti-GFAP (Chemicon International, CA, USA), at a concentration of 1:1000 and the HRP-linked goat anti-rabbit secondary at 1:200 (Chemicon International, CA, USA) were used to stain astrocytes with an avidin-biotin reaction visualised using the DAB substrate (see Chapter 2; section 2.4).

A negative control omitting the primary antibody was performed in all the experiments. Pictures of the immunohistochemistry were obtained with an Olympus BX-60 microscope, using a green fluorescence filter (rhodamine, 530 nm) for the fluorescent-labelled slides. Microscope images were captured with a Zeiss KS-300 colour 3CCD camera.

The qualitative rating was based on a 4-point categorical scale modified from the scale developed by Colburn and colleagues (Colburn et al., 1999). It depends on a
combined evaluation of the level of glial (microglia and/or astrocytes) activation based on cell morphology and immunoreactivity (see Chapter 2; Table 2.1).

3.2.4 Behavioural study: Trace fear conditioning

Figures 3.1, 3.2 and 3.3 show a schematic representation of the entire experimental procedure. Each behavioural testing session was carried out on mice, at time course of 1, 3 or 7 days after the intervention. On the day of testing, the animals were transported to the behavioural room and placed into the conditioning chamber. Training consisted of a 198-second acclimatization period. It was followed by six trials of tone-trace-shock fear conditioning cycles, each lasting 214 seconds. Trace fear conditioning consisted of a tone (16 seconds, 2 kHz), followed by a trace interval lasting 18 seconds and a foot-shock (2 seconds, 0.9 mA). The inter-trial interval, defined as the time elapsing from the end of each tone to the onset of the next, was of 198 seconds (see Chapter 2; section 2.3).

The data were elaborated by a computer-based system (Freezeframe, Actimetrics Software). This system retrieved the information in a video format, recorded by the infrared camera positioned in front of the chamber. Freezing was recognized by the software as a total lack of movement, including absence of fur, vibrissae and skeletal movement (except for breathing), whilst significant muscle tone was shown. The percentage of time spent freezing over the total time spent in the chamber to accomplish the test was used to score the hippocampal-dependent memory and learning abilities. A decrease in the percentage of time spent freezing indicated impairment of these abilities.
Figure 3.1 Schematic representation of the training session for the trace fear conditioning protocol. During the first 198 seconds, or baseline, the mice move freely in the new environment aiming to explore it. The green bars in the motion index section of the graph represent the level of movement. Higher bars indicate more activity, whereas absence of the green graph trace indicates freezing. After the baseline period, mice are exposed to a tone (red bars in the sound section of the graph) and, subsequently, to a shock (black thin bars in the shocker section of the graph). Following the shock, there is a brief sharp increase in the motion index, which is dependent of a rapid increase in motion as the animal tries to escape the shock in search for a safe spot, free from aversive stimuli. The tone-to-shock couplings are repeated here 6 times, with an interval between tone and shock of 18 s. Acquisition and consolidation of the memory trace improves over time and number of trials, as shown by progressive rarefaction of the green bars in the motion index section (which implies more learning and memory), towards the end of the test.
Figure 3.2 Schematic representation of the context test after trace fear conditioning. Mice are exposed to the same environment where the training took place. This context is recognised as aversive. Consequently, the activity in the testing box is reduced, so that many episodes of freezing are displayed, due to the high expectation for the presentation of a shock. No tones or shocks are given in this paradigm.

Figure 3.3 Schematic representation of the acoustic-cued test following trace fear conditioning. Mice are exposed to a new context, which, initially, during the baseline period, does not trigger any freezing reaction. The tone is thereafter presented three times, at regular intervals. As a consequence of recognition of an aversive stimulus, mice freeze in high expectation of a shock. The freezing behaviour is more pronounced in the period during which the tone is played and in the immediate aftermath. No shock is given in this paradigm.
3.3 RESULTS

3.3.1 GFAP expression in the hippocampus was not increased in rats undergoing laparotomy under general anaesthesia.

GFAP and GAPDH protein levels were measured for each of the six groups of rats considered: naive and laparotomy at time course 2, 4, 8, 12 and 24 hours after surgery, respectively. GFAP immunoreactivity appeared as a single band at approximately 50 kDa on the SDS-PAGE gel, whereas GAPDH was visualised as a single band at approximately 38 kDa. The values extrapolated for GFAP optical density were normalized against GAPDH, which was quantified with the same method (see Chapter 2; section 2.5). Results are shown as ratio of GFAP/GAPDH optical density. There were no significant differences between the levels of GAPDH for each experimental group, thereby confirming as acceptable the execution of the protein quantification and gel loading (Fig. 3.4, A). Laparotomy, in aseptic conditions, under general anaesthesia, was not followed by increased levels of GFAP at any of the time courses considered, compared to naive counterparts (Fig. 3.4). However, a trend of increasing levels towards 12 and 24 hours time points was noted (Fig. 3.4 B).
Figure 3.4 Western blotting to measure the time course of GFAP expression in the hippocampus of rats undergoing laparotomy. GAPDH immunoreactivity was used to indicate the level of protein sample loaded in each gel lane, these are comparable between the different experimental samples. Densitometric measurements of the optical density of GAPDH immunoreactive bands were then used to normalise the measurement of GFAP immunoreactivity for each sample (A). The optical density of GFAP immunoreactivity (expressed as a ratio of the GAPDH signal) is demonstrated for hippocampal samples from Wistar rats obtained at 2, 4, 8, 12 and 24 hours after laparotomy under general anaesthesia and in naive animals. There was no significant difference between the GFAP signal from naive animals and those undergoing surgery at any time point (B). A trend towards an increase is observed at 12 and 24 hours post surgery (B). Data are expressed as mean ± SEM of densitometry in arbitrary units (A), and ratio between GFAP and GAPDH, n=4.
3.3.2 GFAP expression in the hippocampus was not significantly different between groups of rats undergoing anaesthesia alone, laparotomy under general anaesthesia, or orthopaedic (tibial) surgery.

Protein levels were measured for GFAP and GAPDH in naive rats, or rats subjected to general anaesthesia (GA) alone, or those undergoing laparotomy under GA or orthopaedic surgery under GA, which were sacrificed 24 hours after each procedure, respectively. Again, the values extrapolated for GFAP optical density were normalized against GAPDH, which was quantified with the same method. Results are shown as ratio of GFAP/GAPDH optical density. There were no significant differences in the levels of GAPDH in all the experimental groups (Fig. 3.5, A). Administration of anaesthetics alone, or laparotomy under GA, in aseptic conditions, was not followed by increased levels of GFAP, compared to naive counterparts (Fig. 3.5, B). A trend for increasing optical density levels in the orthopaedic surgery group at the 24 hour post-surgery time point was noted (Fig. 3.5, B), yet this result was not significant, compared to naive rats.
Figure 3.5 Western blotting analysis of GFAP in the rat hippocampus 24 hours after general anaesthesia (GA) alone, or laparotomy under GA, or orthopaedic surgery under GA. Expression of GAPDH, which was comparable in each lane (A), was used to normalise the results. There was a trend towards an increased expression of GFAP in Wistar rats undergoing surgery of the tibia (orthopaedic) under general anaesthesia with isoflurane 1.5 MAC and buprenorphine 0.1mg/kg, compared to naive littermates but this was not significant. Administration of isoflurane and buprenorphine only (anaesthesia) or laparotomy under the same anaesthetic protocol also did not increase GFAP expression relative to naive samples (B). Data are expressed as mean ± SEM of densitometry in arbitrary units (A), and ratio between GFAP and GAPDH. n=4.
3.3.3 The impairment of consolidation of hippocampal memories after trace fear conditioning is short lived following tibial surgery in mice and the impairment can be mitigated by minocycline treatment.

3.3.3.1 Trace fear conditioning after tibial surgery

Trace fear conditioning (TFC) is a validated test to assess learning and memory in the hippocampus (Chowdhury et al., 2005). To elucidate the effect of surgery on hippocampal-dependent learning and memory abilities, a comparison between groups of mice was carried out in the TFC testing paradigm with regards to the percentage of freezing behaviour over the whole amount of testing time. Figure 3.6 shows the mean (± SEM) percentage of time spent freezing over total testing time, which measures hippocampal memory function. Training took place at time points 1, 3 and 7 days post surgery and memory testing was conducted 3 days later. The results for testing of mice trained at 1, 3 and 7 days (Figure 3.6 A, B and C, respectively) reveal important differences between the groups considered. Mice subjected to anaesthetics alone seem to display the same general levels of freezing as naive mice. Interestingly, freezing time was significantly different in the surgical animals trained 1 and 3 days after surgery, but not 7 days after surgery when compared with both the mice exposed to the anaesthetics alone and those in the naive group, (p<0.05). The administration of minocycline to the animals undergoing surgery affected their behaviour after trace fear conditioning, in that these animals expressed a percentage of freezing time that was comparable to animals in the naive control group, at any time point considered.
Figure 3.6 freezing levels in the acquisition phase of trace fear conditioning (during tone-to-tone inter-trial intervals) are impaired at the 6th trial, when trained 1 day after tibial surgery, but not
at 3 and 7 days after surgery. Mice received trace fear conditioning training at 1 day post-surgery (A), 3 days post-surgery (B) and 7 days post-surgery (C). Three days later mice were placed in the same conditioning chamber where training took place and left undisturbed for the first 198 s (baseline) of training. Six trials of tone-trace-shock fear conditioning cycles; each lasting 214 seconds were administered. Each tone was followed by a trace interval lasting 18 seconds and a foot-shock (2 seconds, 0.9 mA). The inter-trial interval, defined as the time elapsing from the end of each tone to the onset of the next (198 seconds), together with the baseline period, were subjected to an analysis of freezing time. Freezing percentage over the entire length of the test was considered to be marker for acquisition of the engram (learning). Overall, there was no difference between the groups (naive [control], anaesthesia, surgery and surgery with minocycline) in the learning phase of trace fear conditioning, except for the 6th intertrial interval, 1 day after surgery (A), when mice undergoing surgery showed an impaired acquisition of the environmental cues compared to naive, anaesthesia and surgery with minocycline groups. Data are expressed as mean ± SEM of the freezing percentage of baseline and intertrial intervals. *p<0.05; n=12.

3.3.3.2 Contextual fear testing after tibial surgery

Contextual fear memory tests were conducted 3 days after trace fear conditioning training performed at 1, 3 and 7 days after surgery (Figure 3.7 A, B and C respectively). Mice who had undergone surgery and then fear conditioning training 1 or 3 days later, froze significantly less in response to presentation of an aversive environment (the chamber in which they received fear conditioning training) than those receiving anaesthetics or naive mice (Figure 3.7 A and B). Pre-treatment of surgical animals with minocycline prevented the surgical-induced change of memory function measured in the context test. There was no significant difference in freezing when training was performed 7 days after tibial surgery compared with naive or anaesthesia-treated mice (Figure 3.7C).
Figure 3.7 Surgery of the tibia caused an impairment of memory functions assessed by the contextual fear test in animals trained up to three days after surgery. Treatment with
minocycline mitigates the negative effects of surgery on memory function in this test. When exposed to the same environment (or context) where trace-fear conditioning training took place, mice recognise the context as aversive and respond by freezing (in the absence of any shock or tone). In this test, the group of mice undergoing surgery froze significantly less than the other experimental groups, at 1(A) and 3 (B) days after the operation, but not after 7 days (C). Treatment of surgical animals with minocycline prevented the surgical-induced change of memory function from occurring (A, B). There was no difference in freezing time between naive, anaesthesia and surgery with minocycline groups at any time point considered (A, B, C). Data are expressed as mean ± SEM of the freezing percentage of overall exposure time to the context. *p<0.05; n=12

3.3.3.3 Acoustic-cued memory testing after tibial surgery

Tests carried out in a new environment to training (non-aversive context) were conducted to assess freezing in response to the tone stimulus used in fear conditioning behavioural training. Acoustic-cued memory testing was conducted 4 days after trace fear conditioning behavioural training performed at 1, 3 and 7 days after surgery (Figure 3.8 A, B and C, respectively). Mice were played the tone stimulus used in training 3 times in a new, non-aversive testing environment. In this test, there was no significant difference in the freezing behaviour of mice trained at 1, 3 or 7 days after surgery compared with mice treated with minocycline undergoing surgery, anaesthesia-treated, or naive mice (Figure 3.8).
Figure 3.8 Recall of acoustic-cued memories is not impaired after tibial surgery or anaesthesia with trace fear conditioning. When exposed to a different environment (new context) from the one
where the training took place, mice do not recognise the context as aversive, but respond by freezing to another cue, the tone, which is played three times. This tone is the same used during fear conditioning training, where it is coupled with the electric shock. In the acoustic-cued test, there was no difference in freezing time between naive, anaesthesia, surgery and surgery with minocycline groups at any time point considered: training at 1, 3 and 7 days (A, B, C). Data are expressed as mean ± SEM of the freezing percentage of overall exposure time to new context and tone. *p<0.05; n=12

3.3.4 Astroglia are not activated following anaesthesia or surgery of the tibia.

Data from Western blotting experiments suggest that surgery does not cause increased levels of GFAP in the hippocampal formation (section 3.32). However, these findings would not exclude occurrence of astrogliosis in absence of significant increased levels of GFAP. To test this hypothesis in my model of POCD, mice were randomly assigned to one of the following groups: naive (C), received no interventions; anaesthesia (A), were subjected to 20 minutes exposure to isoflurane 1.5 MAC (2.1%) and administration of buprenorphine 0.1 mg/kg at induction of anaesthesia. The surgery group (S) underwent tibial surgery under the same anaesthetic protocol as previously described; and the minocycline group (M) underwent surgery as in group S, but mice were also injected with minocycline 40 mg/kg 2 hours before surgery and once daily thereafter, until euthanized. Immunohistochemistry with anti-GFAP of the hippocampus, which was performed on mice sacrificed at 1, 3 and 7 days after the intervention (surgery, anaesthesia or LPS injection), showed no difference between the groups considered. Strikingly, the inclusion of an additional group, treated with LPS 5mg/kg, also showed no difference in hippocampal GFAP staining compared with naive animals.
Figure 3.9 GFAP immunoreactivity of astroglia in the hippocampus. Mice were subjected to anaesthesia alone (A), peripheral injection of LPS (L), tibial surgery (S), or surgery and minocycline (M). Each picture is labelled according to the time course as the animals were sacrificed at days 1, 3, or 7 post-treatment. Scale bar 30μm.
3.3.5 Microglia activation occurs after orthopaedic surgery of the tibia.

The effects of surgery under general anaesthesia on microglial activation were investigated in the hippocampus of mice using immunohistochemistry with antibodies directed against CD11b, a specific marker for microglia. A previous study suggests that microglial activation becomes evident 3 hours after intraperitoneal (ip) administration of LPS 5mg/kg (Qin et al., 2007). Prior to the experiments, a dose-response curve of microglial staining following LPS administration was performed (Fig. 3.10 A). A time course of CD11b immunostaining in the hippocampus at different time points after surgery was also carried out (Fig. 3.10 B). The earliest detectable microglial activation by means of CD11b immunostaining was found 8 hours after surgery. The lowest most effective dose of LPS to detect the highest microglial activation, 24 hours after injection, was 3 mg/kg. A pilot experiment of CD11b immunostaining with a fluorescence-labelling technique, 24 hours after treatment, is shown (Figure 3.11).

![Figure 3.10 Pilot experiments: dose response effect of LPS i.p. injection on microglial activation as assessed by grading of CD11b staining in the mouse hippocampus 24 hours after injection (A) and time course of hippocampal microglial activation following surgery (B). LPS causes an initial...](image-url)
degree of detectable microglial activation at a dose of 500 \(\mu g/kg\). A ceiling effect is reached with doses in excess of 3 mg/kg (A). The first detectable changes in the morphology of microglial cells is visible 8 hours after surgery and peaks after 24 hours. It appears to return to normal a week after surgery (B).

Data are expressed as mean±SEM; *\(p<0.05\) and ***\(p<0.001\) for comparison with naive group. n=3

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**Figure 3.11 Activation of microglia detected with immunoreactivity for CD11b in the mouse hippocampus.** Microglial activation was induced by systemic lipopolysaccharide (LPS) injection or open tibial fracture with intramedullary fixation (surgery), 24 hrs after intervention. Naive animals (control) were used as a negative control. Scale bar 30 \(\mu m\).
3.4 DISCUSSION

The initial strategy, devised to develop a surgical model for POCD, was intended to find a hippocampal marker of inflammation, based on the possible involvement and activation of glial cells.

The astrocytes were targeted, in the first instance, for many reasons. Astroglia, firstly, represent the most numerous glial cell type in the central nervous system. They are in a continuous cross-talk with neurones; capable of responding to neurotransmitters released at the synaptic cleft by the generation of ion gradients and glutamate exocytosis that signal back to neurones (Pasti et al., 1997). Importantly, reactive astrocytosis occurs prominently in response to all forms of CNS injury or disease. The basic process of reactive astrocytosis is characterised by cellular body enlargement and changes in gene expression, and can include proliferation of this cell type after severe insults. Substantial information is available regarding molecules able to induce reactive astrocytosis, such as cytokines and chemokines, and about the subsequent changes in molecular expression stimulated by reactive astrocytosis (Sofroniew, 2005).

The study was performed, initially, on rats, because these rodents, compared to mice, provide larger amounts of brain tissue, allowing for the use of a limited number of animals; especially when a technique like Western blotting is employed, in which a single animal would provide sufficient tissue for a larger number of blottings.
The astrocytic response to an insult, especially traumatic, is marked by intense immunostaining for GFAP (Nolte et al., 2001). However, a surgical insult did not result in increased expression of GFAP as measured by Western blotting nor did it result in an increased intensity of immunostaining, in my model. Nevertheless, the immunohistochemistry was primarily adopted to investigate possible morphological changes in the astrocytes, possibly occurring after an insult, since activation of astroglia may manifest with morphological changes and not necessarily with increased levels of GFAP protein (Kalman and Ajtai, 2000).

Immunohistochemistry using GFAP to label astroglia resulted in no differences in staining after surgery compared with naive or anaesthetic-treated mice. This represented an important negative finding in my quest for suitable hippocampal markers of inflammation after peripheral surgery and caused a substantial decline in the confidence for the initially formulated hypothesis about POCD.

Before any further attempt to visualise a suitable inflammatory marker in the hippocampus was made, consideration had to be given to a critical aspect of this research, which was the measurement of cognitive dysfunction after surgery. Therefore, the next step was to decide whether or not mice undergoing an orthopaedic procedure involving surgery of the tibia would, in fact, develop some degree of cognitive deterioration. The hippocampus was the main target of this research, because of its important role in the formation of short-term memory (Amadio et al., 2004).
C57BL6 mice were chosen to conduct behavioural tests in the trace fear conditioning paradigm, because this animal type would allow for the future use of knockout animals (with genetic deletions of particular cytokines or other inflammatory markers) if positive behavioural results were obtained.

