The role of neuroinflammatory modulation on POCD development following surgery

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DECLARATION OF ORIGINALITY

The work on which this dissertation is based was performed at the Anaesthetics, Pain Medicine and Intensive Care Section of the Department of Surgery and Oncology at Chelsea and Westminster Hospital, Imperial College, during the years 2007-2011, under the supervision of Dr. Daqing Ma.

This dissertation is exclusively the result of my own research work, except for the delay-fear conditioning experiment described herein which was performed in collaboration with Dr. Mario Cibelli. Assistance in interpretation and comment on data was provided by Dr. Mario Cibelli, Dr. John White, Mr. Faruq Noormohamed, Ms. Laura Benzonana, Dr. Istvan Nagy and Dr. Mervyn Maze.
ABSTRACT

The effects of peripheral surgery-induced inflammation and the role of the pro-inflammatory cytokine interleukin 1-beta (IL-1β) on cognitive function in mouse in several different contexts are explored.

Lipopolysaccharide (LPS)-induced inflammation, but not isoflurane-induced anaesthesia, results in memory impairment in mouse, causing a permanent retrograde amnesia in contextual fear-conditioning tests. Blocking the action of IL-1β reduces the hippocampal memory deficit induced by LPS.

Peripheral orthopaedic surgery results in inflammation in the brain and cognitive impairment in a mouse model of orthopaedic surgery. Such surgery is associated with increased levels of IL-1β in the serum and in the hippocampus. It also induces hippocampal microgliosis without being associated with an increase in apoptosis. Injection of an interleukin 1 receptor antagonist (IL1-ra) results in reduced microgliosis and reduced IL-1β levels in the serum and in the hippocampus.

The inflammatory response to such surgical insult also results in impairment of remote (pre-frontal cortex (PFC)) localised memory in mouse as assessed by two tests of contextual remote memory. Such impairment is not accompanied by an increase in IL-1β in the PFC. There is also a reduction in the level of hippocampal brain derived neurotrophic factor (BDNF) which may contribute to the impairment of memory after such surgery.

The murine anxiety response to peripheral orthopaedic surgery, as assessed using the social interaction test, shows that surgery does not increase anxiety in our animal model of peripheral surgery. Nor does such surgery affect olfactory memory under the conditions presented on the olfactory habituation-dishabituation task.

A sub-pyrogenic dose of LPS alone fails to impair memory function. However, when the same is administered prior to peripheral surgery, it exacerbates surgery-induced cognitive dysfunction as assessed by fear-conditioning tests. It causes a concomitant additional increase in the levels of IL-1β in both plasma and hippocampus of those animals.
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Dedicated to

Janine Streuli, my partner, and

Bruno Fidalgo, my brother,
for their love and support throughout
the years
**Publications arising from this Dissertation**

*Peer-reviewed articles*


Other peer-reviewed publications outside the subject of this Degree


Poster presentations

- **Fidalgo AR, Cibelli, Ma D** (2010). Systemic inflammation enhances surgery-induced cognitive dysfunction in mice. Forum of European Neuroscience Societies Meeting, Amsterdam, Netherlands, 3rd-7th July.


Oral presentations


Invited presentations

This work was performed while reading for the Degree of Doctor of Philosophy of Imperial College in the Anaesthetics, Pain Medicine and Intensive Care Section of the Department of Surgery and Oncology at Chelsea and Westminster Hospital. In today’s ageing population, the risk of surgery-induced cognitive dysfunction is an ever-growing concern. The natural progressive decline of cognitive function as one ages is universally viewed with apprehension. Moreover, Alzheimer’s disease is an increasing problem in a population whose individual members have an increased life expectancy due to modern medicine. In this study I have examined how surgery induced inflammation affects various cognitive functions in a mouse model of surgery induced inflammation.

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Chapter 1

INTRODUCTION

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1. Overview

Post-operative cognitive dysfunction describes the impairment of higher brain functions which frequently occurs after surgery. Patients who develop this condition spend more time in hospital after surgery and therefore have an increased need for care. This creates a huge burden on the health care system, on caregivers and on patients’ families.

Normal brain ageing is characterised by a shift towards a pro-inflammatory environment in the hippocampus, while surgery is associated with the release of pro-inflammatory cytokines. These two factors, advance brain age and surgery, may interact resulting in post-operative cognitive dysfunction which would not manifest itself in a younger person.

Here, I summarise key concepts relating to inflammation and memory. I also briefly review the literature relating to post-operative cognitive dysfunction.

2. Inflammation: relevant concepts

i. Definition and function

Inflammation describes the body’s immune response to tissue damage or disease. It involves the generation and release of a wide range of chemical agents. These inflammatory mediators include: bradykinin; eicosanoids; nerve growth factor (NGF); glial cell-derived neurotrophic factor (GDNF); serotonin; histamine; anandamide; ATP; and cytokines. It plays an important role in several diseases of the brain which are characterised by cognitive impairment, such as AIDS-related dementia complex and Alzheimer’s disease. Acute inflammation has a rapid onset. While inflammation serves a vital protective immunological function, the inflammatory response is, however, poorly controlled. Thus, an excessive or inappropriate inflammatory response may itself result in a pathological condition (White et al, 2010).
Inflammation results in *calor, dolor, rubor, and tumor* (from the Latin: heat, pain, redness and swelling) at the site of injury. The initial *acute inflammatory response* consists of cellular and humoral responses to injury which are found in its aftermath. (Murphy et al, 2008). The acute inflammatory response occurs almost immediately after injury. The initial intense inflammatory response gradually reduces over time, unless the provoking agent, such as bacteria, remains. The signs and symptoms induced by acute inflammation are reversible and tend to disappear with the healing of the tissue or elimination of the pathogen (as the case maybe). The objective of the acute inflammatory response is to return the tissue to the state of homeostasis (Murphy et al, 2008). Vascular changes near the damaged tissue occur at an early stage in acute inflammation, developing at a rate associated with the severity of the injury (Murphy et al, 2008). There is an initial vasoconstriction of arterioles which is followed by vasodilation (*i.e.* an increase in arteriolar diameter). This results in an increase of blood flow in the affected tissues due to the release of histamine from the damaged tissue cells and mast cells. The circulation of blood slows down in the affected area in a process termed stasis. Stasis occurs due to increased microvasculature permeability, loss of fluids, and increased concentration of red-blood cells in small blood vessels resulting in increased blood viscosity. The development of stasis marks the major cellular events associated with acute inflammation such as, *leucocyte margination, rolling* (transient attachment of leucocytes to endothelium), and *migration of leucocytes* through the vascular wall into the damaged tissue (Murphy et al, 2008).

Chronic inflammation is defined by morphological changes in tissue where it occurs. It is characterised by the presence of lymphocytes, macrophages and plasma cells in that tissue. Chronic inflammation may persist for a longer period than acute inflammation (*i.e.* months or years, as is the case in rheumatoid arthritis). In chronic inflammation, macrophages produce pro-inflammatory cytokines which act to perpetuate the inflammatory response (Murphy et al, 2008).

The term “cytokines” describes any protein produced by cells which is capable of affecting the behaviour of other cells that carry appropriate receptors (Murphy et
Cytokines are pleiotropic, as they exert their function over a diverse array of cells. Similarly, cytokines exhibit “redundancy”, as different cytokines are able to exert similar functions (Murphy et al, 2008). Cytokines are also capable of modulating the central nervous system. An excessive production of cytokines in the brain results in sickness behaviour (Danzter et al, 2008), reduced neurogenesis (Monje et al, 1998) and memory deficits (Barrientos et al, 2002; 2009). In the brain cytokines can be synthesised by glial cells, neurones, and endothelial cells (Aloisi, 2001).

**ii. Inflammation within the brain**

Until very recently the central nervous system (CNS) was believed to lack typical inflammatory response mechanisms (Allan et al, 2005). However, it is now accepted view that the CNS exhibits specific immunological responses (Allan et al, 2005). Inflammation in the CNS is accompanied by microgliosis, astrogliosis, and infiltration of circulating immune cells, such as neutrophils and monocytes (Allan et al, 2005). Activated microglial cells (microgliosis) are capable of producing cytokines, including IL-1β and other pro-inflammatory cytokines (Cibelli et al, 2010), reactive oxygen species, excitotoxins (Balcaitis et al, 2003), and neurotoxins, such as β-amyloid precursor protein (Block et al, 2007).

Inflammation within the CNS is generally beneficial. The immune response protects the CNS from pathogens, promotes healing, removes extra-cellular debris and promotes cellular repair (Block et al, 2007). However, an excessive level of inflammation within the brain underlies the pathogenesis of several neurodegenerative diseases (Rivest, 2009). Inflammation and microglial activation have been described in acute conditions such as stroke (Allan and Rothwell, 2001), and in chronic conditions such as Alzheimer’s disease (Block et al, 2007; Rivest, 2009).

A cytokine is termed “pro-inflammatory” if it promotes the expression of inflammatory mediators associated with acute inflammation and/or chronic inflammation (Murphy et al, 2008). Interleukin(IL)-1β is a pro-inflammatory cytokine which has been implicated in the pathogenesis of peripheral and central nervous
system diseases, making the IL-1 system a possible target for therapeutic intervention. IL-1β was first described in the 1940s as a fever-inducing substance (Dinarello, 1996). IL-1β is synthesised as a 31-kDa precursor which can be cleaved into its active form by caspase-1, resulting in a 17-kDa molecule (Dinarello, 1996). IL-1β binds to type I and type 2 receptors (IL1-r1 and IL1-r2). Both of these receptors exist in a membrane-bound and, also, a soluble, form. The IL1-r2 regulates IL-1β mediated activity by binding to IL1-r1 and preventing its signalling. For signal transduction to occur IL-1β has to bind with the IL1-r1, which then has to interact with the IL-1 accessory protein (IL1-racp). IL-1β mediated activity can also be inhibited by the IL1-ra, an endogenous receptor antagonist, that prevents signal transduction by binding to IL1-r1 (Dinarello, 1996).

IL-1β is a proinflammatory cytokine capable exerting of local and systemic responses (Dinarello, 1996). IL-1β levels increase rapidly at the site of injury after stroke and other acute conditions (Allan and Rothwell, 2001). In the brain, the dentate gyrus of the hippocampus has the highest concentration of IL-1β receptors (Pugh et al, 2001), which may account for the adverse impact that high levels of hippocampal IL-1β can exert. Injecting IL-1β into the brain results in sickness behaviour, which is characterised by fever, lethargy, reduced mobility, decreased sexual appetite, anhedonia and changes in slow-wave sleep (Dantzer et al, 2008). IL-1β is also an important molecule for memory processes. Injecting IL-1β intracerebroventricularly has been demonstrated to affect hippocampal memory consolidation in rats, both in the fear-conditioning test (Barrientos et al, 2002) and in the spatial reference task in the water maze (Pugh et al, 2001). IL-1β blocks the induction of long-term potentiation (LTP) in the CA1, CA3 and dentate gyrus areas of the hippocampus (Pugh et al, 2001). Interestingly, injecting IL-1β intra-peritoneally does not have any impact in fear-conditioning tasks, independently of the dose range used - 0.1 to 100 µg/kg (Thomson and Sutherland, 2005).

IL1-ra is a naturally occurring 22 kDa glycoprotein which blocks IL-1β binding. IL1-ra has 19% molecular similarity with IL1-α and 26% molecular similarity with IL-1β (Eisenberg et al, 1990). IL1-ra (anakinra) is prepared from cultures of genetically
modified *Escherichia coli*. The biological activity of the recombinant protein is identical to that of the endogenous human IL1-ra (Yang et al, 2003).

No significant increases in plasma levels of IL-1β, IL-6, and TNF-α are seen during, or after, the intra-venous infusion of IL1-ra in volunteers or controls (Granowitz et al, 1992). In healthy subjects, IL1-ra is well absorbed after subcutaneous bolus injection and has a bioavailability of around 95%. Post-injection plasma IL1-ra levels decline quickly, exhibiting an initial half-life of 21 ± 3 minutes and a terminal half-life of 108 ± 18 minutes (mean ± S.D.). After sub-cutaneous administration of 100 mg IL1-ra maximum serum concentration is seen after 4 hours. Sub-cutaneous administrated IL1-ra has a terminal half-life (5.24 hours) that is longer than that found after intra-venous administration (2.64 hours) (Yang et al, 2003). Such data suggests that IL1-ra is eliminated faster than it is absorbed.

Behavioural studies indicate that over-expression of IL1-ra in mouse impairs contextual fear-conditioning tasks and impairs the acquisition of the spatial reference task in the water maze (Goshen et al, 2007). Similarly, IL1-ra administration during prenatal development results in hippocampal abnormalities and deficits in hippocampal dependent memory (Goshen et al, 2007). Over-expression of IL1-ra in mouse also impairs performance in a T-maze and results in impairment of recognition of previously presented visual stimuli (Spulber et al, 2009). Intracerebroventricular administration of IL-1ra after fear-conditioning results in a memory deficit (Goshen et al, 2007). However, IL1-ra subcutaneously administered prevents LPS-induced amnesia in fear-conditioning tasks (Pugh et al, 1998). Administering IL1-ra prevents the reduction of social interaction after acute LPS or acute stress (Arakawa, 2009).
3. Memory: relevant concepts

i. Introduction

Cognition describes higher order mental processes, such as awareness, attention, decision-making, language, as well as learning and memory. The word cognition is derived from the Latin verb *cognoscere*, meaning to learn, to perceive or to know. Learning and memory can be defined as processes by which organisms acquire knowledge, skills and experience, store it, and subsequently retrieve it, thereby producing adaptive behaviour. Memory involves several processes that can be separated into a series of distinct events, i.e. encoding, consolidation, storage and retrieval. *Encoding* happens when an organism has to convert information into a meaningful structure. The more elaborate and detailed the encoding, the greater is the memory strength (Sternberg, 2000). *Consolidation* was first described by Müller and Pilzecker (1900) by hypothesising that memory needed time to be consolidated. These authors also proposed that memory would be vulnerable to disturbance during the consolidation phase (Müller and Pilzecker, 1900, cit. in Lechner et al, 1999). *Storage* of memories happens after the consolidation process, such storage constitutes the final long-term memory. *Retrieval* of memories, i.e. recollection, is the cognitive process by which stored information is recalled and incorporated in ongoing behaviour (Sternberg, 2009).

Memory can be divided into several psychological systems that process specific types of information. These memory systems are classified into two major forms of long-term memory, namely declarative and nondeclarative (Sternberg, 2009). Declarative, or explicit memory involves conscious recollection of facts and events and includes also knowledge about spatial and temporal contexts. The ability to recollect these types of memories is lost in amnesia (Quinn et al, 2005). Nondeclarative, or implicit memory refers to motor/perceptual skills and is typically expressed as a change in performance, e.g. increased accuracy or reduced response time. This type of memory is usually spared in case of amnesia (Scoville and Milner, 1957). Of major importance is the finding that declarative and nondeclarative memory are dependent on different anatomical substrates (Scoville and Milner, 1957).
ii. Short-term memory

Short-term memory describes the very short time during which one keeps something in mind before either dismissing it or transferring it to long-term memory (Sternberg, 2009). Short-term memory allows recall for a period of several seconds to a minute without rehearsal. Miller published a seminal article in 1956 showing that the average short-term memory capacity was 7±2 items. Modern studies indicate that the capacity of short-term memory is lower, typically of the order of 4-5 items (Sternberg, 2009). Short-term memory capacity can be increased through chunking, that is grouping items in a familiar manner, much like anyone does when remembering a telephone number. Short-term memory is believed to rely mostly on an acoustic code for storing information, and to a lesser extent a visual code (Sternberg, 2009). Research also indicates that one has more difficulty in recalling collections of words that are acoustically similar, e.g.: dog, hog, fog, bog, log (Sternberg, 2009).

iii. Long-term memory

While short-term memory generally has a strictly limited capacity and duration, long-term memory can store much larger quantities of information for potentially unlimited duration of time (Sternberg, 2009). It is not possible to measure long-term memory capacity. For example, given a random seven-digit phone number, we may remember it for only a few seconds before forgetting, suggesting that it was stored in our short-term memory system. However, we can remember telephone numbers for many years through repetition, by using information stored in long-term memory. While short-term memory encodes information acoustically, long-term memory encodes it semantically (Sternberg, 2009).