A significant difference in the training of mice undergoing surgery, 1 day after the operation, suggested an impaired acquisition of the fear memories or, more likely, an impairment of learning. This impairment could be explained by the residual pain from surgery but this pain has previously been demonstrated to exert no significant effects on memory function (Fanselow and Baackes, 1982). Furthermore, decreased freezing levels demonstrated in the fear conditioning test in mice undergoing tibial surgery under anaesthesia, 1 and 3 days after surgery, suggests a short-lived, but significant decrease in hippocampal-dependent consolidation of memory. These results assume a particular significance when the recovery in memory function, after minocycline administration in surgical animals, is taken into due account. Minocycline is a wide spectrum antibiotic, belonging to the tetracycline class, with proven anti-inflammatory effects. Evidence for the normalisation of freezing behaviour in animals undergoing surgery after minocycline treatment, in contrast with the reduced freezing behaviour in littermates undergoing surgery without minocycline administration, has two possible explanations. If surgery is likely to have induced hippocampal inflammation and subsequent memory impairment as a consequence of a peripheral inflammatory effect, then the anti-inflammatory properties of minocycline could have aided the recovery of memory functions after surgery and anaesthesia. The second explanation involves a contamination of the surgical wound by an infective agent that is cleared
by minocycline. Infection is a known cause of sickness behaviour and, consequently, memory impairment (Pugh et al., 1998).

The subsequent finding of microglial activation in the pilot study performed on C57BL6 mice does not completely resolve the above issue. This is because microglial activation also occurs after administration of large doses of lipopolysaccharide, the main component of the cell wall in Gram negative bacteria (Qin et al., 2007) and could be already evident after smaller doses, as shown in my pilot.

Further experiments should, hence, be directed at resolving the question of the possible contamination of the surgical wound by an infective agent. The way to tackle the issue is to make a parallel assessment between two groups of mice undergoing surgery: one treated with minocycline, and the other one treated with an antibiotic with a similar spectrum of bactericidal effects, but devoid of any anti-inflammatory properties.

Moreover, it is necessary to make the immunohistochemistry data robust, in the first instance. A power calculation to estimate the minimal requirements, in terms of sample size for this experiment was conducted. Immunohistochemical identification of activated microglia requires a cohort size of n=7 to show a 30% change with 80% power at 5% significance.

Notwithstanding the shortcomings, the aforementioned results were encouraging. The next chapters will help to further clarify the major aspects of this research, from the early stages of development presented in this chapter.
Another note, before closing this chapter, has to be devoted to the results of trace fear conditioning, especially to the acoustic-cued test, which revealed no difference in freezing between the various groups. An impairment of both contextual and acoustic-cued behaviour seems to be necessary in this particular setup of behavioural testing to return stronger evidence of impaired hippocampal recall of fear memories (McEchron et al., 1998; Quinn et al., 2002; Kinney et al., 2002). The reason why there is no evidence of auditory-cued disrupted freezing behaviour in this setup could be dependent on the short duration of the tone presentation in the particular construct of this test, as it was adapted for these experimental purposes by myself. This could have accounted for a certain degree of weakness in the ability of the test to assess acoustic-cued memory function, which will be corrected in future behavioural experiments presented in the subsequent chapters. The other consideration, relates to the fact that this paradigm tested anterograde amnesia, since training followed surgery. Although evidence for a critical role of the hippocampus in anterograde amnesia was previously presented (Misane et al., 2005) debate over its full validity is still ongoing (Phillips et al., 1994). Therefore, in future experiments I will consider a new setup to investigate retrograde, rather than anterograde amnesia. This is because a large body of evidence confirms the superiority of retrograde over anterograde amnesia in terms of the reliability of fear conditioning tests (Quinn et al., 2002; Sanders et al., 2003; Phillips et al., 1994).
Chapter Four

Systemic and hippocampal IL-1β-mediated inflammation underlie cognitive dysfunction following surgery.
Contents

4.1. Introduction 121

4.2. Methods 122

4.2.1 Animals and surgical procedures 122
4.2.2 Conditioning chamber and fear conditioning 124
4.2.3 Cytokine measurement 129
4.2.4 Immunohistochemistry 130
4.2.5 Analysis of flow cytometry 131
4.2.6 Data analysis 132

4.3. Results 132

4.3.1 Surgery elevates plasma concentrations of inflammatory cytokines IL-1β and IL-6 132
4.3.2 Hippocampal IL-1β increases after surgery but not after anaesthesia alone 133
4.3.3 Surgery is followed by microglial activation 136
4.3.4 Surgery-induced, cell-mediated inflammation is characterised by clonal expansion of polymorphonucleates and Gr-1high subset of monocytes in the blood. These cells also marginate to the lungs in response to surgery. 138
4.3.5 Activated microglial cells in the hippocampus do not increase in number 24 hours after surgery. 144
4.3.6 Contextual fear after delay conditioning is impaired in mice undergoing surgery 146
4.3.7 Memory for both context and tone is impaired after surgery in trace fear conditioning 147
4.3.8 Pre-treatment with IL-1ra mitigates the effects of surgery on memory function 149
4.3.9 Contextual and auditory cued fear memory is not impaired in animals undergoing surgery one or three days after delay fear conditioning 150

4.4. Discussion 152
4.1 INTRODUCTION

Cognition, the mental activity involved in memory, attention and perception, may often decline as a result of illness (Forton et al., 2001; Hopkins et al., 2005; Helfin et al., 2005). An impairment of cognitive functions features prominently in major infective diseases (Capuron et al., 1999); the inflammatory response to infection, associated with elevated cytokines in the periphery, signals to the brain to produce an array of symptoms ranging from lethargy to social withdrawal and memory impairment, which is collectively known as sickness behaviour (Dantzer, 2004). The function of sickness behaviour is to promote recovery from illness and injury (Bolles and Fanselow, 1980).

An impairment of cognition has also been shown to develop as a consequence of surgery (Moller et al., 1998). Termed post-operative cognitive dysfunction (POCD), this features disturbance of memory, attention, consciousness, information processing and sleep-wake cycle, leading to postoperative morbidity and mortality (Bohnen et al., 1994; Monk et al., 2008). The highest incidence of POCD occurs in elderly patients (Moller et al., 1998), but other age groups are also affected (Johnson et al., 2002).

The precise pathogenesis of POCD is not known and may involve perioperative as well as patient-related factors (Newman et al., 2007); general anaesthetics have been implicated because animal studies suggest that anaesthetic-induced changes in the brain outlast the elimination of anaesthetic agents from the body (Fütterer et al., 2004) and are capable of producing long-lasting cognitive dysfunction under certain circumstances (Culley et al., 2003). Yet there appears to be no decrease in the
incidence of POCD after regional anaesthesia (Campbell et al., 1993; Williams-Russo et al., 1995); therefore, a causative role for general anaesthetics appears to be unlikely.

Since interleukin-1β (IL-1β) mediates part of the inflammatory response to both infection and injury (Dinarello, 1996) and exerts local, concentration-dependent, effects on hippocampal memory functions (Goshen et al., 2007), we have hypothesized that a surgical intervention induces systemic cytokine release, which is followed by hippocampal inflammation and memory impairment. Using fear conditioning tests to assess cognitive function, we investigated the role of inflammation in general, and IL-1β signalling specifically, as the possible pathogenic mechanism for hippocampal-dependent memory dysfunction following surgery.

4.2 METHODS

4.2.1 Animals and surgical procedures
All the experiments were conducted under Home Office approved licence and were performed using 12-14 weeks old male C57-BL6 mice (Harlan, Oxon, UK) housed in groups of up to 4 animals/cage, under a 14:10 hours light-dark cycle, in a constant temperature and humidity controlled environment, with free access to food and water. Acclimatization to light/dark cycle for a minimum of 7 days preceded any intervention or assessment. All the animals were checked on a daily basis for signs of infection. Evidence of poor grooming, huddling, piloerection, weight loss, wound dehiscence, muscle twitching, back arching and abnormal activity, were recorded.
Mice were randomly assigned to the following groups: surgery under general anaesthesia (S), surgery under general anaesthesia plus minocycline (M), surgery
under general anaesthesia plus enrofloxacin (E), surgery under general anaesthesia plus interleukin-1 receptor antagonist (IL-1ra) (I), general anaesthesia without surgery (A), and no intervention (naive).

Under aseptic conditions, groups of mice were subjected to an open tibial fracture of the left hind paw with an intramedullary fixation as previously described (Harry et al., 2008). Briefly, isoflurane (Abbot Laboratories Ltd., Queensborough, Kent, UK) 2.1% in air (1.5 MAC) (Engelgardt et al., 2006) and buprenorphine (Reckitt Benckiser Healthcare Ltd, Hull, UK) 0.1mg/kg subcutaneously (sc), were given to provide both surgical anaesthesia and extended post-operative analgesia for the surgical intervention and post-surgical recovery. After shaving the overlying skin and disinfecting with chlorhexidine gluconate 0.5% and isopropyl alcohol 70% (PDI, Orangeburg, NY, USA), a fracture of the tibial shaft was created under direct vision. A longitudinal incision was made through the skin and fascia lateral to the tibia to expose the bone. A 0.5mm hole was drilled just above the proximal third of the tibia to insert an intramedullary 0.38mm diameter stainless steel fixation wire. Subsequently, the fibula and the muscles surrounding the tibia were isolated, the periosteum stripped over a distance of 10mm circumferentially and an osteotomy was performed with scissors at the junction of the middle and distal third of the tibia. The skin was sutured with 8/0 Prolene and intra-operative fluid loss was replaced with 0.5 ml of subcutaneously injected normal saline. Mice in groups M, E and I also received an intraperitoneal (ip) injection of minocycline (Sigma, Poole, UK) 40 mg/kg 2 hours prior to surgery and once daily until assessment of outcome, or enrofloxacin 10 mg/kg 2 hours prior to surgery and twice daily thereafter until assessment of outcome or IL-1ra 100mg/kg prior to surgery, respectively. Mice in group A received anaesthesia
(isoflurane 1.5 MAC for 20 minutes) and analgesia (buprenorphine 0.1 mg/kg) with no surgical intervention or other treatment. All the animals were injected twice daily with experimental drugs or equivalent volumes (0.1 ml) of saline. Mice were allowed free, unrestricted food and water intake following recovery from the procedure. Mice from each treatment group were randomly assigned to two different assessment groups for either harvesting blood and tissue samples or for cognitive behaviour. This was done in order to obviate possible confounding effects of fear conditioning testing (Nguyen et al., 1998) on inflammatory markers.

4.2.2 Conditioning chamber and fear conditioning

Fear conditioning is used to assess learning and memory in rodents, which are trained to associate a conditional stimulus (CS), such as a tone, with an aversive, unconditional stimulus (US), such as a foot-shock (Kim and Fanselow, 1992). Freezing behaviour is an indicator of aversive memory that is measured when subjects are re-exposed to the CS. The environment, or context, in which the animals are trained, represents a more sophisticated example of CS. In delay fear conditioning, where the tone and shock are temporally contiguous, lesions of the amygdala disrupt recall of fear responses to both auditory cue and context, whereas lesions of the hippocampus disrupt context-related but not auditory cue-related memories (Kim and Fanselow, 1992; Philips and LeDoux, 1992).

The behavioural study was conducted using a conditioning chamber (Med. Associates Inc., St. Albans, VT, USA) (see Chapter 2; section 2.3). The back- and the side-walls of the chamber were made of aluminium, whereas the front door and the ceiling were of transparent Plexiglas. The floor of the chamber consisted of 36 stainless steel rods
(1 mm diameter) spaced 0.5 cm apart (centre-to-centre). The rods were wired to a shock generator and scrambler for the delivery of foot shock. Prior to testing, the chambers were cleaned with a 5% sodium hydroxide solution. Background noise (60dB, A scale) was provided by means of a fan positioned on one of the sidewalls. An infrared video camera mounted at the front of the chamber captured animal behaviour and video data were stored in a computer compatible format (Video Freeze, Med. Associates Inc., St. Albans, VT, USA).

In order to make the environment (context) different for acoustic-cued behavioural assessment, 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 surface and background noise was removed. Assessment of the acoustic-cued behaviour of tone-to-shock pairings in the novel context, tests amygdalar, rather than hippocampal function in delay fear conditioning (Quinn et al., 2002). On each day of testing, mice were transported to the behavioural room and left undisturbed for at least 20 minutes before placing them into the conditioning chamber. Freezing was recognized by the software as a total lack of movement excluding breathing but including movement of fur, vibrissae and skeleton. The percentage of time spent freezing over the total time spent in the chamber to accomplish the test was used to score memory and learning abilities. A decrease in the percentage of time spent freezing indicated impairment of these abilities.

In delay fear conditioning, training consisted of placing the mouse in the conditioning chamber and allowing exploration of the context for 100 seconds. Next, an auditory cue (75-80 dB, 5 kHz), the conditional stimulus (CS), was presented for 20 seconds.
A 2-second foot-shock (0.75 mAmp), the unconditional stimulus (US), was administered during the final 2 seconds of the CS. This procedure was repeated with an inter-trial interval of 100 seconds, and the mice were removed from the chamber 30 seconds later (Figure 4.1).

Trace fear conditioning (TFC) differed from delay in that the 2-second foot-shock was administered 20 seconds after termination of the tone. In TFC disruption of hippocampal memories is followed by impaired freezing behaviour in both contextual and auditory-cued tests (Chowdhury et al., 2005). After the termination of each trial, which lasted a total of 270 seconds, each mouse was taken individually to the surgery room, within 30 minutes. All the animals, regardless to the specific intervention, underwent the same handling, including naive animals. After testing, animals were returned to their housing cage. Three days after conditioning, mice were transported again to the behavioural room and left undisturbed for 15 minutes. They were returned into the same chamber where training had occurred for a context test lasting 270 seconds, during which no tones or foot-shocks were delivered (Figure 4.2). Freezing behaviour in response to context was recorded by the software. At the end of the test, mice were individually returned to their home cage. Approximately 3 hours later, freezing was recorded in a novel environment and in response to the auditory cue. The novel environment resulted from modifications to the basic chamber consisting of an opaque Plexiglas triangle; a Plexiglas floor; increased illumination; no background noise from the fan; and a different smell. Mice were placed in this novel environment, and time sampling was used as baseline during which freezing was scored for 135 seconds. The auditory cue was then presented for 135 seconds, and freezing was again recorded (Figure 4.3).
Figure 4.1 Schematic representation of the training session for the delay fear conditioning protocol. During the first 100 seconds, or baseline, the mice move freely in the new environment aiming to explore it. The green bars in the motion index section of the graph represent the level of movement. Higher bars indicate more activity, whereas lower green bars indicate less activity. After the baseline period, mice are exposed to a tone (red bars in the sound section of the graph) and, subsequently, to a shock (black and green thin bars in the shocker section of the graph). Following the shock, there is a brief sharp increase in the motion index, which is dependent on a rapid increase in motion as the animal tries to escape the shock in search for a safe spot, free from aversive stimuli. The tone-to-shock couplings are repeated 2 times here, and there is no interval between tone and shock. In trace fear conditioning, instead, a gap lasting 20 seconds is imposed between tone and shock.
Figure 4.2 Schematic representation of the contextual test after delay or trace fear conditioning. Mice are exposed to the same environment where the training took place. This context is recognised as aversive. Consequently, the activity in the testing box is reduced, so that many episodes of freezing (absence of green bars in the motion index section of the graph) are displayed, due to the high expectation for the presentation of a shock. No tones or shocks are given in this paradigm.

Figure 4.3 Schematic representation of the acoustic-cued test following delay or trace fear conditioning. Mice are exposed to a new context, which, initially, during the baseline period, does not trigger any freezing reaction. The tone is thereafter presented as a single, long event. As a consequence of recognition of an aversive stimulus, mice freeze in high expectation of a shock. No shock is given in this paradigm.
4.2.3 Cytokine measurement

Blood was collected by cardiac puncture into heparin coated syringes whilst animals were under terminal anaesthesia with pentobarbital. Samples were centrifuged at 3500 rpm for 10 minutes at 4°C and plasma was collected and stored frozen at -80°C until assaying. IL-6 and TNF-α were measured in plasma using a commercially available ELISA kit (Biosource, CA), whereas the ELISA kit for IL-1β was from a different manufacturer (Bender Medsystem, CA). The sensitivities of the assays were 1.2 pg/ml for IL-1β, 3 pg/ml for TNF-α and 3 pg/ml for IL-6. Positive controls consisted of animals treated with i.p. LPS (0111:B4, Invivogen, CA).

Under terminal anaesthesia with pentobarbital, each mouse was euthanized and the brain quickly removed following decapitation. The hippocampus was dissected under microscopy on a frosted glass plate placed on top of crushed ice, then snap frozen and stored at -80°C until processing. Each hippocampus was added to Iscove’s culture medium containing 5% fetal calf serum (FCS) and a cocktail of enzyme inhibitors (in mM: 100 amino-n-caproic acid, 10 EDTA, 5 benzamidine-HCl, and 0.2 phenylmethylsulfonyl fluoride). The proteins were mechanically dissociated from tissue by means of sonication in a container plunged in ice. This consisted of 3 cycles of cell disruption each lasting 3 seconds. Sonicated samples were centrifuged at 10000 rpm for 10 minutes at 4°C. Supernatants were collected and stored at -80°C until the ELISA was carried out. IL-1β was measured in the supernatant from hippocampal extracts, which were appropriately diluted prior to measurement to fall on the linear portion of the sigmoid curve, using a commercially available ELISA kit (Bender Medsystem, CA). The sensitivity of the assay was 1.2 pg/ml. The ELISA kit was validated for use with brain tissues. Samples collected from mice, naive or treated
with LPS (011:B4, Invivogen, CA) 3mg/kg ip, were used to confirm reliability of dilution linearity and spike recovery. Serial dilution of samples produced a linear distribution of concentrations read from the standard curve of the assay. Spike recovery was 87.8% ±6.12 with 10μg of IL-1β; 86.3% ±4.24 with 50μg of IL-1β and 87.1% ± 2.69 with 100μg of IL-1β.

4.2.4 Immunohistochemistry

Under terminal anaesthesia with pentobarbital, mice were euthanized and perfused transcardially with ice-cold heparinized 0.1M phosphate buffer solution (PBS) followed by 4% paraformaldehyde in 0.1M PBS at pH 7.4 (VWR International, Lutterworth, Leicester, UK). The brains were harvested and post-fixed in 4% paraformaldehyde in 0.1M PBS at 4°C and cryoprotected in 0.1M PBS solutions containing 15% sucrose for 24 hours (VWR International, Lutterworth, Leicester, UK) and then 30% sucrose for a further 48 hours. Brain tissue was freeze-mounted in OCT embedding medium (VWR International, Lutterworth, Leicester, UK). Coronal sections of the hippocampus (30μm thickness) were cut sequentially in groups of 6 and mounted on Superfrost® plus slides (Menzel-Glaser, Braunschweig, Germany). The rat anti-mouse monoclonal antibody, anti-CD11b (low endotoxin, clone M1/70.15) or goat anti-mouse monoclonal antibody anti- Ionized calcium-binding adaptor molecule 1 (Iba-1) in the concentration of 1:200 (Serotec, Oxford, UK) were used to label microglia for morphological assessment or counting of cells, respectively. Visualization of immunoreactivity for CD11b or Iba-1 was achieved using the avidin-biotin technique (Vector Labs, Cambridge, UK) and a goat anti-rat or a rabbit anti-goat secondary antibody (Chemicon International, CA, USA) at a concentration of 1:200 was employed to visualise CD11b and Iba-1 staining,
respectively. A negative control omitting the primary antibody was performed in all experiments. A positive control group consisted of animals injected ip with LPS 3mg/kg. Immunohistochemical photomicrographs were obtained with an Olympus BX-60 microscope and captured with a Zeiss KS-300 colour 3CCD camera. The assessment of staining, by an observer that was blinded to the interventional group, was based upon a 4-point categorical scale modified from Colburn and colleagues (Colburn et al., 1997) for CD11b, which uses a combined evaluation of the level of microglial activation from both cell morphology and immunoreactivity (see Chapter 2; section 2.4.6). Microglial counting was carried out in the hippocampal region of the brain divided into 4 different sections, with the mean value considered for each animal.

4.2.5 Analysis of flow cytometry

Lung cell suspensions were prepared from the excised lungs by mechanical disruption to release intravascular cells, as described previously (O’Dea et al., 2005). For quantification and characterization of lung monocytes and neutrophils, finely dissected lung tissue was homogenized on a 40μm nylon mesh sieve (BD Falcon, Oxford, UK) with a syringe plunger and flushed through sieves with FACS medium (PBS with 2% FCS, 0.1% sodium azide and 5 mM EDTA). Homogenization and flushing of cells was then repeated in order to ensure maximal recovery of intravascular cells. Lavage of the lungs for recovery of intra-alveolar cells was performed using 750μl of saline, as described previously (Wilson et al., 2003). After centrifugation, lung cells were resuspended in FACS medium supplemented with 20% goat serum (Invitrogen, Paisely, UK). Cells were stained in the dark at 4°C for 30 minutes with fluorophore-conjugated anti-mouse antibodies for CD11b (clone
M1/70), Gr-1 (RB6-8C5) (BD Pharmingen, Oxford, UK), F4/80 (CI:A3-1) (AbD Serotec, Oxford, UK), or appropriate isotype-matched control antibodies. In experiments involving blood, RBC lysis and fixation were performed using FACS-lyse solution (BD Pharmingen). Samples were acquired (a minimum of 1,000 gated monocytes per sample) using a FACSCalibur flow cytometer and Cell Quest software (BD, Oxford, UK). The data were analyzed with Flowjo software (Ashland, OR, USA). Absolute cell counts in samples were determined using microspheres beads (Catlag Medsystems, Towcester, UK), as previously described (O’Dea et al., 2005; Choudhury et al., 2004).

4.2.6 Data analysis

Data are generally expressed as mean ± SEM or, in case of flow cytometry, as mean ± SD. Statistical analysis was performed with analysis of variance followed by the Student-Newman-Keuls test for numerical data. Student’s t test was only used for comparisons between two groups. The non-parametric test of Kruskal-Wallis followed by the Dunn’s test was used for categorical data. A p value of < 0.05 was considered to be of statistical significance.

4.3 RESULTS

4.3.1 Surgery elevates plasma concentrations of inflammatory cytokines IL-1β and IL-6

In order to assess the temporal relationship between peripheral injury and systemic inflammation, plasma concentrations of IL-1β, IL-6 and TNF-α were measured postoperatively with ELISA. Plasma levels of IL-1β and IL-6 were increased 6-fold
(IL-1β: 36.90 pg/ml, SEM ±6.54, n=6, p<0.001) and 5-fold (IL-6: 31.74 pg/ml, SEM ±5.28, n=6, p<0.05), respectively, one day after surgery when compared with naive animals (IL-1β: 6.09 pg/ml, SEM ±1.31, n=6; IL-6: 5.89 pg/ml, SEM ±2.10, n=6) (Figure 4.4 A-B). Although the plasma concentrations of IL-1β and IL-6 were unchanged at 2 hours post surgery, these peaked at 6 hours (Figure 4.4 A-B) increasing by 7-fold (IL-1β: 42.63 pg/ml, SEM ±9.60, n=6, p<0.001) and 20-fold (IL-6: 128.50 pg/ml, SEM ±19.64, n=6, p<0.001) above baseline levels, respectively. TNF-α remained undetectable at all time points under all conditions (data not shown). The administration of anaesthetics alone produced no change from the levels of any of the cytokines observed in naive mice (Figure 4.4 A-B). Pre-operative administration of minocycline, an antimicrobial with anti-inflammatory properties (Anim et al., 1996), reduced the plasma concentrations of cytokines back to pre-surgery levels (Figure 4.4 A-B). Conversely enrofloxacin, a broad-spectrum antimicrobial similar to that of minocycline but with no action against inflammation, did not exert any effect on IL-1β plasma concentrations in mice undergoing surgery, at all the considered time points (Figure 4.4 A).

4.3.2 Hippocampal IL-1β increases after surgery but not after anaesthesia alone.

IL-1β plays a pivotal role in signalling to the brain and in activating microglia (Laflamme and Rivest, 1999). Disruption of IL-1β signalling has been linked to hippocampal-dependent impairment of fear-related memories (Goshen et al., 2007). Since IL-1β was increased in plasma after surgery, I hypothesized a role for hippocampal IL-1β in surgery-induced cognitive dysfunction. The expression of IL-1β in the hippocampus was assessed 6 hours after surgery. There was a 2-fold increase of IL-1β levels (5.53 pg/100µg of proteins, SEM ±0.89, n=7, p<0.05)
compared to naive counterparts (2.73 pg/100μg of proteins, SEM ±0.39, n=7) (Figure 4.1 C). IL-1β expression in the hippocampus was not changed in animals exposed to anaesthesia alone (2.34 pg/100μg of proteins, SEM ±0.21, n=7), compared with naive animals. Administration of minocycline in surgical animals reduced IL-1β expression to naive levels. In contrast, treatment with enrofloxacin did not reduce the surgery-induced increase of IL-1β (5.19 pg/mg of proteins, SEM ±0.77, n=7, p<0.05 vs naive).
**Figure 4.4 Surgery-induced systemic inflammation is associated with increased expression of hippocampal IL-1β and this is reduced by minocycline treatment.** IL-1β and IL-6 levels in plasma were measured by ELISA at 2, 6, 24 or 72 hours post-intervention (n=6 per group). Surgery resulted in increased plasma levels of IL-1β (A) and IL-6 (B) compared to mice receiving the same anaesthetics without surgery (Anaesthesia group) or to naive animals. Treatment with minocycline (40 mg/kg, i.p.), an antibiotic with anti-inflammatory properties, mitigated surgery-induced elevations of IL-1β and IL-6 in plasma. Enrofloxacin, a comparable antimicrobial to minocycline, but devoid of any anti-inflammatory properties, given prior to and after surgery, failed to reduce plasma levels of IL-1β compared to those in the surgery group injected with saline. Six hours after surgery, IL-1β expression in the hippocampus was increased compared to naive and anaesthesia groups (C). Administration of minocycline but not enrofloxacin mitigated surgery-induced, IL-1β-mediated hippocampal inflammation (n=7 per group). Data are expressed as mean ± SEM, ***p<0.001; *p<0.05; for comparison between surgery or enrofloxacin vs. naive, anaesthetics and minocycline groups.

### 4.3.3 Surgery is followed by microglial activation

The finding of increased concentrations of IL-1β protein in the hippocampus following surgery prompted the search for other possible markers of hippocampal inflammation. Therefore, I studied whether microglia, the resident immune cells of the brain, would be activated in the hippocampus following a surgical insult, as is observed following a peripheral injection of lipopolysaccharide (LPS) (Qin et al., 2007). As a positive control, LPS-injected animals exhibited CD11b immunoreactive (ir) microglia with an activated morphologic phenotype characterised by hypertrophy of cell bodies, retraction of processes, an apparent amoeboid morphology, and increased levels of CD11b immunoreactivity. By contrast, in naive animals, CD11b-ir microglial cells remained in a non-activated state characterised by very low levels of immunoreactivity and small cell bodies with thin, long and/or ramified pseudopodia. Surgery-induced morphological changes of CD11b-ir microglia were concomitant
with the transition of these cells from a resting to a reactive state. This change was apparent as early as 24 hours after surgery (Figure 4.5) and, using the immunohistochemical grading technique for microglia (see Chapter 2; section 2.4.6) was significantly different from that of naive (n=7, p<0.01) and anaesthesia-only animals (n=7, p<0.05). Microglia in post-surgical mice displayed increased cell body dimensions, shortened and clumpy processes and had higher levels of CD11b immunoreactivity than naive counterparts. Surgery-induced reactive microgliosis was reduced by day 3 (Figure 4.5 F) after the operation and returned to baseline within 7 days (Figure 4.5 G). Administration of minocycline to surgical mice prevented reactive microgliosis (Figure 4.5 E-F).

**Figure 4.5** Immunohistochemistry of microglia with anti-CD11b (A-D). Hippocampi were harvested 1, 3 or 7 days after treatment (photomicrographs shown refer to tissue harvesting after 1 day). Representative photomicrographs from naive (A); anaesthetics alone (B); surgical (C); and surgical animals pre-treated with minocycline (D). The amoeboid hypertrophy of cell bodies, as well as shortening and clumping of processes seen following surgery (C) is prevented by administration of minocycline (40 mg/kg, i.p.) (D). Scale bar 30μm.

**Median (horizontal bar) with 25th to 75th (box) and 10th to 90th (whiskers) percentiles for immunohistochemical grading (0-3) of microglia (E-G).** One day after surgery, mice showed
significantly higher levels of reactive microgliosis compared to naive, anaesthetics only or surgical mice treated with minocycline (E). Three days after surgery mice continued to show an increase in reactive microglia compared with naive animals (F). By 7 days post surgery, microglial activation had returned to normal (G). *p<0.01; *p<0.05 surgery vs naive animals; ﬂp<0.05 surgery vs anaesthesia group; †p<0.05 surgery vs minocycline group (n=7 per group).

4.3.4 Surgery-induced, cell-mediated inflammation is characterised by clonal expansion of polymorphonucleates and Gr-1high subset of monocytes in the blood. These cells also marginate to the lungs in response to surgery.

Monocyte circulation in the blood and recruitment to the pulmonary circulation after surgery was evaluated by flow cytometric analysis of either whole blood or single lung cell suspensions (Figure 4.6). These were prepared from the excised lungs of mice, as described previously (O’Dea et al., 2005). Time-course experiments were performed to determine whether the surgery-induced elevations of blood or lung monocytes were maintained for prolonged periods and would therefore be likely to have an influence at the whole organ level (Figures 4.7-4.10). Numbers of lung-associated Gr-1high monocytes increased and were maximal at 2 hours post surgery, but the decline thereafter appeared to be sharp, i.e. Gr-1high monocytes were only showing a trend for elevation (non-significant) to up to 6 hours post surgery. At the 18-hour time point, cell counts returned to the levels of naive and anaesthesia only animals. This decrease in the lung Gr-1high monocytes was accompanied by an apparent increase in circulating Gr-1high monocytes, which were significantly higher at 4 hours after surgery (p<0.05).

The kinetics of the polymorphonuclear (PMN) cell response was quite distinct from that of monocytes. There were increased circulating and lung-associated PMN cells as
early as 2 hours postoperatively, with numbers returning to normal in the lungs by 6 hours after surgery. This is in contrast to the counts observed in the blood, where these cells remained high at all the time points considered, showing a possible double peak at 2 and 18 hours post surgery (Figures 4.8, 4.9 and 4.10).

Figure 4.6 Flow cytometric analysis of monocyte subsets in the lungs of mice undergoing surgery or in naive animals. In this example of a flow cytometry experiment, monocytes from naive mice or from mice euthanized 2 hours after surgery were identified as CD11b positive and/or F4/80 positive. Their subsets were defined as either Gr-1low or Gr-1high, according to their gating, which corresponds to physiological cell characteristics. These graphs give an immediate visual representation of the increased levels of monocytes and relative subsets found in the surgical group compared to naive animals.
Figure 4.7 Time course of the Gr-1 high subset of monocyte proliferation in the blood and their margination to the lungs, as measured by flow cytometry. Mice received surgery of the tibia and were sacrificed 2, 4 and 6 hours after surgery. In comparison to naive littermates or to mice exposed to the same anaesthetics without surgery, there was a significant increase of Gr-1high monocytes in the blood 4 hours after surgery. Likewise, a significant increase in the population of lung marginated Gr-1high monocytes was observed 2 hours after surgery, compared to the anaesthesia group or naive animals. Data are expressed as mean ±SD of number of cells. *p<0.05; n= 4-5 per time point.
Figure 4.8 Flow cytometric data demonstrating the time course of polymorphonuclear cell (PMN) proliferation in the blood and their margination to the lungs after surgery. Mice underwent surgery of the tibia and were sacrificed 2, 4 and 6 hours after surgery. In comparison to naive littermates or to mice exposed to the same anaesthetics without surgery, there was a significant increase in the number of PMNs counted in the blood at all time points considered after surgery. Also, a significant increase in the population of lung marginated PMNs was evident 2 and 4 hours after surgery, compared to the anaesthesia group or naive animals. Data are expressed as mean ±SD of number of cells. ***p<0.001; **p<0.01; *p<0.05; n= 4-5 per time point.
Figure 4.9 Time course analysis of cell-mediated systemic inflammation following general anaesthesia (GA) alone or surgery under GA assessed by flow cytometry. These charts show a clonal expansion of the PMNs in the blood with a possible double peak; one at the first at 2 hours and the second 18 hours after surgery. Also, a trend in a progressive clonal expansion of both the monocyte subsets Gr-1 high and low is depicted over time following surgery. The administration of anaesthetics, in absence of a surgical stimulation, did not cause any cellular proliferation. Data are expressed as mean of cell numbers. Variance bars are omitted in order to improve clarity; n= 4-5 per time point.
Figure 4.10 Time course of inflammatory-mediated margination of cells to the lung microcirculation in response to general anaesthesia (GA) alone or surgery under GA. These charts show significant margination of the PMNs in the lungs, peaking in the first 2 hours after surgery (for significance see Figure 4.5). Also, there is a trend for a progressive clonal expansion of the monocyte subset Gr-1 high following surgery. Surgery of the tibia does not seem to cause any increased margination of Gr-1 low monocytes to the lungs. The administration of anaesthetics, in absence of a surgical stimulation, does not cause any cellular margination to the lung microcirculation. Data are...
expressed as mean of cell numbers. Variance bars are omitted in order to improve clarity; n= 4-5 per
time point.

4.3.5 Activated microglial cells in the hippocampus do not increase in number 24
hours after surgery.

The occurrence of microgliosis after surgery posed the question as to whether
systemic humoral and cell-mediated inflammation would prompt clone expansion of
the hippocampal population of microglial cells. Immunohistochemistry with Iba-1
provides intense staining of microglial cell somata, making it possible to effectively
count the microglial cells in the hippocampus. The hippocampal microglial cell count
remained the same in all the experimental groups: naive, mice receiving anaesthetics
alone or mice undergoing surgery, assessed at the 24 hour time point after
intervention (Figure 4.11).
Figure 4.11 Surgery does not cause proliferation of Iba1-labelled microglia when assessed 24 hours after the operation. Cell counting revealed no difference in the number of Iba-1-labelled microglia in the hippocampi of naive, anaesthesia or surgical mice. Panels A, B and C show low magnification photomicrographs of the hippocampi of naive, anaesthesia and surgical mice, respectively. Panels D, E and F show high magnification of naive, anaesthesia and surgical mice, respectively, with inclusion of a detailed view of the staining of a single microglial cell. In panel G, numbers of Iba-1-labelled microglia in the hippocampus are shown. Data are expressed as mean ± SEM of the number of microglial cells visualised in the whole hippocampus. Scale bar 50μm; n= 7 per group.
4.3.6 Contextual fear memory after delay conditioning is impaired in mice undergoing surgery

To elucidate the consequences of surgery on learning and memory abilities, comparisons between the groups were carried out in mice trained with delay fear conditioning and tested for freezing to context and tone. The animals were trained shortly before any treatment (<30 minutes). As expected, there was no difference in freezing time between the groups during training (data not shown). Different groups of mice received one of the following interventions: surgery under anaesthesia, administration of anaesthetics without surgery, pre- and post-operative administration of either minocycline or enrofloxacin with surgery. Three days after treatment, mice were taken back to the conditioning chamber and re-exposed to the same environment as in training. The context test (Figure 4.12 A) revealed a significant reduction of freezing in the surgical group compared with naive animals or with those receiving anaesthesia alone (p<0.05). Minocycline treatment significantly reduced the subsequent surgery-induced impairment (p<0.05; Figure 4.12 A), but had no effect on freezing behaviour in naive animals (data not shown). Conversely, enrofloxacin did not reduce the surgery-induced behavioural effect (Figure 4.12 A). Interestingly, there was no difference in freezing behaviour between naive and animals receiving anaesthetics alone. All the animals displayed the same freezing behaviour when exposed to the tone (Figure 4.12 B).
4.3.7 Memory for both context and tone is impaired after surgery in trace fear conditioning

As opposed to delay fear conditioning, trace fear conditioning imposes a brief gap between the tone termination and shock onset. Trace and delay fear conditioning differ in that, in trace, the fear response to both the tone and context highly depends on hippocampal integrity (Chowdhury et al., 2005). To better establish the role of the hippocampus, trace fear conditioning was adopted to test naive versus surgical animals for context and tone hippocampal–dependent memory function. Naive animals were trained and subsequently divided in two groups. Mice in the surgical group underwent surgery within 30 minutes after training. Freezing time during training was not different between naive and surgical groups (data not shown). When exposed to the context, post-surgical mice demonstrated a significant reduction of freezing behaviour compared with naive littermates (p<0.05), consistent with the results from the delay paradigm (Figure 4.12 C). Moreover, when the auditory-cued test was carried out, there was a significant difference between surgical and naive subjects in auditory cued-dependent freezing behaviour (p<0.05), with a reduction in surgical animals (Figure 4.12 D).
Figure 4.12 Hippocampal-dependent recall of fear memories is impaired after surgery. Mice underwent fear conditioning and 30 minutes later they were divided to receive either anaesthetics (Anaesthesia), or surgery of the tibia under anaesthesia (Surgery), or the same surgical procedure with minocycline (Minocycline) or enrofloxacin (Enrofloxacin) administration, respectively. Naive animals received no treatment. Contextual and acoustic-cued memories were tested three days later. A. Recall of contextual delay fear conditioning memories, as measured by freezing behaviour, was impaired in surgical animals compared to naive and anaesthesia groups. Administration of minocycline, but not enrofloxacin, mitigated the surgery-induced, reduction in freezing. \( *p<0.05 \) surgery or enrofloxacin vs naive, anaesthesia and minocycline groups; \( n=34 \) per group. B. Freezing in the auditory-cued test after delay fear conditioning. There was no difference between the groups in either baseline or auditory-cued-related freezing behaviour, thus suggesting that amygdalar-dependent memory function is intact after surgery \( n=34 \) per group. C. Freezing to context after trace fear conditioning. Mice subjected to surgery exhibited reduced freezing to context when compared to naive animals, confirming that the inflammation induced by surgery disrupts recall of fear contextual memories formed in the hippocampus after trace conditioning. \( *p<0.05; \) \( n=28 \) per group. D. Hippocampal-dependent, surgery-induced memory impairment is shown in the auditory-cued test, in mice trained with trace fear
conditioning. There is a significant difference between the groups in auditory-cued-related freezing behaviour, suggesting disruption of auditory-cued, hippocampal-dependent, retrieval of memories after surgery. No difference was shown in the baseline freezing behaviour. *p<0.05; (n=28 per group).

4.3.8 Pre-treatment with IL-1ra mitigates the effects of surgery on memory function

The finding that IL-1β levels are increased after surgery raised the question of whether IL-1β was causally linked to cognitive dysfunction after surgery. To test this hypothesis, animals undergoing surgery were pre-treated with an IL-1 receptor antagonist (IL-1ra), which inhibits IL-1β binding with its cognate receptors. Delay fear conditioning testing was then performed on these animals. The administration of IL-1ra produced a significant improvement in postoperative freezing behaviour compared with untreated animals undergoing the same surgery (p<0.05) and returned fear memory recall abilities to the level of naive animals (Figure 4.13 A). Results from the auditory-cued test on these groups showed no difference, thereby confirming previous evidence from this study that the amygdala is not functionally impaired by surgery or by IL-1ra administration (Figure 4.13 B).