Long-term memories are maintained by more stable and permanent changes in neural connections which are widely spread throughout the brain (Sternberg, 2009). The hippocampus is necessary for learning new information which is subsequently consolidated from the short-term memory system into the long-term memory system (Sternberg, 2009). Without the hippocampus, new memories are unable to be stored.
into long-term memory, and the subject as a very short attention span (Sternberg, 2009). In humans the hippocampus may be involved in altering neural connections for a period of three months, or more, after the initial learning. Researchers hypothesise that sleep improves consolidation of information.

There are several forms of long-term memory. The two major subdivisions are explicit and implicit (Sternberg, 2009). Explicit memory or “declarative memory” requires a conscious thought process (Sternberg, 2009), such as remembering what one had for breakfast or naming the countries in Europe. Explicit memory is what most people have in mind when they think of “memory”, or how good, or bad, their memory is. Episodic memory is one type of explicit memory. Episodic memory provides us with a vital record of our personal experiences. Our episodic memory allows us to remember a holiday, what we had for dinner last night, or who told us that a friend was getting married. Any past event in which we played a part, and which we remember as an “episode”, is effectively episodic memory. This form of memory appears to be centred in the brain’s hippocampus (Sternberg, 2009). Another type of explicit memory is semantic memory. It accounts for one’s general knowledge about the world. Semantic memory it’s what enables us to say, without knowing exactly when and where we learned, that a zebra is a striped animal, or that Paris is the major city in France. As with episodic memory, semantic memory ranges from strong (recall) to weak (familiarity). Unlike episodic memory, semantic memory is better sustained over time. We are often able to retain a highly functional semantic memory into our sixties, after which it undergoes a slow decline (Sternberg, 2009).

iv. Retrograde Amnesia

Amnesia is termed retrograde when one is unable to recall events that occurred before the onset of amnesia. Retrograde amnesia is caused by brain trauma, and is usually associated with failure in recalling a specific event or previously acquired knowledge (Scoville and Milner, 1957). Retrograde amnesia in contextual fear conditioning tasks exhibits a temporal gradient (Maren et al, 1997). When enough time elapses between the acquisition of fear and an hippocampal lesion, the fear memory is spared (Maren et al, 1997). This indicates that memory for contextual fear is stored in
neocortical areas (Frankland et al, 2004). The retrieval of fear conditioning memories may require hippocampal involvement depending on how long ago the memory was first acquired (Wang et al, 2009). As a general rule, fear memories acquired in the initial 14 days will require hippocampal involvement while, afterwards, fear memories are relocated to neocortical areas (Frankland et al, 2004).

v. Memory Consolidation

Memory is not immediately stored. It requires a process called “consolidation” in which protein synthesis occurs and enables memory to be effectively stored in the hippocampus (Barrientos et al, 2002). There are two forms of memory consolidation, namely, cellular consolidation and, systems consolidation (Morris et al, 2006). Cellular consolidation occurs within the initial hours of acquiring a specific memory (Barrientos et al, 2002). System consolidation involves the reorganisation of memory in different brain circuits (Frankland et al, 2004). System consolidation is a much lengthier process than cellular consolidation. Performing an experimental manipulation, such as injecting an endotoxin, or an NMDA antagonist, or performing surgery within the first day of acquire fear memory by a rodent will affect memory consolidation (Barrientos et al, 2002).

vi. Hippocampus

The hippocampus is regarded as a phylogenetically old part of the brain structure. The hippocampus is an elongated structure, which in humans is located in the ventral-lateral wall of the lateral ventricles of each temporal lobe. In rodents, the hippocampal formation is a C-formed structure extending from the area of the basal forebrain, reaching over, and behind, the diencephalon and then down caudoventrally into the temporal lobes.

The hippocampal region includes the hippocampal formation and the adjacent parahippocampal region. It consists of three cytoarchitectonically distinct regions, namely the dentate gyrus, the hippocampus proper (which is subdivided into CA3, CA2 and CA1; CA means Cornu Ammonis) and the subiculum. The parahippocampal
region includes the entorhinal and perirhinal cortices, together with the postrhinal cortex (in non-primate mammals) or the parahippocampal cortex (in primates, including humans) (van Strien et al, 2009). Information from association areas of the neocortex reaches the hippocampal formation mainly via the entorhinal cortex. The projection from the entorhinal cortex, the perforant path, terminates on the dendrites of the granule cells in the dentate gyrus, i.e. the molecular cell layer. The granule cells give rise to the mossy fibers that project to the CA3 region, from which the Schaffer collaterals projects to the CA1 area of the hippocampus. The pyramidal cells in CA1 then project out of the hippocampus via the subiculum/entorhinal cortex to cortical areas (van Strien et al, 2009). This pathway is referred as the trisynaptic pathway.

Cortical inputs to the hippocampus are the main source of information for hippocampal mnemonic functions. However, there are also afferents from subcortical areas, e.g. the septum, hypothalamus and brain stem, which have a modulatory role in hippocampal function.

vii. Amygdala

This is an almond-shaped structure (amygdalum means “almond” in Latin) located in the anteriomedial part of the temporal lobe and consists of four nuclei: the lateral; the basal; the accessory basal; and the central amygdala nuclei (Phillips and LeDoux, 1992). Several human and animal studies demonstrate an important role for the amygdala, in conjunction with closely related brain regions, such as the hippocampus, in the acquisition, storage and expression of long-term memories for emotional events and in the response to environmental stimuli that signal threat (Phillips and LeDoux, 1992).

Clinical data has demonstrated that a patient with selective bilateral damage to the amygdala does not acquire conditional autonomic responses to visual or auditory stimuli, albeit that he acquires the declarative facts about which visual or auditory stimuli were paired with the unconditional fear stimulus. By contrast, a patient with selective bilateral damage to the hippocampus fails to acquire the facts but does acquire the conditioning. If a patient has a bilateral damage to both amygdala and
hippocampal formation he does not acquire either the conditioning or the facts (Bechara et al, 1995). These findings demonstrate a double dissociation of conditioning and declarative knowledge relative to the human amygdala and hippocampus.

Anatomically, the lateral nucleus of the amygdala receives sensory information from the thalamus, neocortex and olfactory cortex. This information is then projected via the basal nucleus to the central nucleus of the amygdala, which in turn mediates the emotional responses through projections to the brainstem (which modulates behaviour) and the hypothalamus (which modulates autonomic responses) (LeDoux, 1992). Also, there exist projections from the basal and lateral nuclei of the amygdala to the entorhinal cortex, subiculum, CA3 and CA1 areas of the hippocampal formation (LeDoux, 1992).
4. Post-operative cognitive dysfunction

i. Introduction

Post-operative cognitive dysfunction (POCD) refers to impairment of the neurocognitive function. It may involve impairment of attention, perception or memory, or, a combination of these faculties. Patients who develop this condition spend more time in hospital after surgery and subsequently have an increased need for care (Moller et al, 1998).

Post-operative cognitive dysfunction describes two separate disease entities. An initial, stage of confusion after surgery is termed “postoperative delirium” (POD), while a later manifestation of a chronic cognitive impairment is referred to as “POCD”. There is, at this time, no accepted definition of post-operative cognitive dysfunction, as such, because the underlying pathogenesis of these disease states has yet to be determined. “Delirium” develops soon after surgery and is usually self-limiting. Sometimes it is followed by the development of “POCD”, which may be the cause of more severe and longer lasting cognitive impairment. It is clear that patients undergoing orthopaedic or cardiac surgery have a higher incidence rate of post-operative cognitive dysfunction than patients who have undergone other types of surgery (Moller et al, 1998).

With increasing life expectancy, surgical procedures conducted in elderly persons is becoming more common. The prevalence of POCD may mean that some patients will exchange the incapacitating condition that led them to surgery in the first instance for another such condition which has been created by the surgical procedure itself.

ii. Post-Operative Delirium (POD)

Post-Operative Delirium (POD), where it occurs, appears in the first two days after surgery and, by definition, subsides within less than a week (Lipowski, 1989). Delirium can be divided into two different categories on the basis of the degree of alertness exhibited by the patient. Such delirium or POD is characterised in the “hyperalert-hyperactive” category by a psychomotor agitation and sympathetic
nervous system abnormality. The state of disorientation which these patients experience may be dangerous for them and for the attending staff. Despite their non-cooperation in relation to diagnosis, the abrupt change in their behaviour in the context within which it occurs facilitates a reliable diagnosis of their condition.

The second variant of POD is described as the “hypoalert-hypoactive” variant. This is characterised by a decreased responsiveness and lethargy associated with impaired cognition. The diagnosis of this quiet delirium is harder to make than is the case with the preceding type, since the relaxed appearance of the patient does not call for the physician’s attention.

The latest version of the Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV TR) and ICD-10 classification includes “delirium” as a disease and provides several broadly similar criteria for its recognition. These include: disturbance of consciousness and attention; change in cognition; psychomotor and emotional disturbances; sleep/wake cycle changes; and early onset. The Royal College of Physicians and the British Geriatric Society have recently published an update of comprehensive evidence-based guidelines for the diagnosis of delirium. In general, a diagnosis of delirium is the result of an accurate clinical assessment obtained mainly by using cognitive investigating techniques and blood tests.

In the “hyperalert-hyperactive” type of POD, 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 may be serious. Disorientation; increased psychomotor activity; anxiety; fear; nervousness; and increased alertness to stimuli may often cause the patient to self harm or to take out intravenous catheters, central lines and monitoring equipment. There is a particular difficulty therefore in providing these patients with effective nursing care. Moreover, episodes of aggression directed against medical and nursing staff are not unusual. Very often patients will not cooperate even with neuropsychological testing designed to confirm a diagnosis of “delirium”. At the same time, the unexpected change in the patient’s behaviour makes the hyperalert-hyperactive delirium easy to diagnose from its early manifestations.
In the “hypoalert-hypoactive” type of POD, decreased responsiveness to stimuli and lethargy, often associated with impaired cognition and memory disturbances, are the most common features. These patients are described as being detached from reality and uninterested in events happening around them (Pockett, 1999). Since the patient seems to be comfortable and, in the absence of an accurate assessment, this condition can easily be missed. The incidence and prevalence of hypoalert-hypoactive delirium is, therefore, substantially underestimated, despite being the cause of more severe impairment and longer hospitalisation than the hyperalert-hyperactive form (Liptzin and Levkoff, 1992).

Sometimes, delirium may present as a mixed, unspecified form, with unexpected swings from one type to the other (Liptzin and Levkoff, 1992). The term “emergence delirium” as been applied to situations where the onset of delirium occurs within the first 24 hours of a surgical procedure (Lipowski, 1989).

iii. Post-Operative Cognitive Dysfunction (POCD)

Post-operative cognitive dysfunction (POCD) can only be identified after several weeks, or even months, have elapsed since the operation. POCD is associated with a longer hospitalisation, higher mortality rates, and a liability to progress to dementia (Rockwood et al, 1999). Rasmussen has regarded post-operative cognitive dysfunction as a mild neurocognitive disorder, within the definition given by the DSM-IV TR, which states that it is a “cognitive disorder not otherwise specified and for which research criteria have not been suggested” (Rasmussen, 1998).

The detection of POCD is dependent on a careful examination of cognitive function using neuropsychological tests. In the clinical setting, physicians tend to use the Mini-Mental State Examination (Folstein et al, 1975). However, this is a rather crude diagnostic method since the initial impairment of short-term memory tends to remain undetected by it. It is while the patient is performing routine tasks that relatives begin to notice changes in memory, skills and behaviour in his part.
DSM-IV TR does not refer at all to post-operative cognitive dysfunction as a distinct disease entity, although the most severe chronic forms of POCD are similar to the more severe disorder defined, namely, dementia.

iv. Risk factors

Cognitive deterioration after surgery was first reported in *The British Medical Journal* of 1887 in an article written by one George H. Savage. Savage reported on “a series of cases in which the use of anaesthetics, in predisposed patients, has been followed by insanity” (Savage, 1887). These observations constituted a landmark in the subject of post-operative cognitive dysfunction because they provide a description of patients; of their background; and of the symptoms which they displayed.

Notwithstanding Savage’s observations, post-operative cognitive dysfunction failed to achieve recognition as a hazard of surgery. It was only during the period from 1955 to 1967 that it re-surfaced when Bedford, Simpson and Blundell exchanged arguments regarding the possible involvement of anaesthetics in mediating post-operative cognitive dysfunction in elderly patients. However, their studies lacked control groups and had insufficient assessment protocols with the result that data obtained was unreliable (Bedford, 1955; Simpson et al, 1961; Blundell, 1967).

In 1998, a major international research endeavour, sponsored by the *International Study of Postoperative Cognitive Dysfunction* (ISPOCD-1), surveyed 1218 patients aged more than 60 years who had experienced a major non-cardiac surgery under general anaesthesia (Moller et al, 1998). This study incorporated proper controls. It confirmed the existence of POCD as a post-operative disease. Moreover, it identified various risk factors for the development of post-operative cognitive dysfunction, namely: advanced age; the duration of surgery; infection; post-operative complications; and subsequent operations (Moller et al, 1998).

The ISPOCD-2 study performed in 2003 focused on the impact of anaesthesia (Rasmussen et al, 2003). The speculation that post-operative cognitive dysfunction could be avoided by performing surgical procedures under regional anaesthesia motivated this study. A total of 438 elderly patients performed a battery of
neurocognitive tests before the surgery for baseline values. The assessment was then repeated again 7 days and 3 months after surgery.

The incidence of delirium was higher in patients that underwent a surgical procedure under general anaesthesia when compared with regional anaesthesia. However, the incidence of a later post-operative cognitive dysfunction was the same in both groups (Rasmussen et al, 2003). This study, like others before it, had a key limitation. It did not have enough statistical power to differentiate between the types of anaesthesia employed. An estimated 1120 patients would be needed in order to detect a difference in the incidence of later post-operative cognitive dysfunction as between regional and general anaesthesia with a power of 0.9 at 0.05 significance level.

Interest has also focused on the pathogenic mechanisms involved in the disease. It has been suggested that perioperative factors are important, including general anaesthesia, benzodiazepine use, alcohol withdrawal, and patient-related factors, such as polymorphisms and co-morbidities (Heyer et al, 2005; Newman et al, 1995). Again, however, an absence of appropriate controls and the failure to adopt a standardised assessment protocol in these studies make the results difficult to analyse.

Risk factors have been divided in factors arising before, during, and after, surgery. Age, alcoholism, severity of the illness leading to surgery, previous cognitive dysfunction and renal impairment are pre-surgery risk factors (Francis et al, 1990; Herrmann et al, 1992; Schor et al, 1992; Inouye et al, 1993; Pompei et al, 1994). An association of these factors contributes to increase the risk of developing early post-operative cognitive dysfunction (Inouye et al, 1993). Intra-operative factors include: use of anticholinergic medication; psychoactive drugs; type of anaesthetics; and, as well, the type of surgical procedure (Marcantonio et al, 1994).

In the ISPOCD-1 study increasing age, duration of anaesthesia, reduced number of years in formal education, and the need for a second operation, were identified as risk factors for the development of post-operative cognitive dysfunction. Amongst the relevant post-operative factors where respiratory complications and postoperative
infections. However, only advanced age was correlated with late postoperative cognitive dysfunction (Moller et al, 1998). An elegant study shows that with increasing age animals exhibited increased mRNA levels of major histocompatibility complex (MHC) II, CD86, CIITA and IFN-δ increase, while IL-10 and CD200 mRNA molecules, that down-regulate macrophage activation, decrease. This indicates that normal brain ageing is characterised by a shift towards a pro-inflammatory environment in the hippocampus (Frank et al, 2006). If these data can be extrapolated to humans, it would appear that elderly patients undergoing surgery are at greater risk of developing cognitive impairment following surgery.

Apart from age, the number of years in formal education has been consistently reported as a risk factor related to post-operative cognitive dysfunction (Moller et al, 1998; Monk et al, 2008). Low educational attainment is correlated with a higher probability of cognitive impairment, whereas high educational levels seem to provide a protective effect, as described by Newman and collaborators in a study of patients undergoing cardiopulmonary by-pass (Newman et al, 1995).

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 received very little attention in the studies concerning risk factors for post-operative cognitive dysfunction. Again, many brain-related co-morbidities, such as depression and pre-operative dementia, have been frequently excluded from the studies as possible confounding factors. The degree of increased risk related to the presence of mental co-morbidities in past medical history was examined in retrospective study of 975 patients, 57 of whom had post-operative complications (Bernstein and Offenbartl, 1991). Importantly, those patients who displayed symptoms of dementia pre-operatively also developed a fatal complication after surgery (25 of 32 who died had dementia). These findings are consistent with more recent findings relating to post-operative cognitive dysfunction and mortality (Monk et al, 2008).
v. Epidemiology

The frequency of post-operative cognitive dysfunction is variable and can be related to the type of surgery, as well as to several pre-, intra-, and post-operative factors. The incidence of delirium in elective non-cardiac patients was found to be 9% within the first 5 post-operative days (Marcantonio et al, 1994). However, in bilateral knee surgery, the incidence increased to 41% (Williams-Russo et al, 1992) and varies between 35 and 65% after hip-fracture repair (Gustafson et al, 1988). In emergency surgery, such as that for lower limb ischemia, the incidence was found to be 42.3% (Sasajima et al, 2000). The highest reported incidence was found in lung transplant surgery at 73% (Dyer et al, 1995).

The ISPOCD-1 trial comprised 1218 patients aged at least 60 years and reported an incidence of POCD of 25.8% one week after surgery and 9.9% of patients 3 months after surgery (Moller et al, 1998). A subsequent study reported 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).

More recently, a study of 417 patients undergoing major surgery reported that at discharge cognitive impairment was found in 56% of patients, with an equal distribution in type and severity of cognitive deficit. At 3 months after surgery, 75.1% of patients had normal levels of cognitive function, 13.6% of patients experienced memory decline alone, 8.4% of patients showed only executive function impairment, and 2.9% of patients had a decline in both executive and memory domains. Of those with cognitive decline, 46.8% of patients had mild, 32.5% of patients had moderate, and 20.8% of patients had severe, impairment. Where patients exhibited both impairment of executive function and memory, the deficits were found to be more severe resulting in greater levels of functional impairment (Price et al, 2008). Some elderly patients (1%) still experience disabling effects on cognitive function one year after surgery (Abildstrom et al, 2000), while there is an increased risk of death within the following year for those patients whose symptoms are still evident 3 months after surgery (Monk et al, 2008).
vi. Neuropsychological Assessment

Cognitive impairment tends to go unnoticed during a brief clinic visit. Studies indicate that between 29% to 76% of cases of dementia, or some sort of cognitive dysfunction are not diagnosed by physicians (Valcour et al, 2000; Olafsdottir et al, 2000; Chodosh et al, 2004).

A critical element in detecting post-operative cognitive dysfunction depends on the neuropsychological evaluation techniques employed. Different studies use different assessment batteries with the result that major variations are found between studies which may reflect the methodology employed in assessment rather than a true variation in the incidence of disease. Table 1 is an example of several neuropsychological tests employed in the study of human POCD.
### Table 1: Example of neuropsychological tests and cognitive functions examined.

Neuropsychological instruments used in the ISPOCD trials are described below.

Evaluating impairment of memory can be done using recall tasks like asking the patient to remember words and, after a short interval, asking for the patient to repeat the same list of words. After the initial registration of the words employed, the information is then moved into the short-term memory systems and can be used in a multitude of tasks. One can then ask for words presented, or give cues about a given category to which a word may belong, or require it to be used to solve problems.

Executive functioning refers to one’s ability for abstract thinking and planning. Some tests of executive function require the participant to draw an analog clock; reading a coloured word list; or encode a digit to a symbol; or find a sequence of numbers.
Diagnosis of mild neurocognitive impairment is more difficult than the diagnosis of dementia. A study indicates that junior neuropsychologists exhibit a high level of agreement with senior colleagues (>98%) in diagnosing dementia, but in moderate dementia the same diagnosis was only seen in 60% of cases (de Mendonça et al, 2004).

Initial studies looking for evidence of post-operative cognitive dysfunction used global scales to assess cognitive changes between patients undergoing a certain type of surgery or anaesthesia. The Mini Mental State Examination (MMSE) is widely used for screening for dementia. However the MMSE is an insensitive instrument for patients in the initial stages of dementia and, ceiling effects have been reported in patients with mild cognitive impairment. Again, even the use of a comprehensive structured battery like the Wechsler Adult Intelligence Scale (WAIS), has been found to be insensitive to the detection of subtle cognitive changes in patients after cardiac surgery (Newman, 1995).

The test battery used to evaluate patients in ISPOCD1 and 2 followed a full latin square design for most tests except the Stroop test for executive functions. The tests employed focused on learning, memory and executive functioning. The Visual Verbal Learning Test is based on the Rey’s Auditory Recall of Words. It consists of a list of 15 words displayed at a speed of 1 per second. The patient is then asked to recall as many words as he can, either immediately or after an interval in which a different test had been performed. The Word Recall test, records the number of remembered words from the Visual Verbal Learning Test some 15-25 minutes after the task as been performed and after other tests been performed. It measures memory after interference. The Cognitive Flexibility test, using the Concept Shifting Test, Part C, based on the Trail Making Test. It consists of 3 subsets that measure cognitive speed and flexibility. It involves linking a series of circles numbered from 1 to 25. After an initial demonstration of the task, the participant is asked to link the circles as fast as he can without lifting the pencil from the paper. The number of errors and time taken are registered. This test is quite sensitive to general frontal lobe dysfunction. A reduced performance indicates an inability to execute and modify a plan of action.
Distractibility can be measured, using the Stroop Colour Word Interference Test. This test measures attention and cognitive speed. Initially the person is given a sheet of paper with black printed names of colours. The patient it is then asked to read as many words as he can. Afterwards, another sheet of paper is given with the names printed in the corresponding colour. Again, the patient is then asked to read as many words as he can. Finally, a sheet of paper is given, but his time, the colours are printed in a different colour and the participant is asked to name the colour in which the word is printed as fast as he can. Working memory can be assessed using the Letter-Digit Coding test. This is based on the Symbol Digit Substitution Test from the Wechsler Adult Intelligence Scale III. In this test the participant is asked to substitute a given digit for a symbol. Within 60 seconds he should complete as many fields as possible. The number of correct digit-symbol pairs are recorded. A cognitive dysfunction is usually reported on the occurrence of at least 2 deficits out of 7 tests.

As control measures pain and mood inventories are also employed. The Beck Depression Inventory screens for the presence of depression in adults. This test has a high reliability and validity across age, sex and cultures. The State Trait Anxiety Inventory measures state (temporary) and trait (engraved in one's personality) anxiety. The State scale consist of 20 statements that measure how the person feels at the present, while another 20 statements assess how a person generally feels. Numerical Rating Scales for Pain, are used for assessing the extent of pain the patient is experiencing at the moment of assessment. Zero, indicates no pain, while 10 indicates excruciating pain.

Neuropsychological instruments should be selected on the basis of their sensitivity in detecting subtle changes in cognitive function. Likewise, learning effects due to the test-retest that patients must undergo, require the availability of validated parallel versions of the several tests used. Notwithstanding this, learning effects are almost impossible to eliminated. On the other hand, an absence or reduction on the learning gains of repeated testing may be used as a measure of subtle cognitive dysfunction.
Usually, the pre-operative assessment is performed 1 to 2 days before the surgical procedure. Unfortunately, this is a time point at which stress and anxiety may affect the patient’s performance of the task. The duration of the assessment is also an element that needs to be considered. Testing for a long period of time, usually more than 2 hours, will affect performance and will leave the frail patients exhausted.

Studies addressing the incidence of post-operative cognitive dysfunction must have a control group which addresses all of these variable factors. Moreover, the pre-surgical evaluation should be performed quite some time before the surgical procedure in order to establish a reliable baseline for cognitive function. A post-operative assessment should be performed at 1 week, for delirium, or at successive interval of 3 months, for post-operative cognitive dysfunction.

vii. Pathogenesis of post-operative cognitive dysfunction

The mechanisms that initiate or precipitate the occurrence of post-operative cognitive dysfunction remain unknown. Although many researchers suggest on the possible involvement of anaesthetics, it is generally accepted that a patient’s unresponsiveness after a general anaesthetic is transient and reversible. However, recent studies demonstrate a pivotal role for surgery-induced inflammatory response as a key factor in the development of post-operative cognitive dysfunction (Wan et al, 2007).

a. General anaesthetics

General anaesthetics are extensively used in a clinical setting. It has been known for some time that general anaesthesia has a profound effect on brain function, affecting cerebral blood flow, metabolism, neurotransmission, protein expression, ion channels, and neuronal membranes (Franks and Lieb, 1994). Modulation of γ-aminobutyric acid (GABA) receptors or N-methyl-D-aspartic (NMDA) glutamate receptors contributes to the hypnotic effects of anaesthetics, and may also result in interference with neurotransmission (Bittigau et al, 2002).
Ageing, a risk factor for post-operative cognitive dysfunction, causes several structural and morphological changes to the brain, reduction in synaptic density (Morrison and Hof, 1997), an adverse effects on cerebral microvasculature (Riddle et al, 2003), and changes in DNA repair systems (Rutten et al, 2003) may result in a lower ability to cope with the harmful effects of general anaesthetics.

Swelling of mitochondria and the endoplasmic reticulum is believed to lead to a transient vacuolization of cerebral neurones in adult (Jevtovic-Todorovic et al, 2000), and especially in aged, rodents (Jevtovic-Todorovic and Carter, 2005), following administration of ketamine, or nitrous oxide, or a combination of both. There is also evidence that increased production of Aβ-protein, which is involved in the development of Alzheimer's disease and apoptosis. There is also evidence that following exposure to isoflurane there is an increase in Aβ-protein production by neurones (Xie et al, 2006; Xie et al, 2007).

There is evidence of a link between enduring learning deficits (Culley et al, 2004, 2006) and/or persistent changes in hippocampal gene expression (Culley et al, 2004) following general anaesthesia that outlive their intended function. Even in circumstances where the exposure to these anaesthetics gases is limited. Moreover, some degree of post-anaesthetic memory impairment has been reported in young animals (Culley et al, 2003).

b. Inflammation

Surgery is associated with an increased production of both pro-inflammatory and anti-inflammatory cytokines. Hip-replacement results in enhanced production of interleukin-6, interleukin-8, and prostaglandin E₂ both in the cerebrospinal fluid and at the site of surgery (Buvanendran et al, 2006; Yeager et al, 1999). Immediately after pump coronary artery bypass surgery, it is possible to detect an elevated concentration of interleukin-6 in the cerebrospinal fluid (Kalman et al, 2006). After brain injury or hypoxic-ischemic encephalopathy, increased levels of IL-1β and interleukin-6 are found in the serum and cerebrospinal fluid (Aly et al, 2006).
Cibelli and his collaborators (2010) described how C57BL/6J mice and k.o. mice lacking IL-1 receptor (IL-1R<sup>-/-</sup> mice) underwent surgery of the tibia under general anaesthesia (Cibelli et al., 2010). Separate cohorts of animals were tested for memory function with fear conditioning tests, or euthanised at different time points after surgery to assess levels of systemic and hippocampal cytokines, microglial activation, and the effects of interventions designed to interrupt inflammation. Surgery caused hippocampal-dependent memory impairment that was associated with increased plasma cytokines, as well as reactive microgliosis and IL-1β transcription and expression in the hippocampus. Non-specific attenuation of innate immunity with minocycline prevented surgery-induced changes. Functional inhibition of IL-1β, both in mice pre-treated with IL-1 receptor antagonist and in IL-1R<sup>-/-</sup> mice, mitigated the neuroinflammatory effects of surgery and memory dysfunction. These results indicate that a peripheral surgery-induced innate immune response triggers an IL-1β-mediated inflammatory process in the hippocampus that underlies memory impairment. This pathway may represent a viable target for interrupting the development of post-operative cognitive dysfunction (Cibelli et al., 2010).

c. Microglia

Microglia are the most numerous immunocompetent cells in the brain. They are usually found in surveillance mode. They have the ability to transform into an amoeboïd form and display activation on appropriate signalling from cytokines (Aloisi, 2001). Once activated, microglia secrete IL-1β and other pro-inflammatory cytokines (Cibelli et al., 2010), reactive oxygen species, excitotoxins (Balcaitis et al., 2003), and neurotoxins, such as β-amyloid precursor protein (Block et al., 2007). Electrophysiological studies show that, with advanced age, activated microglia are associated with impaired long-term potentiation - LTP (Griffin et al., 2006). Microglial cells are one of the first cells to respond to brain injury or neurological disease (Aloisi, 2001). Once active, microglial cells proliferate, retract their processes, increase their cell size and, if required, turn into phagocytosing cells. The removal of cell debris after brain injury is seen as a neuroprotective action which allows remyelination and neuronal regeneration to occur (Aloisi, 2001).
Chapter 2

EXPERIMENTAL TECHNIQUES AND PROTOCOLS

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1. Introduction

i. Techniques employed

The experimental work required for this dissertation was based on various
techniques in molecular biology (immunohistochemistry, immunoblotting, and
enzyme immunoassays) and behavioural neuroscience (water maze test, fear
conditioning protocols, social interaction test, and olfactory discrimination tasks).

Here the theoretical basis of these techniques is described, as are the protocols
which were employed for their execution.

2. Animals

i. Statutory requirements

All the experiments were performed under Home Office License (PPL N.º
70/6104; PIL N.º 70/20834) and in compliance with the requirements of the Animals
(Scientific Procedures) Act, 1986. All efforts were made to limit the number of
animals used in these experiments and to ensure that no unnecessary suffering was
occasioned to the animals employed.

ii. Conditions for animals

C57BL/6 male mice, aged between 12-14 weeks, supplied by Harlan and
Charles River, UK, were employed in these experiments. Upon arrival, the animals
were housed in IVC cages (4 animals/cage) and allowed to acclimatise to their new
environment for 7 days before being employed in the experiments. Their environment
was maintained in a 14:10 light-dark cycle, under constant temperature and humidity,
with free access to food and water.
3. Surgery

i. General

The surgical procedure was conducted under general anaesthesia in aseptic conditions to establish an open tibia fracture model. The surgical procedure essentially involved opening the animal’s left hind paw by an incision, which exposed the tibia and allowed the insertion of an intramedullary pin in the tibia. The tibia was then fractured at a single point. However, the pin, previously inserted prevented collapse of the tibia and enabled the wound to be sutured.

ii. Technique

Animal received isoflurane 1.5 MAC, corresponding to an air concentration of 2.1% (Engelhardt et al, 2006) and buprenorphine 0.1 mg/kg intra-peritoneally (i.p) injection for surgical-anaesthesia and postoperative analgesia. The surgical field was surrounded with sterile drapes and autoclaved instruments were used throughout surgery. The skin was shaved and then disinfected with chlorhexidine gluconate 0.5% and isopropyl alcohol at 70%. The open diaphyseal surgery of the tibia to the left hind paw was adopted with modifications as reported previously (Hiltunen et al, 1993). A longitudinal incision was made through the skin and fascia laterally to the tibia to expose the periosteum. After that, a 0.5 mm hole was drilled just above the proximal third of the tibia in order to create an insertion site for the subsequent insertion of an intramedullary, stainless steel, fixation wire. Afterwards, the fibula and the muscles surrounding the tibia were isolated and an osteotomy was performed with scissors in the middle of the distal third of the tibia. Once the fracture was made, the skin was sutured and intra-operative fluid loss was replaced via a subcutaneous injection of 0.9% NaCl solution.
4. Water maze

i. Theoretical background

The spatial reference task using the water maze test was first described in 1982
(Morris et al, 1982) and it has become one of the most widely used laboratory tools in
behavioural neuroscience. The apparatus consists of a large circular pool filled with
an opaque liquid in which a partially submerged platform is hidden. Across several
trials, the animals learn to find the platform and escape from the pool.

This behavioural test provides the benchmark for measuring hippocampal-
dependent memory. It is also considered to be more sensitive to a hippocampal insult
than fear conditioning. Also, it has advantages in relation to other maze tests. There
are no local cues, like scents, and, unlike fear conditioning, there is no aversive
stimulation. With the platform submerged underneath the surface of the water, one
can make animals solve the maze using either elemental or configural associations.
This can be done by simply changing the animals' starting points. If the animal is
started in the same position (or quadrant of the pool) at the beginning of each trial,
then it will only need to use elemental associations to find the platform. This is
because the animal can choose one cue in the room and the platform will always be in
the same place in relation to that cue. If the animal is started in a different position for
each trial (different random quadrants), it can no longer rely on finding the platform
in relation to one cue. The animal now must build a cohesive spatial representation of
the room to find the platform. The animal may swim towards the door in one trial, yet
have to swim away from it in the next trial. Because the platform no longer can be
found using one visual cue, the animal must construct configural associations to solve
the task.