Figure 4.13 Surgery-induced impairment of contextual fear memories is prevented by pre-emptive administration of an IL-1 receptor antagonist (IL-1ra). A. IL-1ra, injected before surgery,
significantly improved the surgery-induced reduction in freezing behaviour. *p<0.05 surgery versus IL-1ra or naive; (n=30 per group). B. Freezing in the auditory-cued test after delay fear conditioning.

There was no difference between the groups in either baseline or auditory-cued-related freezing behaviour, suggesting that neither surgery, nor IL-1ra affected amygdalar-dependent memory function (n=30 per group). Data are expressed as mean ± SEM percentage of freezing response.

4.3.9 Contextual and auditory cued fear memory is not impaired in animals undergoing surgery one or three days after delay fear conditioning

Retrograde amnesia for contextual fear when surgery takes place 30 minutes after training, suggests that IL-1β interferes with the consolidation of learning. In order to determine if the memory impairment was indeed caused by interrupted consolidation (as opposed to a more permanent loss of hippocampal function) mice underwent surgery 72 hours after delay fear conditioning. The animals were tested for both tone and context memory 3 days after surgery, following the same surgery-to-context time delay as in the previous tests. The test for contextual and acoustic-cued memory showed no statistical difference between surgical and naive animals (Figure 4.14 A-B).

Figure 4.14 Recall of contextual and auditory-cued memories is not affected if surgery is delayed three days after delay fear conditioning. A. Freezing to the context in mice undergoing surgery three
days after delay fear conditioning. Surgery performed three days after treatment did not affect freezing to context when compared to naive animals. (n=28 per group). B. Freezing in the auditory-cued test after training with delay fear conditioning. There was no difference between the groups in either baseline or auditory-cued-related freezing behaviour. (n=28 per group).

Behavioural experiments were also conducted to investigate if a delay of 24 hours interposed between training and surgery would be long enough to prevent cognitive dysfunction from occurring. Mice were divided in two groups: naive and surgery under GA. Mice exposed to delay fear conditioning, undergoing surgery 24 hours after training, did not show any difference in their freezing time compared to naive littermates when they were tested for contextual fear conditioning three days later (Figure 4.15 A). As expected, acoustic-cued testing did not show any difference between the two groups either (Figure 4.15 B).

![Figure 4.15](image)

**Figure 4.15 Recall of contextual and auditory-cued memories is not affected if surgery is delayed 24 hours after delay fear conditioning.** A. Levels of freezing to the context in mice undergoing surgery 24 hours after delay fear conditioning. Surgery performed 24 hours after training did not significantly affect freezing to context when compared to naive animals. A trend towards reduced freezing in the surgery group is shown B. Freezing in the auditory-cued test after training with delay fear conditioning. There was no difference between the groups in either baseline or auditory-cued-related freezing behaviour. Data are expressed as mean ± SEM of the overall percentage of freezing response; n=27 per group.
4.4 DISCUSSION

Data presented in this chapter suggest that inflammation could play a pivotal role in the development of cognitive impairment in mice after surgery, findings that have important implications for the pathogenesis of POCD. The role of inflammation in cognitive impairment is supported by evidence for the ameliorating effects of the anti-inflammatory agent minocycline on peripheral and hippocampal markers of inflammation as well as hippocampal memory impairment following a surgical insult. Data in this chapter also demonstrate that IL-1β is likely to be a pivotal cytokine involved in systemic signalling from a peripheral surgical site to the brain. Hippocampal inflammation follows peripheral surgery, as demonstrated by a local increase in IL-1β protein in hippocampal tissue as well as reactive microgliosis. Attenuation of the IL-1β response to surgery by means of pre-treatment with an IL-1ra prevents post-operative memory dysfunction. Post-surgical impairment of contextual and auditory-cued memory after trace fear conditioning and contextual but not auditory-cued memory in delay conditioning, also suggests that postoperative dysfunction is derived from the hippocampus rather than other components of the fear circuit, such as auditory thalamic, amygdalar or periaqueductal gray regions (Chapter 2, section).

The finding that hippocampal-dependent contextual fear memory is impaired after peripheral inflammation following surgery is consistent with earlier evidence for impaired contextual fear conditioning following intraperitoneal administration of LPS in a model of peripheral inflammation caused by infection (Pugh et al., 1998). The same study also showed that LPS does not induce disruption of delay auditory-cued memories which is also in accordance with the results from the auditory-cued
behavioural test in the major surgery model used in this thesis. In addition, the critical role of the hippocampus in memory impairments after surgery was further confirmed by the trace conditioning procedure in which impaired freezing responses were observed to both context and tone.

A possible cause-effect relationship between surgery, inflammation and memory impairment was suggested by the effects of minocycline administration in reducing surgery-induced increases in peripheral and hippocampal pro-inflammatory cytokines, reactive microgliosis and behavioural impairment. This is consistent with previous evidence showing that minocycline reduces inflammation, microglial activation, transcription of IL-1β in the hippocampus and the resulting behavioural impairment in a mouse model of LPS-induced inflammation (Henry et al., 2008). Minocycline is a tetracycline derivative that exerts its anti-inflammatory effects both systemically and centrally. Minocycline reduces microglial activation through the inhibition of interferon gamma (IFNγ)-induced PKCa/βII phosphorylation and both PKCa/βII and interferon regulatory factor-1 (IRF-1) nuclear translocation, ultimately converging on the partial down regulation of MHC II proteins (Nikodemova et al., 2007). Importantly, minocycline inhibits the transcription factor p38-MAPK, which plays a pivotal role in the cascade leading to the biosynthesis of cytokines such as IL-1β and IL-6, as well as other pro-inflammatory mediators (Du et al., 2001). Moreover, at a functional level, minocycline appears to improve behavioural performance in a mouse model of spatial learning and memory through its reduction of microglial activation (Fan et al., 2007). In an activated state, microglia are capable of mounting macrophage-type innate immune responses and can secrete pro-inflammatory cytokines, reactive oxygen species, excitotoxins (such as glutamate) and neurotoxins
such as β-amyloid precursor protein (van Rossum and Hanisch, 2004). Activation of microglia has been associated with the cognitive dysfunction that is seen in sickness behaviour and is causally related to impairment of long-term potentiation in advanced age (Griffin et al., 2006). Thus, increased microglial reactivity, and the associated inflammatory processes, are both capable of producing the molecular changes that disrupt signalling pathways involved in memory formation (Maher et al., 2006).

Microglial cells differentiate in the bone marrow from haematopoietic stem cells, the progenitors of all blood cells. During haematopoiesis, some of these stem cells differentiate into monocytes and travel from the bone marrow to the brain, where they settle and further differentiate into microglia if inflammation mounts (Ritter et al., 2006). Based on this premise, I hypothesised that monocyte subsets proliferate, migrate to the brain and replenish the resident population of microglia in response to the altered inflammatory milieu caused by surgery. The clone of Gr-1high subsets of the monocyte population is usually expanded in inflammatory states (O’Dea et al., 2005). In my model, these cells were increased in the blood and demonstrated a certain tendency to marginate in the capillaries of the lungs in response to a surgical insult. Localization of these monocytes within the lungs, having possibly extravasated in the lung tissue, suggested the possibility of monocyte margination in the capillaries of the blood brain barrier with subsequent extravasation into brain tissue and the hippocampus. This possibility was assessed by immunohistochemical analysis with antibodies directed against Iba-1, a marker commonly used to stain microglia (O’Dea et al., 2009). The finding of an unchanged number of microglial cells in the hippocampus was not surprising, but worth investigating. This is because an important difference between microglia and other cells that differentiate from myeloid
progenitors is the turnover rate. Macrophages and dendritic cells are constantly being used up and replaced by myeloid progenitor cells, which differentiate into the needed type. The blood brain barrier presents an element of difficulty for the immune system if microglial populations in the brain require frequent replenishment. Therefore, instead of constantly being replaced with myeloid progenitor cells, the microglia are normally maintained in a quiescent state, and then, following activation, they can rapidly proliferate when necessary, in order to provide an effective response during inflammation. Accumulating evidence has shown, however, that in case of elevated systemic inflammation, the blood-brain barrier becomes relatively more permeable, allowing microglia to be replaced with haematogenous, bone marrow derived cells, such as monocytes and/or macrophages. Once the insult is removed, the normal homeostasis between peripheral blood and central nervous system is re-established so that the blood brain barrier returns to the normal state of impermeability to macromolecules and cells (Gehrmann, 1996; Nottet et al, 1996). In my model of surgery, the insult seems to be insufficient to stimulate further recruitment of cells, whereby a primed activated state appears to be enough to exert the required defensive function.

It is possible that, despite the aseptic technique employed, the advent of post-operative hippocampal inflammation and cognitive dysfunction, and its attenuation by an antimicrobial (minocycline), can be explained by an infective process associated with the surgical intervention. However, in this study, no clinical evidence of infection, such as piloerection, weight loss or lack of movement, was seen in any of the animals, which is consistent with previous studies that used the same murine tibial fracture model involving several hundred animals (Harry et al., 2008) and similarly
reported no clinical or histological evidence of infection. Importantly, in my study, the administration of enrofloxacin, a wide spectrum antibiotic often used in rodents with anti-infective properties but devoid of any anti-inflammatory effects, did not improve any of the surgery-induced effects on either expression of pro-inflammatory markers or behaviour.

It could be argued that pain may be a confounding factor producing immobility and thus influencing the extent of ‘freezing’ in the behavioural tests. However, my model of surgery aimed to reproduce routine clinical settings and, accordingly, administration of an analgesic opiate (buprenorphine) in my experiments should have greatly reduced surgical pain. Nevertheless, if any nociceptive input caused the animals to restrict movement of their affected limb during retrieval of context or auditory-cued memories, one would have expected to see more, and not less, freezing. It is of some importance to note that even pain produced by subcutaneous injection of formalin into a hind paw does not disrupt the freezing seen with contextual fear conditioning (Fanselow and Baackes, 1982). Moreover, a possible interference from pain in the extinction tests was addressed by the results of the delay fear conditioning paradigm in which surgery was delayed by three days after conditioning. Since the delay between surgery and retrieval tests in this experiment was the same as in the other implemented fear conditioning experiments, it is reasonable to conclude, based on results showing no differences between surgical and naive animals in memory retrieval, that there was no interference from post-operative pain in all the fear conditioning retrieval tests adopted in this study.
My data support a pivotal role for IL-1β in surgery-induced cognitive dysfunction (Pugh et al., 1998; Barrientos et al., 2002). Increased systemic and hippocampal IL-1β expression, demonstrated in this study, are consistent with previous evidence suggesting that peripheral cytokines signal to the brain via both blood and neural routes, thereby stimulating cytokine production by brain glial cells (Godbout et al., 2005; Eriksson et al., 2000; Maier et al., 1998), especially in the hippocampus (Ban et al., 1992; Nguyen et al., 1998). Evidence of increased hippocampal levels of IL-1β suggests the possible role of microglia in the de novo production of cytokines in my mouse surgery model. IL-1β interferes with hippocampal long-term potentiation (Cunningham et al., 1996; Verker et al., 2000) that has been viewed as an essential electrophysiological correlate of memory (Morris, 1989). IL-1β acts either directly or indirectly, through microglial activation, on the intracellular neuronal mechanisms, such as protein synthesis, that stabilize the long-term plasticity necessary for memory. Loss of memory induced by IL-1β is unlikely to be caused by permanent damage, retrieval failure or an inability to perform the freezing response. This is because such deficits in memory responses would have also appeared when surgery occurred 3 days after fear conditioning training.

The present data, in the context of two other previous reports (Wan et al., 2007; Rosczyk et al., 2008), provide robust evidence for the occurrence of surgery-induced, inflammatory-mediated cognitive dysfunction. However, the mechanism whereby a peripheral surgical intervention initiates hippocampal neuroinflammation is unclear. I have shown that elevated systemic and brain tissue cytokines, as well as hippocampal microglial activation, are all reduced by peripheral treatment with i.p injected minocycline before and after surgery. These data suggest that humoral, rather than
neural factors are involved in the first instance, or that humoral factors are involved in addition to neural factors (Wieczorek et al., 2005) and further studies are required to discern this.

Attenuation of the neuroinflammatory process with minocycline or by interference with IL-1β signalling prevents post-surgical cognitive dysfunction. This neuroinflammatory process and its initiation, represents a realistic target for therapeutic interventions with a major potential impact for the expanding elderly population. Subsequent studies to elucidate whether the neuroinflammatory response can be modulated by anaesthetic agents or by conventional anti-inflammatory strategies may be helpful in ameliorating the adverse consequences of post-operative cognitive decline.
Chapter Five

Lipopolysaccharide-induced hippocampal inflammation and cognitive dysfunction are attenuated by isoflurane.
Contents

5.1. Introduction 161

5.2. Methods 164

5.2.1 Animals procedures 164

5.2.2 Delay fear conditioning 164

5.2.3 Cytokine measurement in plasma and hippocampal tissue 165

5.2.3.1 Plasma 165

5.2.3.2 Hippocampus 165

5.2.4 Immunohistochemistry 166

5.2.5 Data analysis 167

5.3. Results 168

5.3.1 LPS-induced systemic inflammation (as measured by plasma levels of proinflammatory cytokines) is mitigated by isoflurane administration. 168

5.3.1.1 IL-1β 168

5.3.1.2 IL-6 169

5.3.1.3 TNF-α 170

5.3.2 LPS-induced increases in IL-1β hippocampal expression are reduced by isoflurane administration. 171

5.3.3 Isoflurane does not affect microglial activation in mice injected with a high dose of LPS. 172

5.3.4 Impairment of contextual delay fear conditioning memories after LPS injection was mitigated by isoflurane anaesthesia. 174

5.3.5 Fear memory stored in the amygdala is not affected by LPS injection, isoflurane anaesthesia or a combination of both. 176

5.4. Discussion 178
5.1 INTRODUCTION

In the last few years, mounting evidence has shown that cognitive dysfunction following surgery, as well as delirium during the initial phase of ICU stay, predispose to prolonged cognitive impairment and higher mortality in patients (Jackson et al, 2003; Jackson et al, 2004; Hopkins et al, 1999; Ely et al, 2004; Monk et al., 2008). It hence appears clear that diagnosis, management and interventions aimed at reducing the acute neurocognitive effects of surgery and critical illnesses are of fundamental importance.

Despite the urgent need of treatment strategies, no drugs have yet been approved by the United States Food and Drug Administration for the treatment of delirium and post-operative cognitive dysfunction (POCD). Also, it is not clear whether or not sedatives and anaesthetics would have detrimental or therapeutic effects.

Some of the most commonly used sedatives and anaesthetics have shown contrasting effects on cognitive dysfunction.

The benzodiazepines, which have been indicated to be drugs of choice for the treatment of delirium caused by alcohol and benzodiazepine withdrawal syndromes, are not recommended for the management of delirium in general (Lonergan et al, 2009).

Haloperidol, an antipsychotic, is a neuroleptic agent successfully used to reduce the severity of delirium (Ely et al., 2004), but fails to decrease incidence or improve outcome.
Animal studies show that anaesthetics are capable of producing long-lasting cognitive dysfunction under certain circumstances (Culley et al., 2003). Under particular conditions in vitro and in vivo isoflurane induces apoptosis and increases amyloid beta protein levels in modified human neuroblastoma cells (Xie et al., 2007; Xie et al., 2008). Yet, there appears to be no decrease in the incidence of POCD after regional anaesthesia (Campbell et al., 1993; Williams-Russo et al., 1995); therefore, a causative role for general anesthetics requires further analysis.

Ioflurane, an alogenated, volatile anaesthetic, commonly used in clinical practice, has also been demonstrated to exert neuroprotective effects under numerous conditions. Preconditioning with ioflurane protects the hippocampus from post-ischemic neurologic dysfunction in an animal model of cardiac arrest (Blanck et al., 2000). Preconditioning with ioflurane may also influence experimental traumatic brain injury. When compared with fentanyl infusion for 30 min before traumatic brain injury in rats, ioflurane was associated with improved functional and cognitive performance 5 days after trauma (Statler et al., 2006).

In addition, ioflurane mitigates brain damage resulting from both focal and global experimental cerebral ischemia (Miura et al., 1998; Mackensen et al., 1999) and substantially reduces neuronal death as a consequence of oxygen and glucose deprivation in neuronal cortical cell cultures (Wise-Faberowski et al., 2001).

Recent research has highlighted the anti-inflammatory properties of ioflurane, under different conditions. Several in vitro studies have demonstrated an attenuation of the
immune response to inflammatory stimuli after exposure to isoflurane (Mitsuhata et al., 1995; Giraud et al., 2003; de Rossi et al., 2004); whereas in vivo studies have described blunted systemic inflammatory responses after isoflurane administration (Plachinta et al., 2003; Hofstetter et al., 2005; Reutershan et al., 2006), which culminates in an increase in post-operative survival (Fuentes et al., 2006). Furthermore, anaesthesia maintained with isoflurane reduces surgical-induced systemic inflammation (Inada et al., 2004).

Hippocampal inflammation is known to be associated with cognitive dysfunction and memory impairment following infection (Pugh et al., 1998), sepsis (Barrichello et al., 2006), or surgery (Wan et al., 2007). Mitigation of systemic inflammation has proven effective in improving inflammatory-dependent cognitive dysfunction (Pugh et al., 1998).

I hypothesise that, aside from its systemic effects, isoflurane elicits a potential anti-inflammatory effect in the brain, particularly in the hippocampus. The aim of this study is to explore this property and investigate the possible beneficial effects on subsequent inflammatory-induced cognitive dysfunction after peripheral administration of lipopolysaccharide (LPS).

In my experiments, inflammatory-induced cognitive dysfunction occurred after aseptic tibial surgery (see Chapter 3 and Cibelli et al., 2009) but isoflurane anaesthesia without surgery did not induce cognitive dysfunction. Yet, the question of a possible synergistic effect between anaesthesia and inflammation in the development of
memory impairment is still open. This chapter also aims to address this important point.

5.2 METHODS

5.2.1 Animal procedures
Mice were injected with LPS 100μg intraperitoneally (i.p.), with or without concomitant administration of two different regimens of anaesthesia. One group had isoflurane 1.5 MAC for 20 minutes and another group had the same concentration of isoflurane, but for 120 minutes. Naive mice were employed as negative controls.

The animals were allowed free, unrestricted food and water intake following recovery from the procedure. Mice from each treatment group were randomly assigned to two different assessment groups: one for harvesting blood and tissue samples and the other for cognitive behaviour. This was done in order to obviate possible confounding effects of fear conditioning testing on inflammatory markers (Nguyen et al., 1998).

5.2.2 Delay fear conditioning
The methods used to assess freezing behaviour in a conditioning chamber as an indicator of aversive memory when mice are re-exposed to the CS have been described previously (Chapter 4; section 4.2.2). Three days after conditioning, mice were transported back to the behavioural room and left undisturbed for 15 minutes. They were returned into the same chamber where training had occurred for a context test lasting 270 seconds, during which no tones or foot-shocks were delivered. Approximately 3 hours after the context test, the animals were transported back for
the auditory-cued test. Changing the environment of the conditioning chamber (context) for acoustic-cued behavioural testing was also performed as previously described (Chapter 4; section 4.2.2). Mice were placed in the novel environment, and time sampling was used as baseline during which freezing was scored for 135 seconds. The auditory cue was then presented for 135 seconds, and freezing was again recorded. All the animals, regardless to the specific intervention, underwent the same handling, including naive animals. After testing, animals were returned to their housing cage. As before, video data of the behavioural experiments were collected by an infrared camera positioned in front of the chamber and were stored in a computer for further analysis. The percentage of time spent freezing over the total time spent in the chamber to accomplish the test were used to score memory and learning abilities. A decrease in the percentage of time spent freezing indicated impairment of these abilities.