Male animals are usually found to have better spatial learning. However, when
tested at the age of 6 months, male and female rats performed the same, suggesting
that the difference in performance is due to different maturational rates (Bucci et al,
1995). Alterations in the cholinergic system are supposed to underlie the sexual
differences in the water maze task. Female mice are found to be more sensitive to
cholinergic blockers than male mice (Berger-Sweeney et al, 1995).
Initially designed for rats (Morris et al, 1982), mice are also used in this task despite displaying different behavioural responses. Floating and thigmotaxis (i.e. tendency to swing nearer to the wall) tend to be more pronounced in mice than in rats (Wolfer et al, 1998). The C57BL/6 mice are better suited for spatial navigation tasks (Upchurch and Wehner, 1988).

Performance in the water maze declines with the age of animals (Geinisman et al, 1995). A number of researchers have hypothesised that the age-related decline in spatial learning is due to changes in function and morphology of the hippocampus (Gallagher et al, 2003; Geinisman et al, 1995). Twenty-four months old Fischer rat displayed impaired performance in the acquisition of the escape behaviour and in the probe trial when compared with young mice (Deupree et al, 1991).

It has also been demonstrated that sleep has an effect on the watermaze performance. Rats experience an increased paradoxical sleep after the hidden platform acquisition trials, but not when the platform was visible. In a similar manner, sleep deprivation after training impaired performance in the watermaze (Smith and Rose, 1997).

It has been established that the integrity of the hippocampal circuitry is essential for spatial learning (Morris et al, 1986). However, spatial learning and consequent performance in the watermaze is dependent on the co-ordinated action of different brain regions and several different neural networks (Roozendaal et al, 1996; Mumby et al, 1999). Disconnecting brain pathways impairs performance in the watermaze in a similar manner as destroying relevant brain regions (Whishaw and Jarrard, 1995).

It has been shown that hippocampus-lesioned rats have an impaired performance in the watermaze when the platform is hidden, but this does not happen when the platform is visible (Pearce et al, 1998). In a similar manner, performing the watermaze task always with the same initial starting point does not result in a learning impairment for hippocampal lesioned rats (Pearce et al, 1998). The spatial learning impairment is related to the extent of the damage to the hippocampal formation.
Dorsal hippocampus lesions have a more profound effect than ventral hippocampal lesions (Moser et al, 1993; Moser et al, 1995). Upon performing hippocampal lesions to C57BL/6J mice the acquisition of the hidden platform while learning and subsequent probe trial are impaired, but contextual fear conditioning is still possible in these animals (Cho et al, 1999).

Hippocampal “place-cells” are proposed to be the primary substrate for the spatial acquisition and memory underlying the watermaze tasks (Morris et al, 1986). Reversible inactivation of the hippocampus impairs the watermaze performance in rats (Riedel et al, 1999). Hippocampal inactivation during the acquisition phase impairs acquisition and performance in the probe trial. However, inactivation during the interval between the last acquisition trial and the probe trial impairs performance in the probe trial (Riedel et al, 1999).

Several researchers have suggested that hippocampal long-term potentiation (LTP) is an electrophysiological requirement for hippocampal dependent learning (Bliss and Lomo, 1973). Richard Morris’s group was the first to describe how an intraventricular injection of amino-phosphonovaleric acid impairs performance in the hidden platform watermaze task, while blocking long-term potentiation in the dentate gyrus (Davis et al, 1992). Studies using genetically engineered mice have shown that strains that display an impaired hippocampal plasticity have an impaired performance in the watermaze task (Wilson and Tonegawa, 1997). It has also been shown that pre-training saturation of long-term potentiation in the perforant path synapses of the dentate gyrus by high frequency stimulation impairs the performance in watermaze tasks (Moser et al, 1995).

The water maze test have been successfully employed in several disease models that tried to mimic the pathophysiological mechanisms underlying global ischaemia model (Block and Schwarz, 1998), traumatic brain injury (Hamm et al, 1992), *Alzheimer’s* disease (Nabeshima and Nitta, 1994), and other pathologies.
ii. Water maze apparatus

The apparatus consists of a large circular pool, 110 centimetres in diameter, containing water at around 25°C made opaque by adding dry milk creating a contrast with the dark colour of the C75BL/6 mice allowing image tracking by the computer software. The water in the pool is manually filled and drained daily. The choice of water temperature at around 13°C below body temperature is sufficiently stressful to motivate animals to escape. A video camera is placed above the centre of the pool to capture images of the swimming animals, and is connected to an on-line computer system running specialized tracking software from Ethovision (Noldus, The Netherlands). The top surface of the hidden platform, 10 cm in diameter, is 1 cm to 1.5 cm below the water surface. The pool itself was located in the laboratory room with distinctive 2- and 3-D distal cues that aid orientation. Prior to the commencement of training, mice were individually handled for 2 minutes each day over 3 consecutive days.

iii. Water maze protocol

Three groups of mice (each of 8 animals) were employed in this experiment. One group of animals was subjected to surgery by means described above. The second group of animals (control group) were merely handled and did not undergo surgery. The third group just underwent the habituation phase and performed the probe trial. All mice employed in this experiment underwent habituation training performed three days before the acquisition phase of the spatial reference task. Over the course of one hour each mouse was allowed to swim to a flagged platform. Once the animal reached the platform it was immediately removed. This procedure was repeated 4 times. Two days later, 8 mice were assigned to Control group, and 8 mice underwent the tibial surgery. The acquisition phase consisted of 4 days training on the spatial reference task in the water maze. Each day, the mice had 4 trials. Trials finished once the animal reached the platform or 90 seconds had elapsed. Once the animal reached the platform, it was allowed a further 30 seconds before being recovered, dried and placed in a warm chamber before the next trial. Each trial was performed 10-12 minutes apart.
On day 5 (probe trial), the platform was removed and mice were allowed to swim for 60 seconds before being rescued from the water maze. The probe trial measures hippocampal dependent spatial navigation.

5. Fear conditioning

i. Theoretical background

Fear is an extreme emotional experience. It has served an evolutionary purpose by helping species to survive since the activation of a defensive behavioural pattern system can protect an organism from potentially dangerous threats (Fanselow, 1980). Those threats can be either innate or learned (Blanchard and Blanchard, 1972a; 1972b). When placed near a source of potential injury, like a cat, a rat will become motionless and freeze, displaying only breathing (Bronstein and Hirsch, 1976). Alongside the displayed freezing, the rat shows a fear-potentiated startle response (Brown et al, 1951; Kim and Davis, 1993; Leaton et al, 1985), analgesia (Fanselow and Sigmundi, 1986), a wide range of autonomic changes (Black and de Toledo, 1972; LeDoux, 1992), and increased release of several hormones (Roozendaal et al, 1991).

The behavioural tasks designed for investigating fear can fall into learned or innate categories. Tests relying on unlearned fear use stimuli that naturally provoke fear even without prior training. These include exposure to predators (Blanchard and Blanchard, 1972a), exposure to a novel place which is brightly lighted (Montegomery and Monkman, 1955) or exposure to an elevated place (Graeff et al, 1993). Approaches using learned fear examine conditional behaviours associating an aversive stimulus (usually a foot-shock) with a neutral stimulus, like a light, a tone, or a harmless environment. After pairing the aversive and neutral cues, the neutral cue will itself be able to elicit an aversive reaction from the animal (Fanselow, 1980). The previous neutral stimulus is now a conditional stimulus. The innate reaction to fear in laboratory rodents is immobility, an element that can be scored as an index of memory (Quinn et al, 2005). Even after limited exposure to such conditions, the
animals are able to acquire a robust conditional response either to the tone or to the context (Fanselow, 1980).

When a rodent exhibits conditional fear, a number of physiological phenomena occur concurrently, including defecation, potentiated startle, elevated blood pressure, alterations in the heart rate, changes in skin conductance, supression of ongoing behaviour and freezing (LeDoux, 1992). Freezing is a adaptive reaction specific to mice and rats and is characterised by behavioural immobility (Bronstein and Hirsch, 1976).

Although the use of freezing for examining the neural basis of contextual fear has been criticised (McNish et al, 1997), several reasons support the use of this behavioural index. Animals do not tend to display a high baseline freezing response and most of the experimental treatments, like neurotoxic lesions, do not induce a high baseline freezing response. Freezing is not time limited, and it is observed for several minutes after a conditional stimulus is presented. The freezing behaviour is unambiguously a conditional response to training rather than an unconditional response.

Fear-potentiated startle is another response displayed in conditional fear and is elicited after a sudden stimulus. It has been found in every mammal studied (Landis and Hunt, 1939). A startle response is composed of a fast muscle contraction, particularly prominent in the face, neck and shoulders (Landis and Hunt, 1939). This reaction possibly helps to reduce the latency of a reaction time (Pilz and Schnitzler, 1996). Electromyographical measurements of the startle response latency was found to be around 5 to 10 milliseconds in rats (Caeser et al, 1989). This indicates a short neuronal pathway. It includes the cochlear root neurones, the giant neurones of the caudal pontine nucleus of the reticular formation and spinal neurones (Lee et al, 1996).

Paradigms using fear-potentiated startle were first described in 1951 (Brown et al, 1951). Rats were given several pairings of a light conditional stimulus and a
footshock. After those pairings, the mean amplitude of the acoustic startle response to a loud noise was 50 to 100% higher in the presence of the light conditional stimulus than the noise alone (Fendt et al, 1996). The difference between the light-noise trial and the noise-alone trials represents the fear potentiation of the startle response and serves as a measure of fear.

Data from spatial studies (Black, 1977) and configurational studies (Sutherland and Rudy, 1989) demonstrate that the hippocampus plays an important role in fear acquisition. Contextual fear conditioning is modulated by two key elements: forming a memory of the context; and associating that memory with the shock. Hippocampal damage following fear conditioning contains many parallels to clinical cases of amnesia following hippocampal damage (Kim and Fanselow, 1992). Hippocampal lesions made shortly after acquisition of fear produced a severe retrograde amnesia for contextual fear (Maren et al, 1997). When the lesions were made prior to conditioning, an anterograde amnesia is observed, although not as severe (Maren et al, 1997). Retrograde amnesia for conditional freezing to contextual cues has a temporal pattern. As the interval between the acquisition of memories and the lesion increases, the severity of the retrograde amnesia decreases (Kim and Fanselow, 1992; Maren et al, 1997). There is substantial evidence to support the role of the hippocampus in spatial learning and contextual fear memory obtained through the use of techniques such as surgical lesions (Maren et al, 1997), pharmacological manipulations (Frankland et al, 2004), or genetic manipulations (Frankland et al, 2004). The response of a conditioned animal which has undergone contextual fear conditioning, to the relevant context, is dependent on hippocampal memory.

However, the role of the hippocampus is time-limited. Mice which had the dorsal hippocampus temporarily inactivated with lidocaine, 1 day after acquisition of a contextual fear memory, displayed a reduced freezing to the context. The same procedure performed 28 days after training in the same area did not produce any impairment (Frankland et al, 2004).
The involvement of the amygdala in freezing to shock associated cues was demonstrated in 1972 (Blanchard and Blanchard, 1972a). When lesions are performed to large parts of the amygdala, rats did not display any freezing to a context associated with shock. Phillips and LeDoux (1992) lesioned animals either in the dorsal hippocampus or amygdala after fear conditioning. Animals with amygdala lesions showed a loss of freezing to both tone and context while lesions to the dorsal hippocampus only resulted in decreased freezing to the context (Phillips and LeDoux, 1992). In a similar manner, lesions to the amygdala performed 18 months after acquisition completely abolished rat’s ability to display any conditional fear responses (Gale et al, 2004).

Emotional memories are encoded by the amygdala. A lesion in the amygdala prevents the animals from acquiring fear to the tone, but a damaged hippocampus does not disrupt the acquisition of fear to the tone (Phillips and LeDoux, 1992). Monosynaptic projections from the geniculate nucleus present auditory information to the lateral amygdala that is used to encode an emotional memory event. The extent of such amygdala-dependent memory is assessed using the “tone test”. In the “tone test” the animal, which has already undergone contextual fear-conditioning, is subsequently placed in a novel, non-threatening environment, where the cue (tone) is displayed without the aversive stimulus being generated. The extent to which the animal freezes indicates the extent of amygdala-dependent memory retention (Phillips and LeDoux, 1992). Unlike the contextual assessment, the tone test does not depend on the hippocampus. By testing in a novel environment, the assessment is not tainted by the fear instilled by the acquisition-training environment.

More recently, the role of the neocortex has been investigated as a storage site for remotely acquired fear (Frankland et al, 2004; Wiltgen and Silva, 2007) and spatial memories (Teixeira et al, 2006). This advance in research resulted from the observation that the hippocampus seems to have a time-limited role in memory storage (Kim and Fanselow, 1992; Maren et al, 1997). As memory becomes independent of the hippocampus it is stored in a distributed manner in several areas of the cortex (Zola-Morgan and Squire, 1990; Wiltgen et al, 2004; Frankland and Bontempi, 2005).
Contemporary learning models suggest this reorganization of memory systems reflects the extraction of patterns, and general knowledge from specific experiences (McClelland, 1994). The hippocampus rapidly encodes detailed memories and then replays them so that the cortex can slowly extract features that are common across experiences. In humans, episodic memory retrieval requires a detailed re-experiencing of the original time and place where the event occurred, while semantic memories are remembered as facts and are accompanied by a sense of familiarity (Tulving and Schacter, 1990; Knowlton and Squire, 1995). Neuroimaging approaches have also shown that memory retrieved via recollection activates the hippocampus, while retrieval based on familiarity does not (Eldridge et al, 2000).

In mice it seems that memory for a context becomes less specific with time (Wiltgen and Silva, 2007). These authors conducted a set of experiments designed to assess memory over time. In the first experiment, separate groups of animals were trained with a single shock and tested in the training context or a novel environment 1, 14, 28, or 36 days later. They found a systematic increase in generalisation over that period. Initially, mice froze more in the training context, but fear of the novel environment grew over time until animals eventually froze an equivalent amount in both contexts. The same profile was observed in H-ras mutant mice that exhibit enhanced hippocampal plasticity and learning. These results suggest that context memories are specific early after training when they require the hippocampus, and become more general as they are permanently stored in the cortex (Wiltgen and Silva, 2007).

The establishment of fear memories is dependent on long-term potentiation. Long-term potentiation in the hippocampus and amygdala is coincidental with fear conditioning. An increase in synaptic transmission is observed when a weak pre-synaptic stimulus is paired with a strong post-synaptic depolarization. In fear conditioning, the conditional stimulus activates weak pre-synaptic activity, while the unconditional stimulus activates strong postsynaptic depolarization (Fendt et al, 1996).
ii. Apparatus for fear-conditioning

The memory assessment was performed in a single dedicated conditioning chamber manufactured by Med Associates Inc. (St. Albans, VT, USA). The back and sidewalls of this chamber are made of aluminium, while the front door and ceiling are made of transparent Plexiglass (see Figure 1). The removable floor consisted of 36 stainless steel rods of 1 mm diameter, spaced 0.5 cm apart. This frame was connected to a shock generator and scrambler for the delivery of the foot shock. Before each trial, the tray and floor were cleaned with a 5% sodium hydroxide solution scented with a 0.5% almond extract. A fan positioned on the right side of the chamber provided a background noise of 60 decibels. The infra-red video camera was mounted in the left door of the chamber. The data acquired by the camera was automatically scored for freezing time through a computer programme (Freezeframe, Med Associates Inc.).

Figure 1: Example of the interior of the conditioning chamber

iii. Definition and measurement of freezing response

“Freezing” was defined as lack of movement except that required for respiration. The time spent freezing as a percentage of the total time spent in the chamber during the context phase was used to score for hippocampal-dependent memory resulting from prior learning. A smaller percentage of freezing behaviour is indicative of greater degree of cognitive impairment.

The several fear-conditioning protocols employed are described in each corresponding chapter.
6. Social interaction test

i. Theoretical background

The social interaction test was first developed in 1978 (File and Hyde, 1978) as an ethological test for assessing anxiety, that is, assessing anxiety in a natural environment.

Anxiety is characterized by cognitive, somatic, emotional, and behavioural components (Seligman et al, 2011). When a person is anxious, he typically displays an unpleasant feeling that is typically associated with uneasiness, fear, or worry. Anxiety may occur without an identifiable triggering stimulus. However, typically, the precipitating stimulus will be obvious, for example, the trauma of an accident, fear of future harm to one’s self, one’s family or even one’s friend, and even with persons with whom one is not acquainted (Space Shuttle Colombia or the Hillsborough football disaster). Other factors, as loss of property or other type of financial loss can also precipitate anxiety.

Anxiety disorders affect about 40 million adults in the US alone (Kring, 2007). These are twice as common in women as men. Unlike the mild, brief anxiety caused by a stressful event (like an examination), anxiety disorders last more than 6 months and can get worse if not treated. Anxiety disorders tend to occur in conjunction with other mental or physical illnesses, which may mask anxiety symptoms or make them worse (Kring, 2007). Effective therapies for anxiety disorders are available. These allow people with anxiety disorders lead normal lives (Kring, 2007). Panic disorder is one of the most treatable of all the anxiety disorders, responding in most cases to medication or cognitive psychotherapy, which help to change the thinking patterns that lead to fear and anxiety.

Panic disorder is characterised by sudden attacks of terror, accompanied by an increase in heart rate, sweatiness, weakness, faintness, or dizziness (Kring, 2007). During these episodes, people with panic disorder have an autonomic reaction in which they may flush or feel chilled; their hands may tingle or feel numb; and they
may experience nausea or chest pain. Panic attacks usually produce a sense of unreality, a fear of impending doom, or a fear of losing control (Kring, 2007).

A fear of one’s own physical symptoms during a panic attack is also a symptom of panic disorder. People having panic attacks sometimes believe they are having heart attacks, losing their minds, or that they are on the verge of death. They cannot predict when, or where, an attack will occur, and between episodes many worry intensely and dread the next attack. Panic attacks can occur at any time. An attack usually peaks within 10 minutes, but some symptoms may last much longer.

Panic attacks often begin in late adolescence or early adulthood, but not everyone who experiences panic attacks will develop a panic disorder. Many people have just one attack and never have another. People who have full-blown, repeated panic attacks can become very disabled by their condition and should seek treatment before they start to avoid places or situations where panic attacks have occurred. For example, if a panic attack happens in an elevator, someone with panic disorder may develop a fear of elevators that may affect the choice of a job or an apartment, and restrict where that person can seek medical attention or enjoy entertainment. Some people’s lives become so restricted that they avoid normal activities, such as grocery shopping or driving. Although a minimum level of anxiety is expected in everyday life, it can become a “self-limitating” condition which impairs cognitive processes and social interaction.

The social interaction test uses the rodents’ natural social interactions in varying conditions involving anxiety. Typical stressors employed are, deprivation of food or water, subjecting to noxious stimuli, such as electrical shocks, or imposing a training regimen. Animals of the same sex, are subjected in pairs to a given stressor in a social interaction context in normal activities such as sniffing, following, grooming the partner or, fighting. Their performance in those activities is then assessed.
The social interaction test can be arranged in order to generate different levels of anxiety by manipulating the levels of light and the animal’s familiarity with the test arena. Four conditions can be readily created:

1. low light in a familiar arena (causes the lowest level of anxiety);
2. high light in a familiar arena (which generates moderate levels of anxiety);
3. low light in an unfamiliar arena (causing moderate levels of anxiety);
4. high light in an unfamiliar area (which creates highest levels of anxiety).

Since the behaviour of one mouse influences the other, a pair of mice should be considered as a single observation unit (File and Hyde, 1978). The number of trials should not be inflated by scoring a pair as 2 different observations. An increase in social interaction without an equal increase in motor activity is indicative of an anxiolytic effect, while a decrease in social interaction reveals an anxiogenic effect (File and Hyde, 1978).

Social interaction is highest when mice or rats are tested in a familiar arena lit with low light levels. As the test conditions become more aversive the social interaction decreases (File and Hyde, 1978). Usually, it is easier to see anxiolytic effects, manifested by an increase in social interaction, when the animals are tested in an unfamiliar arena lit with a high level of light, a condition in which the social interaction tends to be low. Conversely, placing rodents in a low level of light while in a familiar arena is the best option to observe anxiogenic effects, as indicated by a decrease in social interaction. These behavioural manifestations make this test a suitable experimental model to examine the neurobiological mechanisms underlying anxiety disorders since the levels of social interaction are readily capable of interpretation and measurement.

When the social interaction test was first validated, it was shown that the reduction in anxiety was not mediated by olfactory cues, since anosmic rats displayed a similar pattern of decreased social interaction across the 4 test conditions (File and Hyde, 1978). These authors also showed that reduced social interaction was not a
result of the animals spending more time exploring the unfamiliar arena (File and Hyde, 1978).

Animals are usually individually housed for a short period before the task. The test requires 5 days of individual housing, a criterion validated subsequently by Niesink and van Ree (1982), where the authors showed that social interaction was maximal after 4 to 7 days of individual housing (Niesink and van Ree, 1982). However, it is important to note that social isolation may modify the animals’ reaction to a given drug, as occurs with diazepam, chlordiazepoxide and nicotine (Wongwitdecha and Marsden, 1996; Vale and Montgomery, 1997; Cheeta et al, 2001). When diazepam was given to male Lister hooded rats, housed individually, it increased aggressive behaviour and avoidance behaviour in animals placed in the high light unfamiliar arena (Wongwitdecha and Marsden, 1996). Chlordiazepoxide reduced the high aggression levels of individually housed rats without changing the high levels of social interaction (Vale and Montgomery, 1997). Administration of nicotine to socially isolated mice, increased the time spent in social interaction without changing locomotion levels, while in socially housed mice it increased motor activity and social interaction (Cheeta et al, 2001).

The test has been validated for male adult rats and there are important sex differences. Female rats do not display an increase in social interaction in a familiar arena (Johnston and File, 1991). Environmental factors may alter social interaction. When rats are housed under noisy conditions for 24 days, they displayed significantly lower levels of social interaction than animals housed in a quiet environment (File, 1994).

Food deprivation does not seem to have an effect on social interaction (Genn et al, 2003). However, a rich diet in soya, a cheap source of protein, reduces social interaction and increases the plasma cortisol response to the social interaction test when compared to rats fed with a soya-free diet (Hartley et al, 2003).

Mice are also used in the social interaction test. Swiss albino mice show the same decrease in social interaction as rats when the light level was manipulated.
However, in the case of mice, the familiarity of the arena is a less reliable predictor of social interaction (de Angelis and File, 1979).

Several brain regions have been implicated in modulating social interaction. The amygdala plays an important role in mediating anxiety. Injecting neurotoxins into this brain structure decreases social interaction and reduces locomotor activity (File and Clarke, 1981). The hippocampus is also known to be implicated in the control of anxiety responses due to the projections from the raphe nucleus. Bilateral injection of midazolam have an anxiolytic effect that is reversed by a benzodiazepine receptor antagonist, flumazenil (Gonzalez et al, 1998). The reciprocal neuronal circuits linking the medial prefrontal context to the amygdala and the hypothalamus may play an important role in mediating fear and responses to stress. Other brain areas involved include the lateral septum (Clarke and File, 1982), the hypothalamus (File and Velucci, 1979), the raphe nuclei (File et al, 1979) and the locus coeruleus (Crow et al, 1978).
ii. Test arena

The social interaction test arena was a plastic box 50×40 cm, with 15 cm walls (see example in Figure 2), and was lit by low light (10 lux). A camera was mounted vertically above the arena. The time spent in social interaction (sniffing, following, grooming the partner, boxing and wrestling) provided the measure of anxiety and was scored by an observer who was blind to the treatment.

Figure 2: example of the arena used for the social interaction test and olfactory discrimination task

iii. Social interaction test protocol

The first group (surgery group) comprises mice upon which the previously described surgery was performed 24 hours earlier. The second group (naïve group) comprises animals treated in a similar manner but on whom surgery had not been performed. While undergoing familiarisation, mice were placed individually, untreated in the test arena for a 5-minutes familiarisation trial at least 3 hours before the social interaction test. Two mice, of the same treatment condition, were placed simultaneously into the centre of the arena for 10 minutes and the time spent in social interaction was scored as a measure of anxiety. This consisted of specific behavioural patterns such as grooming, sniffing, following the partner, crawling under and boxing.
7. Habituation-dishabituation task

i. Theoretical background

Establishing memory in respect of a past event is crucial for survival. Olfactory learning has been employed as an experimental model in offspring recognition in sheep (Kendrick et al, 1997); mate recognition in voles (Pitkow et al, 2001); social recognition in rats (Winslow and Camacho, 1995); and social transmission of food preference in mice (Galef et al, 1994). Olfactory learning shares the following features with human declarative memory: single trial learning; rapid encoding; long-term memory; and high storage capacity. Olfactory memory models represent a simple form of learning. Olfactory learning can be divided into socially and non-socially motivated tasks (Ferguson et al, 2000).

This model is ethologically relevant while studying offspring recognition (Brennan et al, 1995; Kendrick et al, 1997). Offspring recognition ensures that the mother preserves energy by limiting maternal investment to her own offspring. Being able to socially recognise a conspecific (i.e.: a member of the same litter) has functional consequences (Kendrick et al, 1997; Lai and Johnston, 2002; Carr et al, 1979). It is crucial for determining and maintaining a social structure, particularly in small and stable social groups.

Social recognition tests in rodents relies upon the intrinsic motivation that rats and mice have for investigating novel member of the same species while in a familiar place. Under these circumstances, rodents will investigate a novel conspecific more than a familiar one, since they have not yet acquired the odour cues of the novel animal (Carr et al, 1976). Social recognition has been inferred from a decline in olfactory investigation of conspecific intruders during repeated or protracted confrontation with a resident rat. The decline in investigation levels corresponds to criterion for habituation and consequent social memory (Winslow and Camacho, 1995). Introduction of a fresh stimulus increases animal interest in levels of exploration to similar levels of those a first encounter. It also indicates that a generalised habituation does not occur in this task. This paradigm can be used either for measuring short-term memory (Thor et al, 1982) or long term-memory, since it
has been shown that mice housed in group can retain a memory for a familiar individual up to 7 days (Kogan et al, 2000).

The olfactory system in mice and rats is a sensory system designed for obtaining environmental information. The olfactory circuitry is highly developed. The olfactory bulb consists of two major regions: the main olfactory bulb; and the accessory olfactory bulb. The main olfactory bulb discriminates between a wide range of volatile odours present in an environment and links a given odour to a specific feature in the animals’ environment. The accessory olfactory bulb is responsible for sensing pheromonal odours which give rise to stereotypical reactions which do not require higher cognitive processing (Shipley and Adamek, 1984).

Odour learning via the main olfactory bulb is thought to be more distributed and to depend on the nature of the task and temporal configurations. Studies on brain structure activation and reversible inactivation provided much of our current knowledge of this system. Initial memory formation and short-term retention seems to require a distributed neural system involving the olfactory bulb; piriform and entorhinal cortices; and the hippocampus (da Costa et al, 1997).

There have been relatively few studies regarding the involvement of neural substrates in social recognition memory of mice. Mice which lack a receptor for oxytocin show an increase in c-fos expression in the olfactory bulb, piriform cortex and medial amygdala after a brief social exposure (Ferguson et al, 2000). High frequency stimulation of the granulate cell layers of the olfactory bulb results in a selective long-term potentiation in the olfactory bulb and the piriform cortex (Patneau and Stripling, 1992).

Only the olfactory bulb and the piriform cortex appear to be important for social recognition approximately 8 hours after the social exposure (Pitkow et al, 2001). In rodents, lesioning the entorhinal cortex results in deficits of short-term odour memory (Staubli et al, 1984), while lesions to the hippocampus seem to have no effect on short-term recognition memory (Petrulis and Eichenbaum, 2003).
Winslow and Camacho developed the habituation/dishabituation paradigm (Winslow and Camacho, 1995; Dluzen and Kreutzberg, 1993), which offers a model for examining olfactory social recognition memory. These authors examined the decline in olfactory investigation of ovariectomized females by adult male mice. The duration and frequency of olfactory investigation was measured during four 1 minute confrontations with 10-minutes inter-trial intervals. If the same female was presented in each trial, investigation declined to less than 50% of initial levels (Winslow and Camacho, 1995). On the fifth trial, when a novel stimulus was introduced, the investigation level was restored to the original highest level (dishabituation).

The second part of the paradigm, performed 24 hours later, is a social discrimination. In this phase, the test animal is given a binary choice between a previously encountered familiar, and a novel, conspecific (Ferguson et al, 2000). A specific recognition is assessed by comparing the different amount of time spent investigating each stimulus animal. The second part of the protocol allows the recognition memory by comparing the familiar versus the unfamiliar in a single trial. This allows for short-term and long-term memory to be assessed in a single experiment.

**ii. Habituation-dishabituation task protocol**

The first group (surgery group) comprises mice upon which the previously described surgery was performed 24 hours earlier. The second group (naïve group) comprises animals treated in a similar manner but on whom surgery had not been performed.

Two groups of mice \((n = 8\) per group) were used. Naïve and surgery animals were habituated to an arena \((50 \text{ cm} \times 40 \text{ cm} \times 15 \text{ cm} - \text{this was the same arena as used for the social interaction test})\) for 15 minutes before exposure to test stimuli. A video camera recorded the trials. The animals intended to provide a stimulus (stimulus animals) were anaesthetised (sodium pentobarbital 6mg/kg i.p.) 10 minutes before the trials.
The stimulus animal was placed in the arena first. Then, the subject animal was introduced to the arena and allowed to explore the environment there for 2 minutes and then removed and returned to a cage. The subject mouse was re-exposed to the stimulus animal after an inter-trial interval of 10 minutes. This procedure was repeated 4 times.

At the fifth trial, a novel stimulus animal was used to provide a new stimulus. This approach excludes the possibility that the reduction in investigation is due to fatigue or habituation to the task.

In order to assess long-term recognition memory, 24 hours after the last pairing, the subject animal was again exposed, first to the initial stimulus animal and, next, to a new mouse stimulus.

The 3 stimulus animals had been selected from cages other than those of the subject animals in order to avoid odour contamination.

The purpose of the test was to assess the investigatory behaviour of the subject mice under the different conditions to which they were exposed. “Investigation” was defined as sniffing the stimulus animal either at the neck area or at the genital/anus area. A person who was blind to the experimental conditions scored the animals’ behaviours at successive intervals of 5 seconds. Thus, each trial was scored 24 times during the 120 seconds duration. The number of observations was summed and converted to a percentage (number of interactions / total number of observation x 100).
8. Immunohistochemistry

i. Theoretical background

Immunohistochemistry combines anatomical, immunological and biochemical phenomena 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 the appropriate antigen. This first antibody can be labelled but it is more common (and improves the visualisation) to use a second antibody directed against the first (an anti-IgG). This second antibody is conjugated with the biotin-avidin system so that the location of the primary antibody and, thus, the antigen, can be recognised. The term “immunohistochemistry” is frequently used interchangeably with “immunocytochemistry”. Strictly, “immunohistochemistry” refers to the use of this technique in the context of tissues, rather than individual cells to which the term “immunocytochemistry” applies. Antibodies are serum glycoproteins, known as immunoglobulins (Igs), which are secreted by terminally differentiated B cells (lymphocytes). In the vertebrate immune system, antibodies are produced as a defence against foreign substances. Antibodies may be used as biochemical tools to identify and locate specific cell components. Of the five classes of antibodies, IgGs are the predominant class produced in the immune response. Polyclonal antibodies can be made against any antigen by injecting the antigen (isolated cell constituent or molecule) into a host animal (e.g. goat, rabbit or mouse), harvesting the plasma, and removing clotting factors to produce serum containing the antibodies. Animals produce a highly heterogeneous mixture of antibodies in response to challenge by a foreign antigen. These different antibodies come from different antibody producing cells. Polyclonal antibodies have an advantage over monoclonal antibodies in that they are more likely to identify multiple epitopes of the target protein. An alternative is to use “monoclonal antibodies”. Monoclonal antibodies are produced by fusing spleen cells with myeloma cells and growing the resultant hybrids in tissue culture (Cuello et al, 1983; White et al, 2011).
ii. Perfusion and storing for immunohistochemistry

Fixation is necessary to prevent artifactual diffusion of soluble tissue components, to arrest enzymatic activity, to avoid decomposition of the structure, and to protect the tissue against the deleterious effects involved with the various stages of the immunohistochemical process (White et al, 2011).