5.2.3 Cytokine measurement in plasma and hippocampal tissue

5.2.3.1 Plasma

Blood was collected as previously described (Chapter 4, section 4.2.3). IL-6 and TNF-α were measured in plasma using a commercially available ELISA kits. The sensitivities of the assays were 1.2 pg/ml for IL-1β, 3 pg/ml for TNF-α and 3 pg/ml for IL-6.

5.2.3.2 Hippocampus

The hippocampus was harvested and processed for ELISA as previously described (Chapter 4, section 4.2.3). IL-1β was measured in the supernatant from hippocampal extracts, which were appropriately diluted prior to measurement to fall on the linear
portion of the sigmoid curve, using a commercially available ELISA kit (Bender Medsystem, CA). The ELISA kit was validated for use with brain tissues (Chapter 4; section 4.2.3). Samples collected from mice treated with LPS 3mg/kg i.p. were used to confirm reliability of dilution linearity and spike recovery (see Chapter 4; section 4.2.3).

5.2.4 Immunohistochemistry

Methods have been previously described in detail (Chapter 4; section 4.2.4). Briefly, mice were transcardially perfused with PBS and 4% paraformaldehyde fixative. The brains were removed, post-fixed and mounted in embedding media, before being cut on a cryostat to produce a series of 30μm coronal sections freeze-thaw mounted on slides.

Immunohistochemistry for CD11b was performed on slide-mounted hippocampal tissue as previously described (Chapter 4; section 4.2.4). The rat anti-mouse CD11b antibody (1:200) was used to label microglia. Primary antibody labelling was visualised using a goat anti-rat secondary antibody (1:200) and the avidin-biotin amplification technique (see Chapter 2; section 2.4.4). Positive control tissue was derived from animals injected i.p. with LPS 3mg/kg. Negative controls omitting the primary antibody were performed in all experiments. Photomicrographs of CD11b-labelled microglia were obtained and the assessment of staining was made by an observer that was blinded to the interventional group. Assessment was based upon a 4-point categorical scale, which uses a combined evaluation of the level of microglial activation from both cell morphology and immunoreactivity (see Chapter 2; section 2.4.6).
5.2.5 Data analysis

Data are expressed as mean ± SEM. Statistical analysis was performed with analysis of variance followed by the Student-Newman-Keuls test for numerical data. The non-parametric test of Kruskal-Wallis followed by the Dunn’s test was used for categorical data. A p value of < 0.05 was considered to be of statistical significance.
5.3 RESULTS

5.3.1 LPS-induced systemic inflammation (as measured by plasma levels of pro-inflammatory cytokines) is mitigated by isoflurane administration.

Mice were randomly assigned to one of the following experimental groups: naive, which were intraperitoneally injected with vehicle; A20, which underwent vehicle injection followed by induction and maintenance of general anaesthesia with isoflurane 1.5 MAC for 20 minutes in air; A120, as the previous group, but anaesthetised for 120 minutes (2 hours); LPS, which were injected with LPS 100µg/kg; A20LPS, which were injected with LPS 100µg/kg and immediately anaesthetised with isoflurane 1.5 MAC for 20 minutes in air; A120LPS, as the previous group, but remained anaesthetised for 2 hours. Each of the experimental groups was further sub-divided into those undergoing blood withdrawal and euthanasia occurring at either 2 or 6 hours after vehicle or LPS injections.

5.3.1.1 IL-1β

IL-1β plasma levels were significantly increased 6 hours after LPS administration compared to naive and anaesthesia alone groups injected with vehicle. The LPS-injected animals, which were concomitantly exposed to general anaesthesia with isoflurane, still showed a significant increase in the plasma concentration of IL-1β at 6 hours compared to naive and anaesthesia alone counterparts injected with vehicle (Figure 5.1, A: p< 0.001), however this was significantly lower in comparison with LPS-injected only littermates without exposure to anaesthesia (Figure 5.1. A: p<0.05 for LPS vs A20LPS; p<0.01 for LPS vs A120LPS). Animals receiving anaesthesia and vehicle did not show any change in IL-1β concentration from baseline levels.
There was also no change in the plasma concentration of IL-1β measured by ELISA in any of the experimental groups considered, including LPS, at the 2 hour post-injection time point (Figure 5.1, A).

5.3.1.2 IL-6

In contrast, plasma concentrations of IL-6 in animals injected with LPS were elevated earlier at 2 hours post-injection compared with all other experimental groups (Figure 5.1, B; p<0.001). Also, at the 2 hour post-injection time point, A20LPS and A120LPS groups had increased levels of plasma IL-6 compared to naive and anaesthesia only groups (Figure 5.1, B; p<0.001), but had a significantly lower concentration of IL-6 than mice injected with LPS without anaesthesia (Figure 5.1, B; p<0.001). Unlike IL-1β plasma concentrations, the length of exposure to anaesthesia affected plasma levels of IL-6 after LPS injection. Longer isoflurane exposure reduced plasma levels of IL-6 induced by LPS injection at the 2-hour time point (Figure. 5.1 B; p<0.01 for A20LPS vs A120LPS). IL-6 remained elevated in plasma, 6 hours after LPS injection when compared to naive and anaesthesia groups receiving vehicle injections (Figure 5.1, B; p<0.05). At this later 6-hour time point, there was no difference in measured IL-6 levels between the groups receiving LPS and isoflurane for 20 minutes and those receiving LPS without anaesthesia. However, administration of isoflurane for 2 hours significantly reduced plasma levels of IL-6, stimulated by LPS injection and sacrificed 6 hours later. Groups of animals subjected to anaesthesia without LPS showed no difference in IL-6 plasma levels, compared with naive counterparts, at any time point considered.
5.3.1.3 TNF-α

The plasma concentration of TNF-α was increased in LPS, A20LPS and A120LPS groups, compared to naive and anaesthesia only groups, at the 2-hour time point (Figure 5.1, C; p<0.001). Longer duration of exposure to isoflurane, in the A120LPS group, caused a greater reduction in plasma levels of TNF-α compared with the LPS and A20LPS groups of mice (Figure 5.1, C; p<0.001 for A20LPS vs A120LPS). The increased levels of plasma TNF-α were also observed 6 hours after injection in the LPS group (Figure 5.1, C; p<0.05 vs all other groups). This returned to baseline levels when isoflurane was given after LPS injection in both A20LPS and A120LPS groups.

![Graph showing cytokine levels](image)

**Figure 5.1** Isoflurane anaesthesia reduces plasma cytokine levels in mice injected with lipopolysaccharide (LPS).

Administration of LPS 100μg/kg initiates an inflammatory reaction, which culminates in systemic cytokine release. IL-1β is increased 6 hours after LPS injection (A), whereas IL-6 (B) and TNF-α (C) are already increased 2 hours after administration of LPS compared with naive controls. Exposure to
isoflurane 1.5 MAC, concomitantly with LPS, has a significant reducing effect on the plasma levels of all the cytokines measured (A, B, C). In some cases, these levels can be further reduced if the duration of isoflurane administration is increased from 20 minutes to 2 hours. For example, plasma levels of IL-6 and TNF-α measured at 2 hours post-injection are reduced further after prolonged exposure to isoflurane (2 hours, instead of 20 minutes). At 6 hours post-injection, prolonged administration of isoflurane had an equivalent effect to a brief exposure for both IL-1β and TNF-α measured in plasma. Data are expressed as mean ± SEM pg/ml plasma concentration, n= 6-8. *p<0.05; **p<0.01; ***p<0.001; +p<0.05 and +++p<0.001 for comparison with Anaesthesia for 20 min group; ###p<0.001 for comparison with Anaesthesia for 2 hours group.

5.3.2 LPS-induced increases in IL-1β hippocampal expression are reduced by isoflurane administration.

The peripheral administration of LPS caused an elevation of IL-1β protein measured in the hippocampus compared with naive mice injected with vehicle (Figure 5.2). Interestingly, mice injected with LPS and exposed to either 20 or 120 minutes of isoflurane anaesthesia did not show a significant difference in hippocampal expression of IL-1β. The IL-1β levels in the hippocampus measured following LPS injection and concomitant isoflurane administration were significantly reduced from those observed with LPS injection alone.
Figure 5.2 Isoflurane protects against LPS-induced increases in hippocampal IL-1β levels.

Intraperitoneal administration of LPS 100μg/kg increases the levels of IL-1β measured in hippocampal tissue by ELISA. Inhalation of isoflurane for either 20 minutes or 2 hours, concurrently with LPS injection, prevents IL-1β levels from rising above those measured in animals injected with vehicle (naive). Experimental groups were as follows: Naive: vehicle-injected; LPS: LPS-injected; A20: vehicle-injected and exposed to isoflurane inhalation for 20 minutes; A120: vehicle-injected and exposed to isoflurane inhalation for 120 minutes; A20LPS: LPS-injected and exposed to isoflurane inhalation for 20 minutes; A120LPS: LPS-injected and exposed to isoflurane inhalation for 120 minutes. Data are expressed as Mean ± SEM pg/100μg of proteins extracted from the hippocampus, n= 7. ***p<0.001 for comparison with naive, A20 and A120 groups. ##p<0.01 for comparison with A20LPS and A120LPS groups.

5.3.3 Isoflurane does not affect microglial activation in mice injected with a high dose of LPS.

LPS injected i.p. at the dose of 3mg/kg resulted in diffuse activation of hippocampal microglia visualised by CD11b immunohistochemistry (Figure 5.3, D). Activated microglia featured the typical thickening and shortening of cytoplasmatic processes
and enlargement of the cell somata, which were more intensely stained. Contrastingly, no morphological changes were observed in the microglia of animals subjected to vehicle injection with and without anaesthesia with isoflurane for either 20 or 120 minutes (Figure 5.3, A-C). In these animals, microglia featured long and thin pseudopodia and lower levels of CD11b staining in cell somata. The microglia of LPS-injected mice undergoing both short and long exposure to isoflurane anaesthesia, were no different from their LPS-injected littermates not exposed to the anaesthetic (Figure 5.3, E-F).
Figure 5.3 LPS-induced microglial activation in the hippocampus, visualised by CD11-b immunohistochemistry, is not reduced by isoflurane anaesthesia.

Intraperitoneal LPS 3mg/kg injection is followed by microglial activation in the hippocampus (D), the concomitant administration of isoflurane 1.5 MAC for either 20 (E) or 120 (F) minutes with LPS injection does not prevent microglial activation. Vehicle injection together with isoflurane anaesthesia, for either 20 (B) or 120 (C) minutes, does not produce any change in hippocampal microglial morphology or staining intensity if compared to naive animals injected with vehicle (A). Scale bar 30µm.

5.3.4 Impairment of contextual delay fear conditioning memories after LPS injection was mitigated by isoflurane anaesthesia.

Mice received LPS or anaesthesia with isoflurane or both, shortly after delay fear conditioning had occurred. Recall of fear-related memories was assessed 3 days later. Animals injected with LPS froze significantly less than their vehicle injected littermates (Figure 5.4, *p<0.05 for comparison between LPS vs naive); whereas there was no difference in the extent of freezing between vehicle-injected mice
anaesthetised with isoflurane for either 20 or 120 minutes compared to naive (Figure 5.4, A20 and A120 vs naive). Strikingly, when anaesthesia and LPS were given together for either 20 or 120 minutes, there was a significant difference in the level of freezing compared with LPS-injected mice not receiving anaesthesia (Figure 5.2, *p<0.05 for comparison between LPS vs A20LPS or A120LPS). Importantly, the level of freezing recorded with LPS-injected animals undergoing concurrent administration of isoflurane was no different to naive mice (Figure 5.4, A20LPS and A120LPS vs naive).

Figure 5.4 LPS-induced impairment of contextual fear memories is prevented by isoflurane anaesthesia.

Recall of contextual delay fear conditioning memories, as measured by freezing behaviour, was impaired in LPS-injected animals (LPS) compared to naive and anaesthesia groups treated with isoflurane for 20 (A20) or 120 (A120) minutes. When isoflurane anaesthesia was co-induced with the injection of LPS, and maintained for 20 (A20LPS) or 120 (A120LPS) minutes, retrieval of memories
returned to the same level as naive mice. Data are expressed as Mean ± SEM of % of freezing behaviour. *p<0.05 (n=18).

5.3.5 Fear memory stored in the amygdala is not affected by LPS injection, isoflurane anaesthesia or a combination of both.

Assessment of the consolidation of amygdalar-dependent memories, with an auditory-cued test, took place 3 days after conditioning and treatment, and 3 hours after completion of the behavioural context test.

In this test, baseline freezing, scored before a prolonged presentation of the tone, was the same in all the experimental groups (Figure 5.5). Likewise, when the tone was presented, the level of freezing in response to the fear that the tone evokes was the same in all the experimental groups (Figure 5.5).
Figure 5.5 Freezing in the auditory-cued test after delay fear conditioning.

There was no difference between the groups in either baseline or auditory-cued-related freezing behaviour, thus suggesting that amygdalar-dependent memory function is intact after LPS or isoflurane administration. Experimental groups are as follows: A20: isoflurane 20 min; A120: isoflurane 120 min; A20LPS: isoflurane 20 min + LPS 100μg/kg; A120LPS: isoflurane 120 min + LPS 100μg/kg. Data are expressed as Mean ± SEM of freezing percentage; (n=18).
5.4 DISCUSSION

Results from this study strongly suggest that isoflurane plays a protective role in hippocampal IL-1β-mediated inflammation induced by LPS. Evidence for the attenuation of LPS-induced IL-1β expression in the hippocampus, coupled with the demonstration of reduced memory impairment after co-administration of isoflurane and LPS, opens a new prospect for the possible management of inflammatory-mediated cognitive dysfunction, as occurs following sepsis in intensive care.

The implications of these findings go beyond the concept of sickness behaviour following an infective process, as in the sepsis paradigm. Evidence from my research (see chapter 4) is suggestive of an active role of hippocampal inflammation in the development of postoperative cognitive dysfunction in mice. Furthermore, results from the same studies indicate that surgical trauma is likely to be the factor responsible for the elevation of systemic cytokines and subsequent IL-1β-mediated hippocampal inflammation and cognitive impairment. This chapter demonstrates that peripheral infection (modelled by LPS injection) induces a comparable induction of peripheral and hippocampal pro-inflammatory cytokines and impairment of cognitive function as occurs following a peripheral surgical insult.

Isoflurane anaesthesia did not promote either systemic or hippocampal expression of any of the considered pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) following a peripheral infective insult with LPS. This result casts doubt over the hypothesis that anaesthetics create a favourable milieu for inflammation, whereby subsequent cognitive deterioration would be initiated more readily by a synergistic effect between anaesthetics and sepsis. Results presented here with LPS injection experiments are
designed to cover a gap in knowledge, which would have otherwise been left open following the research in chapters 3 and 4 on inflammation and cognitive dysfunction. The only other way to investigate the hypothetical synergistic effects between surgery, inflammation and anaesthesia would have implied the use of a control group undergoing surgery without anaesthetics, which is inconceivable.

The data presented here are consistent with previous evidence of protective anti-inflammatory properties from isoflurane (Plachinta et al., 2003; Hofstetter et al., 2005; Reutershan et al., 2006). One of the mechanisms by which isoflurane exerts its anti-inflammatory activity could be secondary to the inhibition of the transcription factor NF-κB (de Rossi et al, 2004; Boost et al, 2006), which is involved in the regulation of cytokine synthesis (Zhang and Ghosh, 2000). The same mechanisms could explain the limited effect of isoflurane on IL-6 plasma expression, measured at 6 hours in contrast to the effects on levels of IL-1β and TNF-α measured at this time point. Plachinta and colleagues speculated that a higher level of NF-κB inhibition would have been necessary to affect IL-6 expression in their model of increased IL-1β and TNF-α plasma levels after LPS (Plachinta et al., 2003). An alternative explanation is that the effects of anaesthetics were more reliably measured on peak cytokine plasma levels than lower levels of IL-6 measured 6 hours post injection. Other explanations are related to the compensatory effect of anaesthetics on hypotension caused by LPS, possibly leading to an increased cardiac output and reduced acidosis due to either improved tissue perfusion (Plachinta et al., 2003) or improved ventilation (Patel et al., 1998).
However, this study failed to demonstrate an effect of isoflurane on the microglial activation occurring after LPS injection. This is in contrast with previous research showing reduced activation of microglia after isoflurane treatment of cell cultures exposed to LPS, as well as improved cellular viability. The same study proposed inhibition of iNOS and elevation of PKC as likely underlying mechanisms for isoflurane-mediated protection, in which the cellular stimulation was carried out in association with interferon $\gamma$ (Xu et al., 2008).

In the present study, the observed reductions in LPS-induced peripheral and hippocampal production of cytokines by isoflurane did not correspond to mitigation of microglial activation in the hippocampus. A possible explanation for this effect is that the dose of LPS necessary to trigger a visible activation of microglia is much higher than the dose required to activate a peripheral immune response. The milder inflammatory LPS insult used for plasma and hippocampal cytokine measurement would require a less powerful pharmacological effect from isoflurane to produce a measurable reduction in cytokine levels. By contrast, the higher dose of LPS employed to achieve microglia activation visible with CD11b immunohistochemistry is likely to have triggered a supramaximal level of microglial activation, which may have been prohibitive to amelioration by isoflurane. Similarly, any reduction in microglial activation may have been undetectable using the adopted experimental method with CD11b immunohistochemistry.

Interestingly, with cytokine measurements after LPS injection, the extent of protection achieved with isoflurane was sometimes related to the duration of anaesthesia. It is worth emphasizing that most of the differences between long and short-term
anaesthesia were observed in the groups randomized to be sacrificed earlier (2 hours post-injection) rather than after 6 hours. In these particular groups, the administration of isoflurane was uninterrupted for 2 hours. Since these animals were virtually euthanized when still under anaesthesia, one could speculate that the immune system would have been kept ‘restrained’ by the anti-inflammatory activity of isoflurane, whilst in the 6-hour group this restriction was removed 4 hours before the animals were sacrificed, resulting in more inflammation being generated in the intervening period.