The animals were given a terminal dose of sodium pentobarbital. They were perfused through the heart with calcium-free Tyrode solution followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.5) for 15 minutes. Heparin (Braun Medical Ltd, Sheffield, UK) was injected into the left ventricle during perfusion to prevent blood clotting from taking place. The brains were harvested and post-fixed in 4% paraformaldehyde for 24 hours at 4ºC. For cryoprotection, the tissues were placed in 15% sucrose in 0.1M PBS until saturation and subsequently changed into 30% sucrose at 4ºC. until further use.

iii. Brain preparation and cryostat sectioning

A metallic base was allowed to cool down to -20ºC. OCT embedding medium (VWR, West Chester, PA) was applied to the metallic base covering an area which was large enough to accommodate the specimen. The specimen was attached vertically onto the prepared base of embedding medium. Additional embedding medium was placed around the specimen until it was completely covered, rapid freezing aerosol spray could be used to ensure that the specimen maintained its vertical position. The specimen was left in the cryostat for at least 30 minutes until it was completely frozen. Once the specimen was ready, the metallic base was attached firmly onto the cryostat (Bright Instruments Huntingdon, UK). Sections were cut transversely at a thickness of 25 µm and thaw mounted on Superfrost Plus microscope slides (VWR, West Chester, PA). They were later air dried overnight in darkness and stored in -20ºC. until further use (White et al, 2011).
iv. Immunohistochemistry protocol

1. Sections from all of the treatment groups were run together in order to reduce variability.

2. To begin the staining procedure, the sections were left at room temperature for 20 minutes before being transferred to 0.1 M PBS.

3. To reduce background staining caused by endogenous peroxidase the sections were quenched with a solution of 0.3% H₂O₂, 0.3% normal donkey serum (NDS) and 0.1M PBS for 30 minutes.

4. Sections were rinsed 3 times with 0.1 M phosphate buffered saline with triton X (PBST, 0.3% triton X, pH 7.5) for 5 minutes and incubated with 10% NDS in 0.1 PBST for 25 minutes. Blocking the reactive sites in tissues is essential for the development of an immunohistochemical reaction. 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 nonspecific adsorption or by binding of specific, but unwanted, serum antibodies to antigens in the tissue (White et al, 2011).

5. The sections were then incubated overnight at room temperature with polyclonal primary antibody (Cell Signalling, cleaved caspase-3 rabbit antibody, or Serotec, cd11b mouse antibody, as appropriate for Caspase-3 or microglia) at a dilution of 1:200.

6. On the following day sections were washed 3 times for 5 minutes in 0.1 M PBST, and incubated with secondary antibody (Biotin-SP-AffiniPure Donkey Anti-Rabbit IgG, for cleaved caspase-3; or Donkey Anti-Goat IgG for microglia) for 2 hours at room temperature at a dilution of 1:200.
7. After rinsing in PBST, all sections were incubated with avidin-biotin system (ABC Vectastain Elite solution) for 30 minutes. Avidin is a 68,000 molecular weight glycoprotein with an extraordinarily high affinity for the small molecular weight vitamin, biotin. The binding of avidin to biotin (unlike antibody-antigen interactions) is essentially irreversible. 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 (White et al, 2011).

8. DAB was used for the staining and colour was allowed to develop for 1 minute and 15 seconds.

9. The slides were dehydrated in a stepwise manner through 50%, 75%, 90% and 100% ethanol for 10 minutes at each concentration.

10. They were submerged in xylene for a further 10 minutes and mounted under a cover slip using depex polystyrene (DPX) mounting medium (Raymond A Lamb, East Sussex, UK).

11. Slides were air dried overnight under the fume hood in darkness and later kept in plastic boxes for storage.
9. Enzyme immunoassays

i. Theoretical background

Enzyme-linked immunosorbent assay (ELISA) is a plate-based assay employed to search for the presence, and quantify the amount, of peptides, proteins, antibodies or hormones. This technology involves the antigen being immobilised to a solid surface whereupon it is complexed with an antibody linked to an enzyme. The conjugated enzyme activity is measured by incubation with a substrate to produce a measurable product. ELISAs are typically carried out in a 96-well polystyrene plate which can passively bind antibodies and proteins. Horeseradish peroxidase (HRP) and alkaline phosphatase (AP) are the enzyme labels which are usually employed. When the ELISA is performed with an HRP or AP conjugate, there is a large selection of substrates available. The assay sensitivity required and the device available for signal detection (spectrophotometer, fluorometer or luminometer) will determine the choice of substrate (Thermo Scientific Proteomics, 2009; White et al, 2011).

Immobilization of the target antigen can be achieved by direct adsorption to the assay plate. Alternatively, the target antigen can be immobilised using a “capture antibody” that has been attached to the plate. The target antigen is then detected using a labelled primary antibody or using a labelled secondary antibody. The so-called “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 which is both sensitive and robust. In a sandwich ELISA, it is essential that the secondary antibody should be specific for the detection of the primary antibody only and not for the capture antibody. Generally, this is achieved by using capture and primary antibodies from different host species (Thermo Scientific Proteomics, 2009; White et al, 2011).
ii. Sample harvesting

Samples for the IL-1β ELISA (Bender Medsystem, Austria) were extracted from the hippocampus at 6 hours after tibia surgery while blood samples were taken at 24 hours.

Thoracotomy was performed and cardiac blood was withdrawn under deep isoflurane anaesthesia. This procedure was performed at the same time of day to minimize the circadian fluctuation (between the hours of 10:00 and 14:00). Blood samples were collected in pre-heparinized Ependorf tubes.

Under terminal anaesthesia with isoflurane, each mouse was sacrificed and the brain quickly removed following decapitation. The hippocampus was dissected 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 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 hippocampal samples and serum were centrifuged at 84,000g for 15 minutes at 4°C. Supernatants were collected and stored at -80°C until the ELISA was carried out. IL-1β in the hippocampus and plasma was measured as previously described (Nguyen et al, 1998) using ELISA (Bender MedSystems, Austria) and normalised to total protein levels.
iii. IL-1β ELISA protocol

1. Cytokine levels were assessed with sandwich enzyme-linked immunosorbent assay (ELISA). Pre-coated 96-wells ELISA plates were purchased from Bender MedSystems, Austria.

2. Standards and samples were incubated for 120 minutes at room temperature with biotin conjugate (50 µL total volume).

3. After washing all wells four times with washing buffer, samples were incubated with Streptavidin-HRP Working Solution (Bender MedSystems) for 60 minutes at room temperature on a shaker, and then washed again four times. Washes were performed between each step of the ELISA. These washing steps are necessary to remove non-bound reagents and to decrease background, thereby increasing the signal:noise ratio. Inadequate washing allows high background, while too much washing may result in decreased sensitivity caused by elution of the antibody and/or antigen from the well (White et al, 2011).

4. Stabilized chromogen (Bender MedSystems) was added to each well; after 10 minutes of incubation the reaction was stopped and the absorbance read on a micro-platelet reader at 450 nm. The last stage in all ELISA procedures is the detection. Unless a radioactive or fluorescent tag is used, this requires the use of an enzyme substrate. The enzyme converts the substrate to a detectable product. In a properly constructed ELISA, the intensity of the signal produced when the substrate is added is directly proportional to the amount of antigen captured in the plate and bound by the detection reagents.

5. Cytokine levels were calculated by a standard curve of the assessed cytokine. Manufacturer's data reported assay sensitivities of <1.2 pg/ml.
10. Immunoblotting

i. Overview

Western blotting or, protein immunoblot, is an analytical technique used to detect specific proteins in a given sample of tissue homogenate. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions). The proteins are transferred to a membrane, where they are probed (detected) using antibodies specific to a target protein.

ii. Tissue preparation

Animals were rapidly decapitated after being perfused with a saline solution and brains quickly removed. Hippocampal dissections were performed on an ice-cold frosted glass plate and tissues quickly frozen in liquid nitrogen. Hippocampal samples were stored at -80°C. until the time of sonication. In most cases, solid tissues are first broken down mechanically using a blender, for larger sample volumes, and using a homogenizer, for smaller volumes, or by sonication. Cells may also be broken open by one of the above mechanical methods. Assorted detergents, salts, and buffers may be employed to encourage lysis of cells and to solubilize proteins. Protease and phosphatase inhibitors are often added to prevent the digestion of the sample by its own enzymes.
iii. Western blot protocol

1. Protein extracts (15 µg per sample) and a biotinylated molecular weight marker (New England Biolab) were denaturated in sample loading buffer at 90ºC for 5 minutes.

2. The samples were then loaded into a polyacrylamide gel electrophoresis (12%), and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, United Kingdom). Sampled proteins become covered in the negatively charged sodium dodecyl sulphate (SDS) and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilo Daltons, kDa). The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration, the better the resolution of lower molecular weight proteins.

3. The membrane was pretreated with blocking solution (5% non-fat dry milk) in Tween-containing Tris-buffered saline (10 nM Tris, 150 nM NaCl, 0.1% Tween, pH 8.0). Since the PVDF membrane has been chosen for its ability to bind protein, and both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein.

4. The membranes were incubated with BDNF (rabbit anti-mouse, 1:200, Santa Cruz, USA) overnight at 4C°.

5. Horseradish peroxidase-conjugated mouse antibody to rabbit IgG (1:200, New England Biolab) was used to detect the primary antibody. During the detection process the membrane is “probed” for the protein
of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colourimetric reaction and produces a colour. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

6. The bands were visualised with an enhanced chemiluminescence system (ECL, Amersham Biosciences, United Kingdom).

7. Results were normalized with alpha-tubulin (1:10000, Sigma, United Kingdom).
Chapter 3

LPS-INDUCED INFLAMMATION RESULTS IN
ANTEROGRADE AND RETROGRADE AMNESIA IN MOUSE

1. Introduction

2. Methods

3. Results
   i. Anterograde amnesia
   ii. Retrograde amnesia
   iii. Permanent retrograde amnesia
   iv. LPS-induced retrograde amnesia can be prevented by an IL1-ra

4. Discussion
Introduction

Memory is profoundly affected by general anaesthetics. This has been described in animal and human studies alike (Savage, 1887; Ghoneim and Block, 2007; Dutton et al, 2002; Culley et al, 2004; Baxter et al, 2008). More recent human studies indicate that a memory deficit may persist for at least a week in middle-aged patients (Johnson et al, 2002) and for months in elderly patients (Moller et al, 1998). Whether this results from the effects of surgery, general anaesthesia, or other perioperative factors remains unclear.

Anaesthetics have been shown to modulate the behavioural effects of glutamate injections into the hippocampus of rats (Baxter et al, 2008). Fear-conditioning tests have demonstrated that administering a sub-clinical dose of the anaesthetic isoflurane concurrently with fear conditioning results in dose-dependent anterograde amnesia but not retrograde amnesia (Dutton et al, 2002). Again, performance in the radial arms maze is reduced after recovery from general anaesthesia for longer than predicted by the pharmacology of the drugs used, which suggests a long-term deficit in learning and memory may result from anaesthetic exposure (Culley et al, 2004).

Systemic infection results in physiological and behavioural changes in both humans and animals (Dantzer et al, 2008). Cytokines play an important role in the inflammatory response after infection (Pugh et al, 1998). It has been demonstrated that an elevation in the level of the pro-inflammatory cytokine IL-1β in the brain impair contextual, but not auditory-cued, fear-conditioning. Moreover, an increase in brain IL-1β results both in retrograde and anterograde amnesia (Barrientos et al 2002; Barrientos et al, 2009).

The following experiments were intended to determine the impact of isoflurane-induced anaesthesia and LPS, respectively, in the development of anterograde amnesia and retrograde amnesia.
Methods

Subjects

12 to 14 week-old C57/BL6 male mice (30–35 grams; Harlan, Oxon, UK) were group housed, maintained on a 14:10 hours light/dark cycle, given *ad libitum* access to food and water, and handled for 3 to 5 days before behavioural experiments. Experiments were performed in strict compliance with the Home Office approved License.

Fear-conditioning

Twenty to 30 min before fear-conditioning, subjects were taken into the room where the behavioural testing would occur.

In the *anterograde amnesia assessment* three group of mice (*n* = 6 per group) were employed. One group received LPS. The second group was subjected to isoflurane anaesthesia. The third group received a saline injection. Each treatment was administered 24 hours before contextual fear-conditioning. Mice received an unsignalled foot-shock (0.85 mA, 2 second) after 3 minutes and then remained in the context for an additional minute. At day 4, mice were placed back into the training environment and freezing behaviour was measured.

In the *retrograde amnesia assessment*, three groups of mice (*n* = 6 per group) were employed. One group received LPS. The second group was subjected to isoflurane anaesthesia. The third group received a saline injection. Each treatment was administered 24 hours after contextual fear-conditioning. Contextual fear response was assessed at day 4. The contextual fear conditioning parameters are similar to parameters use in the anterograde amnesia protocol.

The *permanent amnesia assessment* (see Figure 1) was designed to study a longer-term effect of LPS on memory. Four groups of naïve mice (*n* = 6 per group), underwent fear conditioning and subsequently two groups received LPS immediately after undergoing contextual-fear conditioning. The remaining animals were given a saline injection and constituted the control groups. Contextual fear response was assessed at either day 3 and or day 7. At each time-point an LPS group and a control
group were employed. The contextual fear conditioning parameters are similar to parameters use in the retrograde amnesia protocol.

**Acquisition**  
Day 1  

**Assessment**  
Day 3 or Day 7

*Retention interval*

(0.85 mAmp)

![Experimental manipulation within 30 minutes]

**Figure 1.** *Contextual-fear conditioning for the assessment of permanent amnesia.* Training consisted of exploration of the conditioning box for 180 seconds followed by an unsignalled 2 seconds foot-shock (0.85 mAmp). Mice were removed from the chamber 60 seconds later. Immediately after being removed from the conditioning box, mice received an injection of LPS. Either at day 3 or 7, rodents were returned to the same chamber for 240 seconds exposure to the context. Overall percentage of time spent freezing was recorded.

In contextual fear-conditioning protocol designed to study the effect of an IL1-ra in mitigating LPS-induced memory decline, three groups of mice (*n* = 8 per group), underwent fear-conditioning training and subsequently were injected with saline, LPS, or LPS with a IL1-ra immediately after undergoing contextual-fear training. Contextual-fear response was assessed at day 3. The contextual fear-conditioning parameters are similar to parameters used in the retrograde amnesia protocol.

**Injections**

LPS (Escherichia Coli endotoxin; 0111:B4, InvivoGen, USA) was dissolved in normal saline and injected intra-peritoneally at a dose of 100 µg/kg, which has been previously shown to provoke an immune response which impairs hippocampal memory consolidation (Fidalgo et al, 2011a). IL1-ra (Amgen, Anakinra 100 mg/kg, Netherlands) was given subcutaneously prior LPS injection. Control animals were injected intra-peritoneally with saline.
**Anaesthetic induction**

Mice were anaesthetised by isoflurane 1.5 MAC, corresponding to an air concentration of 2.1% and buprenorphine 0.1 mg/kg intra-peritonially were given and maintained during 10 to 15 minutes of deep-anaesthesia mimicking the length of anaesthesia underwent by mice which received the orthopaedic tibia surgery in our model.

**Data analysis**

All data are expressed as mean ± SEM and analysed by one-way ANOVA followed by the Bonferroni post-hoc test. A $p$ value $< 0.05$ was considered to be of statistical significance.
**Results**

*i. Anterograde amnesia*

LPS-induced inflammation, but not isoflurane induced-anaesthesia, results in anterograde amnesia. This was evidenced by reduced freezing levels in the LPS-treated animals as compared to those in the control group (see Figure 2). The control animals were found to freeze longer which showed that they retained a recollection of the context of the previous aversive event to which they had been reintroduced (52.5% ± 5.27). Anaesthetic induction did not impaired contextual fear-memory (47.17% ± 8.71, \( p > 0.05 \) compared to control). LPS injected 24 hours before the aversive event, significantly reduced the animal’s recollection of the aversive event when measured at Day 4 (26.0% ± 3.13, \( p < 0.05 \) compared to control). This data corroborate the previous finding that inflammation within the brain results in a reduced freezing response (Barrientos et al, 2009).

![Figure 2](image)

**Figure 2. Contextual-fear conditioning retrieval test for the anterograde amnesia assessment.** Rodents were exposed to the same context where aversive conditioning was previously carried out. Mice which were administered with LPS 24 hours before contextual fear conditioning (n = 6) froze significantly less then their Control counterparts (n = 6). Anaesthesia administration 24 hours before contextual fear conditioning did not impaired contextual freezing (n = 6). Data are expressed as mean ± SEM, * \( p < 0.05 \) for statistical comparisons.
**ii. Retrograde amnesia**

Injection of LPS at 24 hours after the aversive event significantly reduced the animal’s recollection of the aversive event when measured at Day 4 (35.0% ± 4.29, \( p < 0.05 \) compared to control). The control animals were found to freeze longer which showed that they retained a recollection of the context of the previous aversive event to which they had been reintroduced (54.5% ± 4.12). Anaesthetic induction did not impaired contextual fear-memory (49.67% ± 6.87, \( p > 0.05 \) compared to control). Again, this data corroborate the previous finding (Barrientos et al, 2002) that an elevation in hippocampal IL-1\( \beta \) levels impairs memory processes which underlie the formation and storage of fear memories, resulting in a reduced freezing response (Barrientos et al, 2002) (see Figure 3).

![Figure 3. Contextual-fear conditioning retrieval test for the retrograde amnesia assessment.](image)

Rodents were exposed to the same context where aversive conditioning was previously carried out. Mice which received LPS (n=6) froze significantly less then their Control counterparts (n = 6). Anaesthesia administration 24 hours after contextual fear conditioning did not impaired contextual freezing. Data are expressed as mean ± SEM, * \( p < 0.05 \) for statistical comparisons.
iii. Permanent amnesia

Injection of LPS immediately after the aversive event significantly reduced the animal’s recollection of the aversive event when measured 3 days later (52.5% ± 5.27 for control 3d vs. 29.67% ± 5.46 for LPS 3d, \( p < 0.05 \)) and 7 days later (49% ± 2.33 for control 7d vs. 29.33% ± 4.86 for LPS 7d, \( p < 0.05 \)). The control animals were found to freeze longer which shows that they retained a recollection of the context of the previous aversive event to which they had been reintroduced (52.5% ± 5.27 for control 3d and 49% ± 2.33 for control 7d) (see Figure 4).

![Figure 4](image)

**Figure 4.** *Contextual-fear conditioning retrieval test for the permanent amnesia protocol*: thirty minutes after undergoing contextual fear conditioning mice received an LPS injection. Three and seven days later rodents were exposed to the same context in which fear conditioning was previously carried out. Contextual fear response reveals hippocampal-dependent memory impairment both at day 3 and 7. Data are expressed as mean ± SEM (n = 6), \( * p < 0.05 \) for statistical comparisons.
iv. LPS-induced retrograde amnesia can be prevented by an IL1-ra

In the context test, the extent of freezing in the acquisition environment for the 3 different groups was measured. The difference between control group and LPS (without IL1-ra) group was significant (47.33% ± 2.75 for the Control vs. 21.17% ± 4.00 for the LPS, $p < 0.05$). So also was the difference in freezing time between the LPS without IL1-ra group and the LPS with IL1-ra group (21.17% ± 4.00 for the LPS vs. 39.67%, ± 7.46 for the LPS+IL1-ra, $p < 0.05$). No significant difference was found between the control group and the LPS with IL1-ra group (47.33% ± 2.75 for the control vs. 39.67% ± 7.46 for the LPS+IL1-ra, $p > 0.05$) (see Figure 5).

![Figure 5. Contextual fear-conditioning retrieval test for the contextual fear-conditioning protocol.](image)

Within thirty minutes following training, mice were injected with saline, LPS or LPS with IL1-ra, respectively. Three days later, rodents were exposed to the same context in which fear conditioning was previously carried out. Contextual fear response reveals a hippocampal-dependent memory impairment in the LPS group when compared to control group. Pre-treatment with IL1-ra significantly ameliorated the memory retention at day 3 when compared to LPS alone. Data are expressed as mean ± SEM (n = 8 per group), * $p <0.05$ for statistical comparisons.
Discussion

The present findings indicate that LPS-induced inflammation, but not isoflurane-induced anaesthesia, results in memory impairment in mouse. This memory impairment causes a permanent retrograde amnesia. Blocking the action of IL-1β is sufficient to reduce the hippocampal memory deficit induced by LPS. Taken together with previous experiments (Barrientos et al, 2002, 2009), which demonstrate that an elevation of IL-1β in the hippocampus results in cognitive deficits in rat, this data supports the hypothesis that inflammation within the brain may disrupt the consolidation of fear-memories.

This is not the first study which demonstrates that inflammation within the brain results in reduced freezing responses in fear-conditioning tasks (Barrientos et al, 2002, 2009). Stimulating the immune system with LPS to mimic a Gram-negative bacterial infection induces the expression of inflammatory cytokines in the brain (Barrientos 2009) affecting particularly the hippocampus (Barrientos et al, 2002; Csölle and Sperlágh, 2010). The increased expression of IL-1β in the hippocampus is derived from local production of microglial cells (Griffin et al, 2006). Elevated IL-1β levels in the hippocampus impairs the ability to induce and maintain long-term potentiation - LTP (Vereker et al, 2000; Griffin et al, 2006). It has also been shown to reduce neurogenesis (Monje et al, 2003), and to reduce the expression of neurotrophins (Guan and Fang, 2006). The disruption of memory processes by increased levels of IL-1β has been demonstrated in cases of external administration of LPS or E.coli (Barrientos et al, 2002, 2009) and in over-expressing knock-out mice (Avital et al, 2003; Goshen et al, 2007). IL-1β induced memory deficit is more evident in hippocampal memory tasks such as the context test (Barrientos et al, 2002), as reported by Barrientos and colleagues (2002, 2006, 2009). The tone test appears to be unaffected by either intra-peritoneal LPS or E.Coli administration or even hippocampal injection of IL-1β (Barrientos et al, 2002; 2006; 2009).

The reduced levels of freezing response cannot also be attributed to sickness behaviour (Barrientos et al, 2009). Because sickness behaviour is characterised by reduced mobility, pilo-erection, lethargy, anhedonia and decreased social interaction as a result of a pathophysiological elevation of cytokines in the hypothalamus
(Barrientos et al, 2009). Also, the assessment was performed after day 3 in order to avoid possible sickness behaviour confounding responses.

The disruption of memory induced by anaesthetic gases has been previously demonstrated (Culley et al, 2003, 2004) Culley and collaborators (2004) used a 2 hours administration of anaesthetics (Culley et al, 2004). The protocol in this study required a 15-minute administration of isoflurane. This length of time was chosen because it reflected the time under anaesthetic in the tibial surgery model used in subsequent experiments in this dissertation. It is possible that such a short anaesthetic protocol may be ineffective in detecting anaesthetic-induced cognitive dysfunction. A power analysis calculation indicates that a sample of 43 animals per group would be required to detect a statistical significant difference ($\alpha$ of 0.05 and power of 0.9) if we assume the values reported in the retrograde amnesia experiment.

In summary, I have demonstrated that LPS induced inflammation impairs contextual fear conditioning. This occurs by inflammation-induced interference with the memory consolidation processes that occur after acquisition of a fear memory (Barrientos et al, 2002). This disruption of memory processes induces a permanent retrograde amnesia for the contextual fear memory to which the mice were exposed.
Chapter 4

IL1-RA REDUCES POST-OPERATIVE INFLAMMATION-INDUCED COGNITIVE DEFICITS IN MOUSE

1. Introduction

2. Methods

3. Results
   i. IL-1β levels in plasma after surgery
   ii. IL-1β levels in hippocampi after surgery
   iii. Microgliosis after surgery
   iv. Apoptosis (caspase-3) levels in the brain after surgery
   v. Water maze: spatial reference task
   vi. Delay fear-conditioning: Antagonist trial
   vii. Contextual fear-conditioning: Antagonist trial
   viii. Contextual fear-conditioning: IL1 ra injection

4. Discussion
Introduction

Postoperative cognitive dysfunction (POCD) is a term employed to describe problems with memory, learning and the ability to concentrate following surgery. Data indicates that this condition is exacerbated in the elderly (Moller et al, 1998). The International Study of Post-Operative Cognitive Dysfunction (ISPOCD-1) demonstrated that patients over 60 years of age are more prone to develop a cognitive dysfunction. The ISPOCD-1 study compared 1218 patients (age >60 years) undergoing major surgery with a control group matched for age. Cognitive dysfunction, measured by means of a battery of neuropsychological tests at a three month post-operative visit, was found in three-times as many patients as controls (9.9% vs. 2.8%) (Moller et al, 1998). The precise pathogenesis of POCD is not known and may involve perioperative factors and patient-related factors (Newman et al, 2007). However, several studies have indicated a possible role for inflammation as playing a pivotal role in the development of surgery-induced cognitive dysfunction (Buvanendran et al, 2006; Wan et al, 2007; Rosczyk et al, 2008; Frank et al, 2010). In normal ageing, an immune challenge induces a sensitised and protracted neuroinflammatory response in parallel with memory impairments (Rosczyk et al, 2008), while a peripheral Escherichia coli infection increases central pro-inflammatory cytokines resulting in memory deficit (Frank et al, 2010). As interleukin(IL)-1β has been identified as a mediator of the inflammatory response to peripheral infection and physical injury (Dinarello, 1996; Barrientos, 2002, 2009) and exerts an important role in memory function (Goshen et al, 2007), we hypothesised that peripheral orthopaedic surgery induces systemic cytokine release which results in hippocampal inflammation and associated memory deficits.

The following experiments were intended to determine the impact of surgery-induced inflammation on memory dysfunction following peripheral orthopaedic surgery. We have investigated whether surgery increases IL-1β levels in the plasma and in the hippocampus. We have also examined whether surgery is associated with hippocampal microgliosis. We have also investigated whether surgery-induced inflammation impairs hippocampal dependent long-term memory using watermaze and fear-conditioning tests. We also examined if the administration of an IL1-rationealorates surgery-induced cognitive dysfunction.
Methods

Subjects

12 to 14 week-old C57/BL6 male mice (30–35 grams; Harlan, Oxon, UK) were group housed, maintained on a 14:10 light/dark cycle, given ad libitum access to food and water, and handled for 3–5 days before surgery or behaviour. Experiments were conducted in strict compliance with the Home Office approved License.

Surgery

Mice were anaesthetised by isoflurane 1.5 MAC, corresponding to an air concentration of 2.1%. Buprenorphine 0.1 mg/kg was given intra-peritoneally for analgesia. Both were given and maintained during 10 to 15 minutes of deep-anaesthesia. The surgical procedure essentially involved opening the animal’s left hind paw by an incision, which exposed the tibia and allowed the insertion of an intramedullary pin in the tibia. The tibia was then fractured at a single point. However, the pin, previously inserted, prevented collapse of the tibia and enabled the wound to be sutured. This procedure is described at length in Chapter 2 (Methods).

IL-1β assessment by ELISA

Samples for the IL-1β ELISA (Bender Medsystem, Austria) were extracted from the hippocampus at 6 hours after tibia surgery while blood samples were taken at 24 hours. Under terminal anaesthesia with isoflurane, each mouse was sacrificed and the brain quickly removed following decapitation. The hippocampus was dissected 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 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 hippocampal samples and plasma were centrifuged at 84,000g for 15 minutes at 4°C. Supernatants were collected and stored at -80°C until the ELISA was carried out. IL-1β in the hippocampus and plasma was measured as previously described (Nguyen et al, 1998) using ELISA (Bender MedSystems, Austria) and normalised to total protein levels.
Assessment of microgliosis and apoptosis through immunohistochemistry

Sections from all of the treatment groups were run together in order to reduce variability. To begin the staining procedure, the sections were left at room temperature for 20 minutes before being transferred to 0.1 M PBS. To reduce background staining caused by endogenous peroxidase the sections were quenched with a solution of 0.3% H₂O₂, 0.3% normal donkey serum (NDS) and 0.1M PBS for 5 minutes. Sections were rinsed 3 times with 0.1 M phosphate buffered saline with triton X (PBST, 0.3% triton X, pH 7.5) for 5 minutes and incubated with 10% NDS in 0.1 PBST for 25 minutes. The sections were then incubated overnight at room temperature with polyclonal primary antibody (Cell Signalling, cleaved caspase-3 rabbit antibody, or Serotec, cd11b mouse antibody, as appropriate for Caspase-3 or microglia) at a dilution of 1:000. On the following day sections were washed 3 times for 5 minutes in 0.1 M PBST, and incubated with secondary antibody (Biotin-SP-AffiniPure Donkey Anti-Rabbit IgG, for cleaved caspase-3; or Donkey Anti-Goat IgC for microglia) for 2 hours at room temperature at a dilution of 1:200. After rinsing in PBST, all sections were incubated with ABC Vectastain Elite solution, but using a dilution of 1 in 200 for reagent A and B, instead of that suggested in manufacturers the user guidelines. DAB was used for the staining and colour was allowed to develop for 1 minute and 15 seconds. The slides were dehydrated in a stepwise manner through 50%, 75%, 90% and 100% ethanol for 10 minutes each. They were submerged in xylene for further 10 minutes and mounted under a cover slip using depex polystyrene (DPX) mounting medium (Raymond A Lamb, East Sussex, UK). Slides were air dried overnight under the fume hood in dark and later kept in plastic boxes for storage.
**Behavioural testing**

**Water maze**

**Water maze apparatus**

The apparatus consists of a large circular pool, 110 centimetres in diameter, containing water at around 25°C made opaque by adding dry milk creating a contrast with the dark colour of the C75BL/6 mice allowing image tracking by the computer software. The water in the pool is manually filled and drained daily. The choice of water temperature at around 13°C below body temperature is sufficiently stressful to motivate animals to escape. A video camera is placed above the centre of the pool to capture images of the swimming animals, and is connected to an on-line computer system running specialized tracking software from Ethovision (Noldus, The Netherlands). The top surface of the hidden platform, 10 cm in diameter, is 1 cm to 1.5 cm below the water surface. The pool itself was located in the laboratory room with distinctive 2- and 3-D distal cues that aid orientation. Prior to the commencement of training, mice were individually handled for 2 minutes each day over 3 consecutive days.

**Water maze protocol**

Three groups of mice (each of 8 animals) were employed in this experiment. One group of animals was subjected to surgery by means described in Chapter 2 (Methods). The second group of animals (control group) were merely handled and did not undergo surgery. The third group just underwent the habituation phase and performed the probe trial. All mice employed in this experiment underwent habituation training performed three days before the acquisition phase of the spatial reference task. Over the course of one hour each mouse was allowed to swim to a flagged platform. Once the animal reached the platform it was immediately removed. This procedure was repeated 4 times. Two days later, 8 mice were assigned to Control group, and 8 mice underwent the tibial surgery, as described in detail in the Chapter 2 (Methods). The acquisition phase consisted of 4 days training on the spatial reference task in the water maze. Each day, the mice had 4 trials. Trials finished once the animal reached the platform or 90 seconds had elapsed. Once the animal reached the platform, it was allowed a further 30 seconds before being recovered, dried and
placed in a warm chamber before the next trial. Each trial was performed 10-12 minutes apart. On day 5 (probe trial), the platform was removed and mice were allowed to swim for 60 seconds before being rescued from the water maze. The probe trial measures hippocampal dependent spatial navigation.

**Fear-conditioning**

*Apparatus for fear-conditioning*

The memory assessment was performed in a single dedicated conditioning chamber manufactured by Med Associates Inc. (St. Albans, VT, USA). The back and sidewalls of this chamber are made of aluminium, while the front door and ceiling are made of transparent Plexiglass. The removable floor consisted of 36 stainless steel rods of 1 mm diameter, spaced 0.5 cm apart. This frame was connected to a shock generator and scrambler for the delivery of the foot shock. Before each trial, the tray and floor were cleaned with a 5% sodium hydroxide solution scented with a 0.5% almond extract. A fan positioned on the right side of the chamber provided a background noise of 60 decibels. The infra-red video camera was mounted in the left door of the chamber. The data acquired by the camera was automatically scored for freezing time through a computer programme (Freezeframe, Med Associates Inc.).