The results presented in this chapter have demonstrated that isoflurane anaesthesia has an effect of reducing pro-inflammatory processes both systemically and in the brain of mice injected with LPS to mimic a peripheral infection. The concentrations of pro-inflammatory cytokines measured in the blood and in the hippocampus after LPS injection are decreased by concomitant isoflurane anaesthesia. Impairment of cognitive function consequent to neuroinflammation measured by trace fear conditioning after the LPS insult is also ameliorated by isoflurane administration. Although the role of anaesthetics in surgery-induced inflammation and cognitive dysfunction cannot be measured directly, experiments with the LPS model suggest that isoflurane anaesthetic has an anti-inflammatory rather than pro-inflammatory effect on both cytokine levels and cognitive impairment.
Chapter Six

Effect of Inflammation, Age, Gender and Estrogens on Post-Operative Cognitive Dysfunction
Contents

6.1 Introduction 184

6.2 Methods 186

6.2.1 Animals and surgical methods 186

6.2.2 Checking the phase of the oestrus cycle 187

6.2.3 Oestrogen treatment 189

6.2.4 Delay fear conditioning 189

6.2.5 Cyokine measurement 190

6.2.6 Immunohistochemistry 191

6.2.7 Data analysis 191

6.3 Results 192

6.3.1 Surgery induces increased levels of plasma IL-6 but not IL-1β or TNF-α in young adult female mice. 192

6.3.2 Hippocampal tissue levels of IL-1β is not significantly increased and microgliosis does not occur after surgery in young adult female mice. 195

6.3.3 Contextual and auditory-cued fear memories after delay conditioning are not impaired in young adult female mice undergoing surgery. 197

6.3.4 Surgery elevates plasma concentrations of inflammatory cytokines IL-1β, IL-6 and TNF-α in old female mice. 199

6.3.5 Treatment with E2 reduces the surgery-related increase in plasma concentrations of inflammatory cytokines IL-1β, IL-6 and TNF-α measured in old female mice. 200

6.3.6 Hippocampal IL-1β concentrations are elevated in old female mice after surgery compared with naive mice but this effect is reduced by E2 treatment. 204

6.3.7 Surgery is followed by microglial activation in old female mice. 205

6.3.8 Recall of contextual fear memories after delay conditioning is impaired in female old mice undergoing surgery, E2 treatment has no effect. 208

6.4 Discussion 211
6.1 INTRODUCTION

Evidence from previous studies supports neuroinflammation as a possible pathogenic mechanism in the development of POCD (see Chapters 3 and 4; Wan et al., 2007), whereas age has been suggested to be the main risk factor (Moller et al., 1998).

Microglia, the resident inflammatory cells of the brain, appear to be primarily responsible of promoting hippocampal inflammation in response to surgery-induced systemic cytokines release. An important role has been proposed for IL-1β as an inflammatory signal communicating to the brain from the periphery and as the most likely signalling molecule to generate the self-perpetuating cycle of microglial activation, local cytokine release and cognitive dysfunction.

Resident microglia ‘sense’ the environment by exhibiting a typical ‘resting’ phenotype, with highly ramified pseudopodia and low expression of membrane receptors, serving important immunological functions. When some kind of disturbance of the surrounding milieu occurs, microglia would rapidly change the configuration of the inner cytoskeleton resulting in the enlargement of the somata as well as shortening and thickening of the cytoplasmatic processes (Stence et al., 2001). This enlarged, macrophage-like, configuration confers phagocytic properties to the cells, which are designed to engulf and remove noxious molecules and cellular debris (Beyer et al., 2000). The neuroprotective activity of microglia, however, may turn neurotoxic when excessive activation occurs, as is often sustained by an enduring inflammatory milieu. This detrimental activity appears to be responsible for the inflammatory-mediated pathogenetic mechanisms of several neurodegenerative
diseases of old age, including those causing dementia such as Alzheimer’s disease (Minghetti, 2005).

The female gender appears to be protective against inflammatory-mediated neurodegenerative disorders as indicated by experimental studies, in which oestrogens mitigate the inflammatory response in the brain (Vegeto et al., 2006). This anti-inflammatory hypothesis is corroborated by evidence that menopause, the period of life in which oestrogen levels are drastically reduced, is associated with an increased incidence of inflammatory conditions in a number of different organs, including the brain (Bonomo et al., 2009).

The administration of physiological doses of oestrogens prior to the onset of disease produces a substantial anti-inflammatory effect that can be demonstrated by the reduced expression of cytokines, chemokines and their receptors in an experimental model of inflammation with lipopolysaccharide (LPS) (Vegeto et al., 2003). Oestrogens have also been shown to regulate microglial activation both in vitro (Vegeto et al., 2001) and in vivo (Vegeto et al., 2006).

I hypothesised that the female gender, in young adulthood, protects from the surgery-induced systemic and hippocampal inflammation and the consequent cognitive impairment that follows microglial activation. I have therefore studied the interaction between age and gender on the effect of anaesthetics and surgical trauma in a mouse model of POCD. The possible therapeutic use of oestrogens in old animals was also investigated.
6.2 METHODS

6.2.1 Animals and surgical methods

All the experiments were conducted under Home Office approved licence and were performed using 12-14 weeks old and 78-82 weeks old female C57-BL6 mice (Harlan, Oxon, UK) housed in groups of up to 4 animals/cage, under a 14:10 hours light-dark cycle, in a constant temperature and humidity controlled environment, with free access to food and water. Acclimatization to light/dark cycle for a minimum of 7 days preceded any intervention or assessment. All the animals were checked on a daily basis for signs of infection. Evidence of poor grooming, huddling, piloerection, weight loss, wound dehiscence, muscle twitching, back arching and abnormal activity, were recorded.

Mice were randomly assigned to the following groups: surgery under general anaesthesia, general anaesthesia without surgery, and no intervention (naive). The old animals were also assigned to an additional surgical group, which was injected daily with 10µg of subcutaneous (sc) 17β-estradiol (E2) for 6 weeks prior to surgery and thereafter until they were sacrificed (Vegeto et al., 2006). Under aseptic conditions, groups of mice were subjected to an open tibial fracture of the left hind paw with an intramedullary fixation as previously described (Harry et al., 2008 and Chapter 4; section 4.2.1). Briefly, mice received isoflurane 2.1% in air (1.5 MAC) and buprenorphine 0.1mg/kg (sc) to provide both surgical anesthesia and extended post-operative analgesia for the surgical intervention and post-surgical recovery. A fracture of the tibial shaft was created under direct vision. A longitudinal incision was made through the skin and fascia lateral to the tibia to expose the bone. A 0.5mm hole was
drilled just above the proximal third of the tibia to insert an intramedullary 0.38mm diameter stainless steel fixation wire. Subsequently, the fibula and the muscles surrounding the tibia were isolated, the periosteum stripped over a distance of 10mm circumferentially and an osteotomy was performed with scissors at the junction of the middle and distal third of the tibia. The skin was sutured and intra-operative fluid loss was replaced with 0.5 ml of subcutaneously injected normal saline. A group of animals received anaesthesia (isoflurane 1.5 MAC for 20 minutes) and analgesia (buprenorphine 0.1 mg/kg) without surgical intervention or other treatment.

6.2.2 Checking the phase of the oestrus cycle

Young adult mice had surgery in the diestrus phase of the oestrus cycle when oestrogen levels are at their minimum. In order to check the phase of the cycle, vaginal smears samples were collected in saline. The saline cell suspensions were smeared onto slides fixed with 95% ethanol and dried. Smears were subsequently de-salted with a distilled water rinse and dipped for 4 minutes in an aqueous solution of 0.1% toluidine blue stain, made slightly basic with dilute sodium hydroxide. The slides were then rinsed again and air dried. Slides were analysed under light microscopy in order to determine the phase of the oestrus cycle (Figures 6.1 and 6.2).
Figure 6.1 Pattern of the 4-day oestrus cycle in the female adult mouse. In this diagram, the concentrations of serum 17β estradiol and progesterone are shown as they relate in time to the surge of luteinizing hormone (LH). Ovulation usually happens in the early morning hours of oestrus, approximately 10-12 hours after the peak of LH. Timing for surgery occurred approximately in the diestrus 1 phase of the cycle, as shown. Shaded segments at the bottom of the figure indicate the dark timing of a 14:10 hours light:dark photoperiod (modified from Goldman et al., 2007).

Figure 6.2 Photomicrographs of unstained vaginal secretion from adult female mice. Proestrus (A), predominantly consisting of nucleated epithelial cells; oestrus, with anucleated cornified cells (B); diestrus, consisting predominantly of leucocytes (C).
6.2.3 Oestrogen treatment

All the animals were injected daily with E2 or equivalent volumes (0.1 ml) of saline. Mice were allowed free, unrestricted food and water intake following recovery from the procedure. Rodents from each treatment group were randomly assigned to two different assessment groups for either harvesting blood and tissue samples or for cognitive behaviour, in order to obviate possible confounding effects of fear conditioning testing (Nguyen et al., 1998) on inflammatory markers.

6.2.4 Conditioning chamber and delay fear conditioning

The methods used to assess freezing behaviour after delay fear conditioning, in a conditioning chamber, as an indicator of aversive memory when mice are re-exposed to the tone, context or auditory cue in a new context have been described previously (Chapter 4; section 4.2.2).

Briefly, all the animals, regardless to the specific intervention, underwent the same handling, including naive animals. After testing, animals were returned to their housing cage. Three days after conditioning, mice were transported again to the behavioural room to undergo context testing. Both young and old female mice were returned into the same chamber where training had occurred for a context test lasting 270 seconds, during which no tones or foot-shocks were delivered. At the end of the test, mice were individually returned to their home cage. Approximately 3 hours later, freezing was recorded in a novel environment and in response to auditory cue. Mice were placed in this novel environment, and time sampling was used as baseline during which freezing was scored for 135 seconds. The auditory cue was then presented for 135 seconds, and freezing was again recorded.
On each day of testing, mice were transported to the behavioural room and left undisturbed for at least 20 minutes before placing them into the conditioning chamber. Recording of freezing behaviour was collected by an infrared camera positioned in front of the chamber and was stored in a computer compatible format. Freezing was recognized by the software as a total lack of movement excluding breathing but including movement of fur, vibrissae and skeleton. The percentage of time spent freezing over the total time spent in the chamber to accomplish the test was used to score memory and learning abilities. A decrease in the percentage of time spent freezing indicated impairment of these abilities.

6.2.5 Cytokine measurement

Blood was collected by cardiac puncture into heparin coated syringes whilst animals were under terminal anesthesia with pentobarbital. Positive control samples consisted of animals treated with i.p. LPS. Samples were processed as previously described (Chapter 4; section 4.2.3). IL-6 and TNF-α were measured in plasma using a commercially available ELISA kit (Biosource, CA), whereas the ELISA kit for IL-1β was from a different manufacturer (Bender Medsystem, CA). The sensitivities of the assays were 1.2 pg/ml for IL-1β, 3 pg/ml for TNF-α and 3 pg/ml for IL-6.

Harvesting and processing of mouse hippocampal tissue was carried out as described previously (Chapter 4, section 4.2.3). IL-1β was measured in the supernatant from hippocampal extracts using a commercially available ELISA kit (Bender Medsystem, CA). Samples were appropriately diluted prior to ELISA measurement, so that concentrations could be measured along the linear portion of the sigmoid curve. The
sensitivity of the assay was 1.2 pg/ml. The ELISA kit was validated for use with brain tissues (see Chapter 4; section 4.2.3).

6.2.6 Immunohistochemistry

Methods have been previously described in detail (Chapter 4; section 4.2.4). Briefly, mice were transcardially perfused with PBS and 4% paraformaldehyde fixative. The brains were removed, post-fixed and mounted in embedding media, before being cut on a cryostat to produce a series of 30μm coronal sections freeze-thaw mounted on slides. The rat anti-mouse monoclonal antibody, anti-CD11b (low endotoxin, clone M1/70.15) in the concentration of 1:200 (Serotec, Oxford, UK) was used to label microglia. Visualization of immunoreactivity for CD11b was achieved using the avidin-biotin technique (see Chapter 2; section 2.4.4) and a goat anti-rat secondary antibody at a concentration of 1:200. A negative control omitting the primary antibody was performed in all experiments. A positive control group consisted of animals injected i.p. with LPS 3mg/kg. Immunohistochemical staining was visualised with an Olympus BX-60 microscope and photomicrographs captured with a Zeiss KS-300 colour 3CCD camera. The assessment of microglial CD11b staining was carried out by an observer that was blinded to the interventional group and was based upon a 4-point categorical scale modified from Colburn and colleagues (Colburn et al., 1997) (see Chapter 2; section 2.4.6), which uses a combined evaluation of the level of microglial activation from both cell morphology and immunoreactivity.

6.2.7 Data analysis

Data are expressed as mean ± SEM. Statistical analysis was performed with analysis of variance followed by the Student-Newman-Keuls test for numerical data. Student’s
t test was only used for comparisons between two groups. The non-parametric test of Kruskal-Wallis followed by the Dunn’s test was used for categorical data. A p value of < 0.05 was considered to be of statistical significance.

6.3 RESULTS

6.3.1 Surgery induces increased levels of plasma IL-6 but not IL-1β or TNF-α in young adult female mice.
Consistent with experiments on male adult mice (Chapter 4, section 4.3.1), surgery of the tibia on young female adult mice was followed by significantly increased plasma concentrations of IL-6, occurring 6 hours after the procedure, when compared to animals in naive and anaesthesia only groups. Elevated IL-6 levels had returned to normal 24 hours post surgery, when assessed in comparison with naive/anaesthesia only counterparts (Figure 6.3). Contrastingly, when the profiles of other cytokines were assessed, the levels of IL-1β (Figure 6.4) and TNF-α (Figure 6.5) were not different from the naive group. As expected, the administration of anaesthetics, in absence of any surgical stimulation, did not exert any effect on any of the measured cytokines, IL-6, IL-1β or TNF-α (Figures 6.3-5).
Figure 6.3 Plasma levels of IL-6 are increased in young female mice 6 hours after surgery. Plasma IL-6, measured by ELISA, increased 6 hours after surgery, compared to plasma measurements in naive and anaesthetic only groups of mice at the same time point. IL-6 levels returned to the same level as naive or anaesthesia groups 24 hours after surgery. Mice subjected to anaesthesia alone showed no change at both 6 and 24 hour time points. Surgery was carried out in the diestrus phase of the oestrus cycle to standardize as much as possible for the level of circulating oestrogens. Anaesthesia: isoflurane 1.5 MAC for 20 minutes + buprenorphine 0.1mg/kg; Surgery: as in Anaesthesia group but with surgery of the tibia. Data are expressed in pg/ml as mean ± SEM; n=6. **p<0.01 for comparison with naive or anaesthesia at the same time point.
Figure 6.4 Plasma levels of IL-1β in young female mice were not changed after surgery. Plasma levels of IL-1β, as assessed by ELISA, did not increase at time points 6 or 24 hours after surgery as compared to naive. Surgery was carried out in the diestrus phase of the oestrus cycle to standardize as much as possible for the level of circulating oestrogens. The administration of anaesthetics alone did not cause any IL-1β-mediated inflammation 6 hours after anaesthesia when compared to naive mice or to surgical littermates at the same time point. Naive: no treatment. Anaesthesia: isoflurane 1.5 MAC and buprenorphine 0.1 mg/kg for 20 minutes. Surgery: as Anaesthesia but with surgery of the tibia. Data are expressed in pg/ml as mean ± SEM; n=6.
Figure 6.5 Plasma concentration of TNF-α in young female mice is not affected by either surgery or anaesthesia. Plasma levels of TNF-α did not increase at any of the considered time points after administration of anaesthetics alone or in combination with surgery, compared to naive. Surgery was carried out in the diestrus phase of the oestrus cycle to standardize as much as possible for the level of circulating oestrogens. Anaesthesia: isoflurane 1.5 MAC for 20 minutes + buprenorphine 0.1mg/kg. Surgery: as in Anaesthesia group but with surgery of the tibia. Data are expressed as mean pg/ml ± SEM; n=6.

6.3.2 Hippocampal expression of IL-1β is not significantly increased and microgliosis does not occur after surgery in young adult female mice.

Young adult female mice undergoing surgery did not have significantly increased expression of IL-1β in hippocampal tissue as measured by ELISA, 6 hours after surgery, as compared with naive littermates (Figure 6.6) or male adult mice (Chapter 4; section 4.3.2). Furthermore, unlike male adult mice (Chapter 4; section 4.3.3) staining hippocampal tissue with CD11b failed to show morphological changes in the microglia 24 hours after surgery, when compared to naive animals (Figure 6.7). Also,
the intensity of the staining was not significantly increased in surgical mice, from baseline (Figure 6.7).

**Figure 6.6 Concentrations of hippocampal IL-1β remain unchanged in young female mice after surgery.** The young adult female mice did not show any change in IL-1β levels measured in hippocampal tissue, 6 hours after surgery as compared with naive littermates. Surgery was carried out in the diestrus phase of the oestrus cycle to standardize as much as possible for the level of circulating oestrogens. Naive: no treatment. Surgery: surgery of the tibia under isoflurane 1.5 MAC + buprenorphine 0.1mg/kg anaesthesia. Data are expressed as mean pg/100µg of proteins from hippocampal extracts ± SEM, n=8.

**Figure 6.7 Immunohistochemistry of CD11b-stained hippocampal microglia in young adult female mice: naive and after surgery.** Mice subjected to surgery under general anaesthesia with
isoflurane 1.5 MAC and buprenorphine 0.1mg/kg (B) did not show significant evidence of reactive microgliosis in the hippocampus of young adult female mice, compared to naive littermates (A). Scale bar 30μm.

6.3.3 Contextual and auditory-cued fear memories after delay conditioning are not impaired in young adult female mice undergoing surgery.

To further investigate the possible consequences of surgery on learning and memory abilities, comparisons were carried out in young female adult mice trained with delay fear conditioning and tested for freezing to context and tone. Mice were trained 30 minutes before surgery. As expected, there was no difference in freezing time between the groups during training (data not shown). Two different groups of mice received either surgery under anaesthesia with isoflurane and buprenorphine or no treatment. Three days after surgery, the animals were taken back to the conditioning chamber and re-exposed to the same environment as in training. The context test (Figure 6.8 A; n=20 per group) revealed no difference in freezing in the surgical group compared with naive animals. All groups of animals displayed the same freezing behaviour during baseline testing and when exposed to the tone in the auditory-cued behavioural test (Figure 6.8 B; n=20 per group).
Figure 6.8 Contextual fear and auditory-cued fear memories are not disrupted by surgery in young female mice. Rodents underwent fear conditioning and half an hour later they were divided to receive surgery of the tibia under anaesthesia with isoflurane 1.5 MAC and buprenorphine 0.1mg/kg (Surgery) or no treatment (Naive). Contextual and acoustic-cued memories were tested three days later.

A. Recall of contextual delay fear conditioning memories, as measured by freezing behaviour, was not impaired in animals receiving surgery (n=20) compared to the naive group (n=20).

B. Freezing in the auditory-cued test after delay fear conditioning: There was no difference between the groups in either baseline or auditory-cued-related freezing behaviour, thus suggesting that amygdalar-dependent memory function is intact after surgery. Data are expressed as mean ± SEM percentage of freezing response, n=20 per group.
6.3.4 Surgery elevates plasma concentrations of inflammatory cytokines IL-1β, IL-6 and TNF-α in old female mice.

Plasma concentrations of IL-1β, IL-6 and TNF-α were measured postoperatively with ELISA, in order to draw a temporal relationship between peripheral injury and systemic cytokine release in aged female animals. Plasma levels of IL-1β, IL-6 and TNF-α were all increased above the level of naive animals, but in a different temporal order (Figure 6.9-6.11). IL-1β was not elevated 6 hours after surgery, yet peaked at 24 hours and remained significantly higher than naive counterparts for 72 hours after surgery. IL-1β returned the level of naive animals only one week after the operation and did not change from naive levels in mice exposed to anaesthetics alone, at any time point considered (Figure 6.9). IL-6 levels peaked at 2 hours, remained elevated at 72 hours and had returned to naive levels by one week post surgery (Figure 6.10). Mice that were anaesthetised but did not receive surgery did not display increased plasma concentrations of IL-6 at any time point, from 2 to 168 hours after the operation, compared with naive mice. Unlike the results in young adult female mice, TNF-α concentrations in old adult females increased 6 hours after surgery and returned to the levels of naive animals after 24 hours (Figure 6.11). Consistent with young adult female mice undergoing surgery, TNF-α was also unchanged in old surgerised animals at 2 and 72 hour time-points post surgery compared to naive. It was also unchanged from baseline in mice undergoing administration of anaesthetics with no surgery, 6 hours after treatment (Figure 6.11).
6.3.5 Treatment with E2 reduces the surgery-related increase in plasma concentrations of inflammatory cytokines IL-1β, IL-6 and TNF-α measured in old female mice.

Interestingly, when the cytokine levels were measured in mice treated with E2 for 6 weeks and thereafter until euthanized, the trend was significantly changed in all the cases. IL-1β was still increased 24 hours postoperatively but significantly less than in the surgical group with no E2 treatment and levels returned to those of naive mice 72 hours after surgery rather than at the 168-hour time point (Figure 6.9).

Likewise, IL-6 plasma concentrations were still increased in the E2-treated surgical group compared to naive mice (following a similar time-course as that of surgical counterparts) but were significantly lower when compared to the latter. Also IL-6 values returned to naive levels earlier in E2-treated surgical mice (72 hours after surgery) when compared to mice undergoing surgery without E2 treatment (Figure 6.10). TNF-α did not increase at any of the time points considered when surgery was preceded by E2 treatment (Figure 6.11).
Figure 6.9 Surgery under anaesthesia, but not anaesthesia alone induces increased plasma levels of IL-1β in old female animals. Treatment with 17β-estradiol attenuates the effect of surgery on IL-1β plasma concentrations. Plasma concentrations of IL-1β were significantly increased 24 hours after surgery in old female mice compared with mice receiving anaesthesia alone and this difference persisted for up to three days post surgery. Administration of anaesthetics alone to the animals did not produce any effects on plasma IL-1β levels compared with naive animals (time point zero). Treatment with 17β-estradiol (oestrogens) mitigated the effect produced by surgery on plasma IL-1β levels. The IL-1β plasma concentrations in the surgery group were significantly elevated above those in the surgery + oestrogen treatment group for up to 72 hours. Naive: received no anaesthesia or surgery. Anaesthesia: isoflurane 1.5 MAC and buprenorphine 0.1 mg/kg for 20 minutes + vehicle. Surgery: as Anaesthesia but with surgery of the tibia; Oestrogens: as Surgery but with 17β-estradiol 10μg i.p. for 6 weeks prior to surgery and thereafter, until euthanized. ***p<0.001 for comparison of surgery vs anaesthesia or surgery + oestrogens groups. ###p<0.001 for comparison of surgery + oestrogens with anaesthesia group and naive (time point 0). Data are expressed in pg/ml as mean ± SEM, n=6.
Figure 6.10 Surgery under anaesthesia, but not anaesthesia alone, triggers increased plasma concentrations of IL-6 in old female animals. Administration of 17β-estradiol attenuates the effect of surgery on plasma IL-6 levels. The plasma concentration of IL-6 was increased 2 hours after surgery in old female mice compared with naive mice and this increase persisted for up to three days. Administration of anaesthetics alone to the animals did not produce any effects on IL-6 concentrations relative to naive mice. Treatment with 17β-estradiol mitigated the effect produced by surgery and disappeared 72 hours after the operation. Anaesthesia: mice receiving anaesthetics only (isoflurane 1.5 MAC + buprenorphine 0.1mg/kg). Surgery: mice undergoing surgery under anaesthesia. Surgery + Oestrogens: as in Surgery, but treated with 17β-estradiol for 6 weeks before surgery until euthanized. ***p<0.001 for comparison of surgery versus anaesthesia or surgery + oestrogens groups. ###p<0.001 for comparison of surgery + oestrogens group versus anaesthesia or naive (time 0) groups. **p<0.01 and #p<0.05 for comparison with anaesthesia or naive groups. Data are expressed in pg/ml as mean ± SEM, n=6.
Figure 6.11 Plasma levels of TNF-α are increased above naive animals in old female mice at 6 hours after surgery but not after 17β-estradiol treatment. Plasma TNF-α concentrations, as measured by ELISA, increased 6 hours after surgery compared to naive animals and to those receiving anaesthetics. TNF-α levels went back to naive or anaesthesia group levels 24 hours after surgery. Mice subjected to anaesthesia only, showed no change in TNF-α concentrations 6-hour post surgery compared with naive mice. Treatment with 17β-estradiol prevented the 6-hour post surgery increase in TNFα. Naive: vehicle injected. Anaesthesia: isoflurane for 20 minutes + buprenorphine 0.1mg/kg + vehicle; Surgery: as in Anaesthesia group but with surgery of the tibia; Surgery + Oestrogens: as in Surgery but treated with 17β-estradiol 10 μg i.p. for 6 weeks prior to surgery and thereafter, until mice were sacrificed. Data are expressed in pg/ml as mean ± SEM; n=6. ***p<0.001 for comparison of the surgery group at 6 days post surgery with naive, anaesthesia only or surgery + oestrogens at the same time point.
6.3.6 Hippocampal IL-1β concentrations are elevated after surgery in old female mice compared with naive mice but this effect is reduced by E2 treatment.

IL-1β expression was measured in hippocampal tissue because in this site IL-1β contributes to the development of local inflammation and microglial activation (Laflamme and Rivest, 1999), and its dysregulation has been linked to impairment of hippocampal-dependent memory function (Goshen et al., 2007). Since E2 has been shown to possibly reduce inflammation and microglial activation, I hypothesized that E2 effectively reduces both IL-1β hippocampal expression and microglial activation. The concentration of IL-1β in the hippocampus was assessed 6 hours after surgery, and found to be increased compared to naive animals. It was not changed in animals exposed to anaesthesia alone. Pre-treatment with E2 in surgical animals significantly reduced IL-1β concentrations in hippocampal tissue, but not to naive levels (Figure 6.12).
Figure 6.12 Surgery is associated with increased concentrations of hippocampal IL-1β in old female mice. Surgery-induced increases in hippocampal IL-1β are reduced by oestrogens. IL-1β concentrations in hippocampal tissue measured by ELISA were increased 6 hours after surgery compared to naive and anaesthesia groups. 17β-estradiol treatment for 6 weeks prior to surgery and thereafter until the animals were sacrificed, mitigated surgery-induced increases in hippocampal IL-1β. In this group the concentration of IL-1β in the hippocampus was still significantly higher than naive or anaesthesia-alone-treated mice. Naive: vehicle injected. Anaesthesia: isoflurane 1.5 MAC and buprenorphine 0.1 mg/kg for 20 minutes + vehicle. Surgery: as Anaesthesia but with surgery of the tibia; Oestrogens: as Surgery but with 17β-estradiol 10μg i.p. for 6 weeks prior to surgery and thereafter, until mice were sacrificed. Data are expressed as mean pg/100μg of protein extracted from hippocampal tissue ± SEM, (n=7 per group) ***p<0.001 and **p<0.01 for comparison of surgery or surgery + oestrogens vs naive or anaesthesia groups. #p<0.05 for comparison of surgery versus surgery + oestrogens group.

6.3.7 Surgery is followed by microglial activation in old female mice

The finding of increased hippocampal IL-1β levels after surgery which are effectively reduced with a long-term treatment with E2, left open the question as to whether microglia would be activated and possibly affected by E2 treatment in the hippocampus of old female animals. Interestingly, surgery-induced morphological changes to CD11b-immunoreactive microglia included increased cell body dimensions, shortened and clumpy processes and higher intensities of CD11b immunoreactivity; consistent with the transition of these cells from a resting to a reactive state. This change was apparent as early as 24 hours after surgery (Figure 6.13 C) and was significantly different from that of naive and anaesthesia-only-treated old female mice (Figure 6.13 A,B). Surgery-induced reactive microgliosis was reduced by day 3 (Figure 6.13 F) after the operation and returned to baseline within 7 days (Figure 6.13 G) in old animals not exposed to E2 treatment.
Treatment with E2 did not significantly affect reactive microgliosis immediately (1 day) after surgery (Figure 6.13 D), but microgliosis was significantly reduced in E2-treated mice 3 days after surgery when microglial staining in this group was no longer different from naive and anaesthetic only-treated mice (Figure 6.13 H).
Figure 6.13 Photomicrographs of microglia stained with CD11b in hippocampal tissue of old female mice. Hippocampi were harvested 1, 3 or 7 days after treatment. Neither naive (A) nor mice
treated with anaesthetics alone (B) showed evidence of reactive microgliosis. Reactive microglia were found in animals undergoing surgery, one day after the procedure, featuring amoeboid hypertrophy of cell bodies with shortened and clumped processes (C). Reactive microgliosis persisted up to 3 days after surgery (E) and returned to pre-surgical levels after 7 days (G). Mice were also treated with 17β-estradiol 10μg i.p. for 6 weeks prior to surgery and thereafter, until sacrificed. One day after surgery, mice treated with 17β-estradiol exhibited a reactive morphology of their hippocampal microglia, which was similar to that of surgical littermates with no other treatment, one day after the procedure (D). Microgliosis in the oestrogen-treated group was reduced compared to surgical littermates, but not completely abolished, at the same time point, 3 days after surgery (F). Microglia in the oestrogen-treated group returned to naive levels within 7 days after surgery (H). Scale bar 30μm (n=7 per group).
Figure 6.13.1 Median (horizontal bar) with 25th to 75th (box) and 10th to 90th (whiskers) percentiles for immunohistochemical grading (0-3) of microglia in old female animals. One day after surgery, old female mice showed significantly higher levels of reactive microgliosis compared to naive or anaesthetics only, but not with surgical old mice receiving oestrogens (A). Three days after surgery old female mice continued to show an increase in reactive microglia compared with naive and anaesthesia animals (B). By 7 days post surgery, microglial activation had returned to normal (C). **p<0.05 surgery or surgery+oestrogens groups vs naive and anaesthesia group; *p<0.05 for group comparisons as indicated in the graph; (n=7).

6.3.8 Recall of contextual fear memories after delay conditioning is impaired in female old mice undergoing surgery; E2 treatment has no effect.

Since elevated levels of hippocampal IL-1β and reactive microgliosis both depended on age in female mice and were significantly reduced after treatment with oestrogens, the hypothesis that surgery-induced inflammation causes memory impairment in old female mice and can be reduced by oestrogens, was tested. Mice were trained with delay fear conditioning and tested for freezing to context and tone. The animals were trained shortly before any treatment (<30 minutes), and no difference was shown in freezing time between the groups during training (data not shown). Different groups of mice received one of the following interventions: surgery under anaesthesia, or administration of anaesthetics without surgery, or pre- and post-operative administration of E2. Three days after treatment, rodents were taken back to the conditioning chamber and re-exposed to the same environment as in training. The context test (Figure 6.14) revealed a significant reduction of freezing in the surgical group compared with naive animals or with those undergoing anaesthesia alone (p<0.05). Administration of E2 did not improve surgery-induced impairment of memory for context (p<0.05; Figure 6.14). Moreover, there was no difference in
freezing between naive and animals receiving anaesthetics alone. Animals in all experimental groups displayed equivalent freezing behaviour when exposed to the tone in the auditory-cued test (Figure 6.15).

![Freezing behaviour graph](image)

**Figure 6.14** Memory for context is not affected after anaesthesia, but is impaired after surgery in old female mice conditioned with a delay fear paradigm. Administration of 17β-estradiol does not exert any effect on surgery-induced, hippocampal-dependent memory dysfunction. Rodents underwent delay fear conditioning and 30 min later they were divided to receive anaesthetics (Anaesthesia), or surgery of the tibia under anaesthesia (Surgery), or the same surgical procedure with 17β-estradiol administration (Oestrogens), respectively. Naive group received no treatment. Contextual and acoustic-cued memories were tested three days later. Recall of contextual delay fear conditioning memories, as measured by freezing behaviour, was impaired in surgical animals compared to naive and anaesthesia groups. Administration of 17β-estradiol for 6 weeks prior to surgery and thereafter until the animals were sacrificed, did not affect the surgery-induced reduction in freezing. Data are expressed as
mean percentage of freezing ± SEM, ***p<0.001 surgery vs naive group; #p<0.05 and ##p<0.01 surgery or surgery + oestrogens groups vs anaesthesia group. (n=18 per group).

Figure 6.15 Acoustic-cued fear memory is not impaired after surgery in old female mice trained with delay fear conditioning. There were no differences between any of the experimental groups in either baseline or auditory-cued-related freezing behaviour, thus suggesting that amygdalar-dependent memory function is intact after surgery. Naive: vehicle injected; Anaesthesia: isoflurane and buprenorphine for 20 minutes + vehicle; Surgery: as Anaesthesia but with tibia surgery; Oestrogens: as Surgery but treated with 17β-estradiol 10μg i.p. for 6 weeks prior to surgery and thereafter, until mice were sacrificed. Data are expressed as mean percentage of freezing ± SEM, (n=18 per group).
6.4 DISCUSSION

Findings from this study propose that young age coupled with female gender have a protective role in the development of systemic and hippocampal inflammation. The concentration of systemic pro-inflammatory cytokines, hippocampal IL-1β and the extent of reactive microgliosis in young female mice undergoing surgery remained at the same level of naive littermates, with the only exception of plasma IL-6 concentrations. This contrasts with the results of the same experiments conducted in adult male mice (Chapters 3 and 4) and in old female mice. The subsequent evidence that hippocampal-dependent memory functions remain intact after surgery in these animals was not surprising, as this logically relates to the reduced inflammatory phenotype displayed by young adult female mice. Consistently with any other condition examined in this thesis, amygdalar memory function was not affected by either surgery or oestrogenic treatments in old female mice (Figure 6.15).

As supported by previous findings in an LPS model (Pugh et al., 1998), pro-inflammatory cytokine production has long been viewed as the link between peripheral and central inflammation and cognitive dysfunction, where IL-1β plays a primary role. The reason why young female animals are protected in this model of POCD could therefore be dependent on the anti-inflammatory properties of the oestrogens (Vegeto et al., 2006). But the mechanism by which the oestrogens putatively exert their protective effect against surgery-induced cognitive dysfunction in young adult mice could also be related to the improved cognitive performance that these compounds exert in numerous conditions in different tissues. A large body of evidence demonstrates that the oestrogens, as well as other gonadal and adrenal steroid hormones, control neuronal and glial functions (Schumacher et al., 2000). This
is because specific receptors for oestrogens, which have been found in many brain regions, including amygdala, cortex and hippocampus (especially on glial cells) contribute to the regulation of cognitive and memory functions in both physiological and pathological conditions (Sherwin, 1997). These activities are mediated by the binding of oestrogens to the two known types of oestrogen receptor, ER\textgreek{a} and ER\textgreek{b}, which are expressed in neural tissues. Receptor signalling occurs through the activation of cAMP and MAPK pathways, ultimately converging on kainate and IGF-1 receptors (McEwen and Alves, 1999); or by modulating G-protein coupling and calcium ion channels activities (Brinton, 2001) protecting neurones from toxicity of free radicals and excitotoxic damage (McEwen et al., 2001). In addition, some ‘neurosteroids’, including oestrogens, can be secreted by neuronal and glial cells, after endogenous or exogenous stimulation of the central nervous system (Baulieu, 1997). These multi-faceted pharmacodynamic mechanisms linked to the anti-inflammatory properties of the oestrogens, may also explain the reason why premenopausal women are relatively protected from cerebrovascular diseases, such as ischaemic stroke (Sudlow and Warlow, 1997).

However, the postmenopausal physiological and progressive exhaustion of oestrogenic ligands and consequent lack of protective neuro-modulation points to the contribution of age in the progressive deterioration of cognitive functions and development of a neuroinflammatory predisposing milieu.

Hence, this research was also aimed at scrutinizing the possible consequences of age, and to unveil the putative therapeutic effects of oestrogens, on the development of inflammatory, IL-1\textbeta-mediated, POCD. Data from these investigations support age as
a main risk factor for POCD and suggests that a treatment with oestrogens can reduce the extent of the surgery-induced inflammation, but it could be insufficient to tackle the consequences of a residual inflammatory response to surgery on memory function. This is because the ageing brain is normally subjected to a shift from the fine homeostatic balance between pro-inflammatory and anti-inflammatory cytokines to a pro-inflammatory state (Tha et al., 2000). This has been suggested to be responsible for the reduced threshold of reactivity to pro-inflammatory noxious stimulation or stress in the aged brain (Buchanan et al., 2008).

Evidence of post-operative microgliosis and increased levels of hippocampal IL-1β in old but not in young female animals from this study, strengthens the argument for a protective role of young age and adds to the body of evidence supporting the anti-inflammatory properties of the oestrogens both systemically and on the microglial reactivity. Access to age-matched male mice to test along-side the old female animals could have strengthened this evidence, however, unfortunately, these were not available. Nevertheless, this study provides additional evidence for the acute anti-inflammatory effects of oestrogens by presenting data demonstrating a reduction in the levels of surgery-induced hippocampal IL-1β following E2 administration in old female animals together with a partial ameliorating effect on surgery-induced microgliosis.

However, the protection achieved in this model by oestrogen treatment was not complete, that is, it failed to guard against the memory impairment that follows surgery in the old female animals. A possible explanation is that acute oestrogen treatment is insufficient to prevent cognitive impairment in the aged brain, which is
already in a pro-inflammatory state in which a greater proportion of resident microglia are primed for activation in response to stimuli (Sparkman and Johnson, 2008). Another possible explanation is that the critical number of IL-1 receptors in the hippocampus were still saturated with the cognate ligand, IL-1β, in old mice treated with oestrogens. This is consistent with the partial reduction of surgery-induced IL-1β levels in the hippocampus produced by oestrogen treatment.

The level of protection from pro-inflammatory insults afforded by oestrogens is still far from being completely unravelled. Further studies are needed to improve our understanding of this complex aspect linking age and gender to POCD.
Chapter Seven

General discussion

and conclusions
Postoperative cognitive dysfunction (POCD) is certainly one of the most common and incapacitating conditions of the elderly hospitalised population. Approximately one quarter of surgical patients aged 65 or older will be expected to develop this debilitating condition. If the prediction is made amongst specific major surgical settings, such as cardiac surgery, orthopaedic surgery and few others, then these alarming prospective figures may go up to 40-60%. Furthermore, the rates of POCD and postoperative delirium (POD) are likely to increase exponentially. This is because the continuing development of surgical techniques is geared towards offering surgery to an increasing proportion of the expanding elderly population; in order to achieve the ever-extending targets for life span and healthy ageing (Perls, 2004). A projection of American population growth shows that the number of Americans due to be aged 65 or older by 2030 will be close to 62 million (US Census Bureau, 2004). According to a study dated 1993, the surgical population in the age range between 45 and 60 in need of an operation was around 12% of the total, whereas in the group of those aged 65 or more, the percentage went up to 21% (Ergina et al., 1993). In addition, many more people will be 85 or older as the US Census Bureau figures show that this age group will be almost doubled to reach 9.6 million by 2030 (US Census Bureau, 2004). Strikingly, health care expenditure associated with the diagnosis and treatment of POD alone (not including medical delirium or POCD) is expected to exceed $4 billion (1994 dollars) per year (Franco et al., 2001).

Thus, an effective treatment for post-operative cognitive impairment, or some efficient preventative measures, are urgently required, in order to tackle the human cost and economic burden that these complications will represent for health care in the
future. Regrettably, the quest for an appropriate treatment will continue to be extremely difficult until the factors involved in pathogenesis are discerned.

This study represents a contribution in the direction of unravelling some of the major elements involved in POCD pathogenesis. The results presented describe a peripheral and central cytokine response to surgery (likely to be part of a wider inflammatory cascade), in which the upstream and downstream mediators are all yet to be discovered.

Differences in the incidence of POCD between the various surgical settings seem to be supportive of an inflammatory mechanism as part of a multifactorial pathogenesis, which is likely, given the multifaceted nature of POCD. Orthopaedic and cardiac surgeries are the settings in which inflammation is possibly more damaging than ever. The production of inflammatory mediators in these two surgical situations has been proved to be the highest (Buvanendran et al., 2006; Gormley et al., 2002). Coincidentally, the observed incidence of POCD in cardiac and orthopaedic surgeries is also the highest (van Dijk et al., 2002; Gustafson et al., 1988).

It is now widely accepted that inflammation in the brain strongly contributes to the cognitive impairment that develops with ageing. Hallmarks of an induced inflammatory phenotype in brain are the elevation of local pro-inflammatory cytokines and microglial activation. The baseline expression of several cytokines is augmented in brain tissue, as a function of ageing; even in healthy individuals. For example, the aged population shows a progressive increase in brain concentrations of IL-1β (Wilson et al., 2002). In fact, IL-1β expression is associated with hippocampal-
dependent cognitive functioning in both physiological and pathological conditions (Rachal Pugh et al., 2001). The reduction of long term potentiation (LTP), a defined neural signalling mechanism underling memory formation that can be studied in vitro and in vivo, has been linked to a long-lasting increase of IL-1β gene expression, which is blocked by an N-methyl-D-aspartate (NMDA) receptor antagonist (Cavallaro et al., 2002). It is now accepted that the age-related decline in cognitive functioning is associated with an imbalance in brain homeostasis producing a pro-inflammatory milieu (Gathan et al., 1999). Using an experimental model of infection, increased levels of IL-1β in brain tissue have been correlated with both normal ageing and with the development of cognitive dysfunction (Rachal Pugh et al., 2001).

Peripheral inflammation can be evoked by the tissue damage sustained by surgery as well as by the invasion of a foreign pathogen or infective agent. In a model of sickness behaviour produced by peripheral injection with lipopolysaccharide (LPS) (Verker et al., 2000), an increase in the levels of IL-1β in the hippocampus was demonstrated to have a detrimental effect on LTP generation (Katsuki et al., 1990). This appeared to be orchestrated by reducing the local concentration of glutamate (Verker et al., 2000). The critical involvement of IL-1β in the modulation of LTP was confirmed by the abolition of this effect with IL-1Ra (Schneider et al., 1998). This is likely, since the hippocampus is known to be one of the areas of the brain with the highest concentration of IL-1β receptors (Cunningham et al., 1992; Parnet et al., 1994; Wong & Lucinio, 1994; Liu et al., 1996). IL-1β has also been reported to exert synaptic inhibition on hippocampal GABAergic transmission, which was sufficient to prevent postsynaptic depolarization and reduce LTP (Zeise et al., 1992). Moreover, some effects of IL-1β in the hippocampus have been demonstrated on CA1 pyramidal
neurones where it dose-dependently induced depression of inward, voltage-dependent calcium currents; an effect that was reversed with IL-1Ra (Plata-Salaman & Ffrench-Mullen). Consistent with the increased hippocampal concentrations of IL-1β produced by the peripheral surgical insult and subsequent neural inflammation in my experimental model, IL-1β is also likely to be responsible for modulation of LTP leading to the impairment in fear memory formation that I observed following tibial surgery in mice. In common with the LPS experiments, surgery-induced elevations in hippocampal IL-1β levels and memory impairment were both ameliorated by IL-1Ra treatment. This suggests that IL-1β modulation of LTP may also play a role in the surgery-induced memory dysfunctions observed in my model.

The induction of IL-1β signalling by LPS is based on a change of gene expression involving up to 90 genes (O’Neill & Greene, 1998), thereby inducing the transcription of mRNA for several other cytokines, adhesion molecules and acute-phase proteins (O’Connor & Coogan, 1999). Thus, in my study, an indirect pathway leading to the IL-1β-dependent effects on memory in the hippocampus could have involved other cytokines or adhesion molecules, which, in turn, could also have contributed to the amplification of the IL-1β signal, perhaps in coordination with the activity of other effectors, such as microglia.

Data from my studies support a link between surgery, systemic cytokine release and increased levels of hippocampal IL-1β, in line with evidence of a bidirectional communication between peripheral immunity and the central nervous system, which occurs following a peripheral challenge with either ethanol (Qin et al., 2008) or LPS (Henry et al., 2009).
In the LPS model, the upstream cascade linking LPS injection with inflammation is triggered by the activation of Toll-like receptors (TLRs), which bind to LPS. In this mechanism, the immune system relies on its recognition of evolutionary conserved microbial structures, or ‘patterns’ that are present on the pathogens. These so-called pathogen-associated molecular patterns (PAMPs) are identified by a group of antithetic receptors, named pattern recognition receptors (PRRs), of which the family of TLRs is a member (Akira et al., 2006; Medzhitov et al., 1997). PAMPs represent a class of relatively invariant molecules amongst entire classes of pathogens, in that they are essential for the survival of the pathogen, hence, easily distinguishable from self-molecules (Huang et al., 1993).

However, pathogens are not the only structures capable of inducing tissue or cell damage and of triggering an inflammatory response. Evolution has provided a most articulated programme to deal with any kind of damage, including tissue trauma. Tissue can be damaged by mechanical forces, by excessive heat or cold, chemicals or radiation. Surgery is the prototypic source of tissue damage, to which the immune system is adequately prepared to respond. In surgery, PRRs are able to recognize host factors as ‘danger signals’ (or danger associated molecular patterns, DAMPs) and mount an inflammatory response when these molecules are present in aberrant locations, or arranged in atypical structures or morphological configurations (Beg, 2002; Schroeder et al., 2005). These self-DAMPs or alarmins, are the equivalent of PAMPs, but are endogenous structures. Most of the alarmins have a role in inflammation or immunity and their similarity to PAMPs is that they often share a common downstream molecular cascade, ultimately leading to cytokine release and
the priming of inflammatory cells, such as antigen presenting cells (APCs), including monocytes, microglia, or dendritic cells (Bianchi, 2006).

High molecular group box 1 (HMGB1) could be the prototypical alarmin. It is synthesised by most of the cells (more often by myeloid and NK cells), which, when activated, secrete their nuclear HMGB1 in response to inflammatory stimuli or to the detection of PAMPs (Bianchi, 2006). Also, HMGB1 is released in the extracellular space from the secretory lysosomes of necrotic cells, as a result of non-programmed cell death (Gardella et al, 2002; Semino et al, 2007); whereas, in apoptotic cells, it remains tightly bound to chromatin and is ultimately not released (Scaffidi et al., 2002). In Alzheimer’s disease (AD), HMGB1 has been found in the brain bound to Abeta (1-42) protein, as a stabilizer for the oligomerization (Takata et al., 2003). Also, protein levels of HMGB1 were significantly increased in both the cytosolic and particulate fractions of AD brains, and HMGB1 and Abeta were co-localized in senile plaques associated with microglia, suggesting an involvement of HMGB1 in regulating the homeostasis of extracellular Abeta (1-42) and Abeta oligomerization. Strikingly, from the same study, HMGB1 inhibited the microglial uptake of Abeta (1-42) in the presence and absence of TGF-β, which is suggestive of an important interplay between microglia, HMGB1 and dementia (Takata et al., 2003).

HMGB1, therefore, appears very interesting in the context of POCD; especially because it can be released by both neurones and microglia, which, once activated, produce neurological impairment that is reduced by minocycline (Hayakawa et al., 2008).
In my mouse model of POCD, the administration of minocycline resulted in the reduction of both inflammation (systemic and CNS) and memory impairment. Reduced expression of systemic and hippocampal cytokines by minocycline is likely to be highly dependent on the inhibition of p38-MAPK; a regulator of gene expression, which is an upstream effector in the biosynthetic pathway of several cytokines, including IL-1β (Du et al., 2001). I speculate that tissue damage caused by surgery is followed by the release of alarmins, which bind to PRRs. The cascade would then continue with the activation of several post-receptoral intermediates, including p38-MAPK to phosphorylate transcription factors, which, in turn, enter the nucleus to initiate mRNA transcription leading to the biosynthesis of cytokines.

Another interesting possible site of action for minocycline is the blood brain barrier (BBB), as previous evidence shows that minocycline decreases BBB permeability (Ryu and McLarnon, 2006).

The BBB stands as a cornerstone in the homeostatic regulation of the brain microenvironment and preserves the immune-privileged condition of the brain by restricting the entry of inflammatory mediators. This property of the BBB relies on the existing tight junctions between the endothelial cells. Astrocytes, pericytes and perivascular microglia contribute to maintain BBB homeostasis by interacting with the endothelium. Inflammation, especially following infections, creates a breach into the BBB. The disruption of BBB normal function during an inflammatory process is multifactorial, and may involve enhanced production of inflammatory cytokines together with other factors, such as tissue-type plasminogen activator (tPA), vascular endothelial growth factor (VEGF) and nitric oxide (NO). Following inflammation,
monocytes can cross the BBB and travel into the brain, where they exert detrimental effects. This requires the active participation of brain endothelial cells to rearrange their cytoskeleton and tight junctions, processes that involve systemic clonal cell expansion of leucocytes (especially monocytes) and local inflammation and intracellular signalling events (Reijerkerk et al., 2008; Gurney et al., 2006). Furthermore, evidence shows that, under inflammatory conditions, systemic monocytes can infiltrate the BBB and transform into microglia (Flugel et al., 2001). Flow cytometry results from my experimental model, confirmed the clonal expansion of some subsets of monocytes and proliferation of polymorphonucleates (PMNs), but failed to show increased numbers of microglia in the hippocampus. This could be explained by the known propensity for expansion of the locally resident pool of microglial cells in the adult brain (in spite of a more complicated migration of cells from the periphery), which would take place only in conditions of severely increased inflammatory levels (Lassmann and Hickey, 1993). Therefore, in my model of POCD, inflammation was primed to such an extent as to only allow macromolecular signalling between the outside and the inside of the BBB. Experimental studies have reported beneficial effects of minocycline in ameliorating the inflammatory-induced, increased BBB permeability. An effect of minocycline on BBB permeability (Ryu and McLarnon, 2006) would interfere with the messaging between periphery and CNS, mediated by cytokines, thereby inhibiting microglial activation.

However, minocycline is an antibiotic. Microbial contamination of the surgical wound could be a plausible explanation for the memory impairment occurring in my animal model. The experiments that disproved this hypothesis involved the use of enrofloxacin, an antibiotic with a similar antibacterial spectrum as minocycline, but
devoid of any anti-inflammatory properties. All the adverse consequences of surgery in my studies, such as increased cytokine concentrations in the blood and in the hippocampus, in addition to microglial activation and memory impairment, were effectively attenuated by minocycline, but not by enrofloxacin. Surgery with or without enrofloxacin was the same in all the explored aspects of my research. This supports the conclusion that surgery-induced cognitive deterioration is caused by an inflammatory mechanism independent of an infective process.

The anaesthetic agents adopted in the experiments from my study did not cause inflammation after surgery. Isoflurane used alone or in combination with buprenorphine did not disrupt consolidation of hippocampal memories, nor did it cause any inflammatory reaction, in either the periphery or in the brain regardless of age or gender. Yet, synergism between anaesthesia and surgical trauma might have further complicated the outcome. However, it is difficult to investigate whether or not anaesthesia had a synergistic effect on the inflammation primed by surgery. The introduction of a control group undergoing surgery without anaesthesia to compare with counterparts undergoing surgery and anaesthesia would be the only way to clarify this aspect of the research. This is an insuperable limitation, in my studies, due to obvious ethical implications. Nonetheless, it is possible to generate an inflammatory response, with common characteristics to that induced by surgery, by means of an LPS injection. Indeed, evidence from the studies presented in this thesis implicates an anti-inflammatory effect (anergism) of isoflurane. Results of generalized reduced levels of cytokines and inhibition of microgliosis as well as protected memory function after isoflurane administration in LPS-treated animals
contradict the hypothesis of a synergistic effect of isoflurane in exacerbating the observed inflammatory-dependent memory impairment after surgery.

Data from this thesis corroborate the existing evidence by presenting age, in the female gender, as the main risk factor for POCD (Moller et al., 1998), and adds to the possible beneficial effects of the oestrogens on both systemic and central inflammation. The limited effectiveness of acute oestrogen treatment in preventing the surgery-induced POCD in old female animals is highly suggestive of a critical role for age in the development of this syndrome. The oestrogens, however, may have not achieved their expected therapeutic goal for many reasons, one possibly being the reduced threshold of reactivity to noxious stimulation and stressors typical of the aged brain (Buchanan et al., 2008), which is nevertheless characterized by a shift of the normal delicate physiological equilibrium towards an imbalanced pro-inflammatory state (Tha et al., 2000).

Taken together, the results presented in this thesis suggest the existence of a bidirectional communication between the site of surgical injury and the brain, mediated by inflammation. However, some limitations need to be acknowledged and further discussed. In the first instance, limitations with the model must be considered. This study is based on an experimental mouse model, where the interpretation of findings needs to be correlated to human beings. The adopted measure of memory function had to be conducted with behavioural testing based on pavlovian paradigms. This involves the use of an electric shock as an aversive stimulus, which is stressful for the animals. Other behavioural tests, such as the Morris Water Maze (MWM) would have been less stressful and, if used in conjunction with fear conditioning,
would have strengthened the results. The choice of an intramedullary stainless-steel fixation wire in the orthopaedic surgery of the tibia mimics the surgical procedure in the clinical environment. However, in fear conditioning, the fixation wire may have caused an unpredictable change in ambulation and/or motion of mice, and therefore of freezing behaviour, thereby possibly altering the overall scoring of memory function adopted in this work, in favour of more freezing in the surgical group. Nevertheless, the amount of freezing observed during the baseline period of the acustic-cued tests shows no difference in freezing time between naive mice and those subjected to surgery. My choice to conduct the analysis of memory performance with fear conditioning lies on the main characteristics of an easy controllable and objective (computer-scored) technique, which returns data of rapidly-acquired, well defined and localizable memory engrams in the neural path. The fine-tuning of a fear conditioning system can be challenging, but my efforts of finely combining US magnitude with CS duration and intensity has provided me with an optimized amount of conditioning. This is why, despite a natural tendency towards increased freezing from injured animals, which translates in a measure of increased memory retention, these tests still show a significant difference between experimental and control groups. I believe this is an implicit point of strength in the fear tests. In fact, the same difference would certainly be greater, had the animals frozen solely in consequence of the mounting hippocampal inflammation, generated in the periphery as a result of surgery. Also, some clarification is needed in the observation of microglial activation. These cells could be activated either to cause further production of cytokines, as part of a damaging mechanism that impairs memory formation and storage, or else their activation might have a protective effect, without which the observed cognitive impairment could be worse. In my model, nevertheless, the effect of minocycline on
both microglial activation and memory function seems to point towards a detrimental effect of microglial activation on memory function. Experiments in this thesis show that IL-6 blood levels were increased after surgery. IL-6 has been shown to have facilitating effects on IL-1β in mediating inflammation and causing hippocampal-dependent memory impairment (Sparkman et al., 2006), an effect that was not investigated since it was beyond the scope of this project, which was focused on IL-1β, but that merits further attention in future studies.

Another limitation is that I have considered the concentration of oestrogens in the old female animals to be low. However, the best approach would have been to measure the levels of oestrogens by routinely sampling blood in a separated group of aged female animals.

Finally, this work failed to demonstrate a protective effect of the anaesthetic isoflurane against microglial activation caused by the inflammation that follows LPS administration. This is a limitation, because persistence of microglial activation, in this case, depended on the high concentration of LPS that was necessary to produce visible levels of microglial activation. Therefore, an answer to the question as to whether LPS-induced microglial activation could be mitigated by the anaesthetics simply has not been given.

More than 10 years after the publication of the landmark article in the Lancet about POCD, by the international study group on POCD in 1998 (Moller et al., 1998), new contrasting data make their appearance in the literature, some casting doubts on the existence of POCD itself, despite the many shortcomings that strongly limit the impact of this message (Avidan et al., 2009).
New studies exploring POCD and POD are needed to clarify the many aspects that still escape our knowledge.

Despite some limitations, the work described in this thesis contributes to unveil some of the uncertainty surrounding POCD. The results presented here suggest that inflammation could be a plausible pathogenic mechanism underlying POCD. This conclusion entails a series of consequent implications. Firstly, a possible explanation for the higher incidence and seriousness of symptoms following major surgery could be dependent on the higher level of inflammation generated by a higher degree of surgical damage, like in orthopaedic and cardiac surgery. Also, in cardiac surgery, the use of the cardiopulmonary bypass is an additional source of inflammation (Jungwirth et al, 2010) and, in common with orthopaedic surgery, systemic fat or clot embolization is more likely; a phenomenon capable of priming further exacerbation of the inflammatory reaction at any organ level. Hypoxia and hypoperfusion could be considered stand-alone risk factors for the development of POCD, but in the context of inflammation these factors would assume an additional significance. In fact, hypoxia as well as hypoperfusion could be important contributors in the progression towards a pro-inflammatory state.

The tendency towards the maintenance of a pro-inflammatory state in the brain with age, makes the elderly more prone to an uncontrolled higher inflammatory response, and explains the increased susceptibility of elderly patients for POCD. Likewise, before menopause, the anti-inflammatory effect of oestrogens could further contribute to the observed differences in the incidence of POCD in young, middle and old age in women.
This dissertation may represent a starting point and offer an opportunity for some interesting considerations in view of future research on POCD. In the first instance, the creation of an animal model expressing measurable postoperative memory impairment will be useful for future studies where it could be adopted to investigate other possible pathogenic mechanisms. In addition, with a link to the present research, it could be used to further characterize upstream and downstream pathways linking IL-1β to POCD. Furthermore, the validated use of C57BL6 mice in my experiments, rather than rats, could prompt research with the use of knockout mice. Importantly, translational research may help explore IL-1β as the likely target to develop an effective preventative treatment for both short- and long-lasting POCD. IL-1Ra is successfully used in the care of some types of rheumatic diseases otherwise refractory to treatments (Dybowski et al, 2008; Calabrese, 2002). Due to its advantageous safety profile, this drug might easily be employed in clinical research to investigate the inflammatory-mediated theory of POCD that critically involves IL-1β signalling. If successful, it could provide an initial, yet important contribution in the treatment of POCD, but would also help in the future development of research aiming at the full disclosure of POCD pathogenesis.
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Appendix

Manuscripts, articles and presentations


2. White JM, Cibelli M, Nagy I. The Agents of inflammatory pain. (Accepted for publication). Pharmacology and therapeutics (IF: 9.5)

3. White JM, Cibelli M, Nagy I. ERKs in peripheral pain. (Submitted, under review). British Journal of Pharmacology (IF:3.8)

4. White JM, Cibelli M, Fidalgo A, Paule CC, Noormohamed F, Urban L, Maze M, Nagy I. Role of transient receptor potential and acid-sensing ion channels in peripheral inflammatory pain. Anesthesiology Accepted for publication; manuscript number 200 8110 67R4 (IF 5.2)


Abstracts


6. Jing Xu, M.D., Yanje Wen, M.D., **Mario Cibelli, M.D.,** Daqing Ma, M.D., Ph.D., Mervyn Maze, M.D., F.R.C.A. Postoperative Cognitive Dysfunction: A Role for Cytokine-Mediated Inflammation in the Hippocampus. American Society of Anesthesiologists Congress, ASA, Chicago, IL, USA, 2006

**Invited lecture**


**Congress attendance**


2. American Society of Anesthesiologists (ASA), Orlando, FL, USA, October 2008

3. American Society of Anesthesiologists (ASA), San Francisco, CA, USA, October 2007

4. American Society of Anesthesiologists (ASA), Chicago, IL, USA, October 2006