*Delay fear-conditioning paradigm*

Three groups of mice (*n* = 32 per group) were employed in this experiment. The first group underwent tibial surgery as previously described herein. The second group were given a dose of IL1-ra (100 µg/kg injected sub-cuetaneous) immediately prior to undergoing the same surgery. The final group consisted on the control which were handled and given a saline injection without surgery. Three groups of mice were trained and tested on 2 separate days. Each mouse was transported by cage to the behavioural assessment room and left in its cage for 15 minutes before starting.

Training consisted of placing the subject mouse in a chamber and allowing exploration by the animal 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 for the final 2 seconds of the CS. This procedure was repeated, and the mice were removed from the chamber 30 seconds later. With the termination of the trial, every mouse was taken
individually within 30 minutes to the surgery room. Three days after conditioning, mice were returned for 270 seconds to the same chamber in which training had occurred and their freezing behaviour in response to the context only was recorded by the software (0.5 seconds intervals). "Freezing" was defined as lack of movement except that required for respiration. At the end of the 270 seconds context 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 cue. The novel environment consisted of modifications to the 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 to score freezing for 135 seconds. The auditory cue (CS), namely, the training tone, was then presented for 135 seconds, and freezing was again scored. The time spent freezing as a percentage of the total time spent in the chamber during the context phase was used to score for hippocampal-dependent memory resulting from prior learning. A smaller percentage of freezing behaviour is indicative of greater degree of cognitive impairment.

**Contextual fear-conditioning protocol**

Mice were place in a conditioning box and received 1 unsignalled foot-shock (0.85 mAmp). Within 30 minutes mice received either a saline injection, surgery, surgery and IL1-ra (100 µg/kg s.c.) or IL1-ra (100 µg/kg s.c.) alone. Hence there were 4 groups of animals (n of 12 per group in experiment vii and; n of 6 per group in experiment viii). At day three mice were placed in the acquisition environment and freezing was recorded.
**Statistical analysis**

*ELISA:* IL1-β levels in the plasma and hippocampus were compared by one-way analysis of variance (ANOVA) and Student-Newman-Keuls *post-hoc* test.

*Immunohistochemistry for microglia:* photomicrographs were taken from the hippocampal CA1 region (scale bar 50 µm, 20X). Photomicrographs were blindly scored and microgliosis was graded on a scale 0 (lowest) – 3 (highest). Non-parametric data are presented with Kruskal-Wallis followed by Dunn’s *post-hoc* test.

*Immunohistochemistry for caspase-3:* photomicrographs were taken from the hippocampal CA1, CA3 and dentate gyrus regions (scale bar 200 µm, 5X). Photomicrographs were blindly scored and positive cells counted. Overall cell numbers were compared using a one-way ANOVA.

*Spatial reference task in a water maze:* latency of escape to platform in the training phase (short-term memory) and time spent in the target quadrant (long-term memory) were compared by one-way analysis of variance (ANOVA) and followed by the Bonferroni *post-hoc* test.

*Contextual fear-conditioning test:* the freezing time in the context test and tone test were compared by one-way analysis of variance (ANOVA) and followed by the Bonferroni *post-hoc* test.

In all cases $p < 0.05$ indicated a statistically significant level.
Results

i. IL-1β levels in the plasma after surgery

Surgery increased IL-1β levels in the plasma when measured at 24 hours. IL-1β expression in this cohort was compared with its expression in animals which were not subjected to surgery. IL-1β expression was also compared in animals that received surgery after an IL1-ra injection. An increase in IL-1β levels in surgery animals was found when compared to control animals ($p < 0.05$). Administration of IL1-ra prior to surgery reduced IL-1β when compared to animals which received surgery without this antagonist ($p < 0.05$) (see Figure 1).

![IL-1β levels in the plasma after surgery](image)

**Figure 1. IL-1β levels in the plasma after surgery.** Surgery increased IL-1β levels in systemic circulation at the examined time-point when compared to control group (*$p < 0.05$). Injection of IL1-ra before tibial surgery successfully reduced IL-1β levels at the same time-point when compared to surgery alone (*$p < 0.05$). IL-1β levels in the animals that received IL1-ra just prior to surgery are not statistically significant from control group ($p > 0.05$).
ii. IL-1β levels in the hippocampi after surgery

Surgery increased IL-1β levels in the hippocampus when measured at 6 hours. IL-1β expression in this cohort was compared with its expression in animals which were not subjected to surgery. IL-1β expression was also compared in animals that received surgery after an IL1-ra injection. An increase in IL-1β levels in surgery animals was found when compared to control animals ($p < 0.05$). Administration of IL1-ra prior to surgery reduced IL-1β when compared to animals which received surgery without this antagonist ($p < 0.05$) (see Figure 2).

![Figure 2. IL-1β levels in the hippocampi after surgery.](image)

_Surgery increased IL-1β levels in the hippocampus at the examined time-point when compared to control group (* $p < 0.05$). Injection of IL1-ra before tibial surgery successfully reduced IL-1β levels at the same time-point when compared to surgery alone (* $p < 0.05$). IL-1β levels in the animals that received IL1-ra just prior to surgery are not statistically significant from control group ($p > 0.05$)_.


Surgery-induced inflammation resulted in activation of microglial cells when measured at 24 hours. Microglial activation in control was compared with that in animals in were subjected to surgery. Comparison was also made in microglial activation in animals that received surgery after an IL1-ra injection. An increased hippocampal microgliosis was found in surgery animals when compared to control animals ($p < 0.05$). Administration of IL1-ra prior to surgery reduced microgliosis when compared to animals which received surgery alone ($p < 0.05$) (see Figure 3).

**Figure 3. Microglial activation after surgery.** Surgery (slide C) increased microglial activation when compared to control (Slide A) ($p < 0.05$). Injection of IL1-ra (Slide B) just before surgery decreased microgliosis when compared to surgery ($p < 0.05$). However, the decrease in microgliosis was still statistically different from control group ($p < 0.05$).
iv. Apoptosis (caspase-3) levels in the brain after surgery

Despite the hippocampal inflammation there was no increase in caspase-3 positive cells in the surgery treated animals. Caspase-3 expression was compared in surgery treated animals with its expression with animals in were not subjected to surgery. Caspase-3 expression was not increased in surgery animals when compared to control in either the neocortex or the hippocampus of mice ($p > 0.05$) (see Figure 4).

Figure 4. Surgery induced inflammation does not increase apoptosis. Surgery does not increase apoptosis as measured by caspase-3 when compared to control group ($p > 0.05$).
v. Water maze: spatial reference test

Surgery induced-inflammation did not impair the acquisition of spatial reference memory as naïve and surgery-treated animals had similar latencies in finding the platform. Figure 5 indicates the latency for day 4.

![Figure 5](image)

**Figure 5. Spatial reference task in a water maze.** By day 4 animals had become proficient in finding the hidden platform. Animals in the control group reach a plateau, while mice in the surgery group had a slight improvement from Trial 1 to Trial 4. This was not statistically significant ($p > 0.05$).

However, in the probe trial performed twenty-four hours later, with the platform removed, surgery was found to impair long-term spatial memory when compared to control animals ($p < 0.05$) (see Figure 6). The surgery group performed better than untrained animals which only underwent the habituation phase ($p < 0.05$).

![Figure 6](image)

**Figure 6. Probe trial in a water maze.** At day 5 the platform was removed and animals swam for 60 seconds before being rescued. Animals in the control group had a higher percentage of time spent in the target quadrant when compared to surgery group (*$p < 0.05$). Surgery group
had a better performance than animals which just underwent the habituation phase (* \( p < 0.05 \)).

vi. Delay fear-conditioning: antagonist trial

Using a delay fear-conditioning paradigm, the extent of freezing in the acquisition environment for the 3 different groups was measured. The difference between control and surgery groups was statistically significant (\( p < 0.05 \)). So also was the difference in freezing time between the surgery without IL1-ra group and the surgery with IL1-ra group (\( p < 0.05 \)). No significant difference was found between the control group and the surgery with IL1-ra group (\( p > 0.05 \)) (see Figure 7).

![Figure 7. Contextual fear retrieval test for the delay-fear conditioning protocol. Rodents were exposed to the same context where aversive conditioning was previously carried out. Mice undergoing surgery under general anaesthesia (n=32) froze significantly less then their control counterparts (n=32). Pre-surgical treatment with IL1-ra (n=32) improved surgery-dependent impairment of retrieval of hippocampal fear memories. Data are expressed as mean ± SEM, *\( p < 0.05 \) for statistical comparisons.](image)

The tone test involves a comparison of the baseline (defined as the freezing to a novel context) and tone (freezing to the acquisition tone presentation, in such new context). The baseline freezing between the control group and the surgery without IL1-ra group was not significant (\( p > 0.05 \)). Again, no significant difference was found between the surgery without IL1-ra group and the surgery with IL1-ra group (\( p > 0.05 \)) and between the surgery with IL1-ra group and the control group (\( p > 0.05 \)) (see Figure 8).

Freezing in response to the training tone did not differ between the naïve group and the surgery group (\( p > 0.05 \)). No difference was found between the surgery without IL1-ra group and the surgery with IL1-ra group (\( p > 0.05 \)). Again, no
significant difference was found between the control group and the surgery with IL1-ra group (see Figure 8).

Figure 8. Auditory-cued retrieval test for the delay fear-conditioning protocol. Rodents were exposed to a different context of that where aversive conditioning was conducted. There was no statistical difference between control mice (n=32), mice undergoing surgery under general anaesthesia (n=32) and those receiving IL1-ra in addition (n=32). They froze the same to control littermates in both baseline and tone presentation phases of the test. This suggests that surgery does not impair the amygdalar component of fear related memories. Data are expressed as mean ± SEM.
vii. Contextual fear-conditioning: antagonist trial

Using a contextual fear-conditioning protocol, the extent of freezing in the context test for the 3 different groups was measured. The difference between control and surgery groups was significant \( p < 0.05 \). So also was the difference in freezing time between the surgery without IL1-ra group and the surgery with IL1-ra group \( p < 0.05 \). No significant difference was found between the naïve group and the surgery with IL1-ra group \( p > 0.05 \) (see Figure 9).

![Figure 9](image_url)

**Figure 9.** Contextual fear-conditioning retrieval test for the contextual fear-conditioning antagonist trial. Rodents were exposed to the same context where aversive conditioning was previously carried out. Mice undergoing surgery under general anaesthesia froze significantly less than their control counterparts. Pre-surgical treatment with IL1-ra improved surgery-dependent impairment of retrieval of hippocampal fear memories. Data are expressed as mean ± SEM, * \( p < 0.05 \) for statistical comparisons.
viii. Contextual fear-conditioning: IL1-ra injection

In the absence of inflammation an IL1-ra injection on its own resulted in memory deficit. Using a contextual fear-conditioning protocol, the extent of freezing in the context between control and IL1-ra injection groups was significant ($p < 0.05$) (see Figure 10).

![Figure 10. Contextual-fear conditioning retrieval test for the antagonist injection trial. Rodents were exposed to the same context where aversive conditioning was previously carried out. Mice which received an IL1-ra injection after contextual fear conditioning froze significantly less than their Control counterparts. Data are expressed as mean ± SEM, * $p < 0.05$ for statistical comparisons.](image-url)
Discussion

We have employed a model of neuroinflammation in mouse in which peripheral inflammation caused by orthopaedic surgery of the tibia results in inflammation in the brain and cognitive impairment. Surgery is associated with increased levels of IL-1β in the plasma and in the hippocampus. Surgery also induces hippocampal microgliosis without being associated with an increase in apoptosis. An injection of IL1-ra results in reduced microgliosis and reduced IL-1β levels in the plasma and in the hippocampus. Although surgery did not affect the acquisition in the spatial reference memory task, nevertheless, surgery animals spent less time in the target quadrant in the probe trial. In fear conditioning tasks, mice in the surgery group displayed memory impairment, which was reduced to normal levels with the injection of IL1-ra before surgery. Injection of IL1-ra alone resulted in memory deficit. These findings indicate that the cognitive impairment found results, in part, at least, from an effect of surgery on the hippocampal neural network which underlies water maze tasks and fear-conditioning contextual tests. Moreover, this impairment may be abolished by administration of an IL1-ra.

IL1-ra has been usefully used for more than 15 years in diseases where IL-1β plays an important role (Furst, 2004). The use of an IL1-ra in clinical trials for patients with rheumatoid arthritis resulted in the improvement of clinical symptomatology with very few side effects, and these side effects were usually restricted to patients on a daily dose of 150 mg. The major side effects were: infections (17% of patients in the treatment group), mainly respiratory; injection-site related reactions; pain; inflammation; erythema (redness of the skin) or ecchymosis (bruising); and, in a small number or cases, neutropaenia - unusual low number of neutrophils (Furst, 2004). Although the injection-site related reactions tend to lighten with continued daily use (Swart et al, 2010), several sickness behaviour symptoms, such as fever, weight loss, fatigue, and headache are commonly reported throughout IL1-ra treatments (Gran et al, 2011). Data from animal studies indicate that the administration of an IL1-ra may cause temporary memory deficits and should never be given to pregnant women since it may result in permanent memory deficits in the child when born (Goshen et al, 2007).
My findings are consistent with those of Goshen (Goshen et al, 2007) and Spulber (Spulber et al, 2009). This present a particular problem in the context of POCD which is a disease which itself involves cognitive impairment. However, the IL1-ra in all probability acts to prevent the development of cognitive impairment so that it should not act to aggravate cognitive impairment resulting from POCD itself. Nevertheless, it is well known that those who are particularly vulnerable to POCD frequently suffer from pre-surgery cognitive impairment. Thus there may well be a real risk that the intended cure may cause more harm than that is intended to alleviate.

In this study we were able to define the hippocampus as the main site of action for IL1-β. The basolateral amygdala is involved in the acquisition and expression of induced conditional fear and has been identified as a possible site for fear memory acquisition (Gale et al, 2004). Lesion studies suggest that if the amygdala had been affected by the consequences of the surgery, at any time point due to surgery, a severe reduction in freezing behaviour would have been evident (Gale et al, 2004). Of course, we cannot say for certain whether the action of cytokines on the amygdala has the same effect as a lesion, so the value of deduction from these lesion experiments is limited.

Barrientos and colleagues (2002) examined the effect of microinjection of IL-1β into the dorsal hippocampus of rats. They found that although the rats displayed impairment in contextual fear conditioning, they demonstrated normal auditory-cue conditioning freezing (Barrientos et al, 2002). Our study corroborates their findings that an elevation in hippocampal IL-1β levels impairs the memory processes which underlie the formation and storage of fear memories, resulting in reduced freezing responses (Barrientos et al, 2002; 2009).

This study was not able to determine whether a causal relation exist between microgliosis and memory decline after surgery. However, microgliosis has described as impairing long-term potentiation [LTP] (Griffin et al, 2006), and producing neurotoxins, excitotoxins and reactive oxygen species which may result in increased neuronal apoptosis (Van Rossum and Hanisch, 2004; Semmler et al, 2005).
We were unable to determine whether a causal relation exist between anaesthesia and memory dysfunction using fear-conditioning protocols (Cibelli et al, 2010). As the anaesthetic regimen employed in our studies required a 15-minute administration of isoflurane it is possible that such a short anaesthetic protocol may be ineffective in detecting anaesthetic-induced cognitive dysfunction. Further studies are needed to examine the possible role of anaesthetics in cognitive dysfunction.

In summary, we have demonstrated that peripheral orthopaedic surgery is associated with an increase in IL1-β in the plasma and in the hippocampus. This increase results in hippocampal memory deficits that can be ameliorated with an IL1-ra. However, caution must the taken when considering the use of an IL1-ra.
Chapter 5

**Peripheral Orthopaedic Surgery Impairs Contextual Remote Memory While Reducing Hippocampal BDNF Levels in Mouse**

1. Introduction

2. Methods

3. Results
   i. IL-1β levels in the hippocampus and PFC after peripheral surgery
      a. Hippocampus
      b. PFC
   
   ii. BDNF levels in the hippocampus

   iii. Assessment of remote memory after peripheral inflammation
        a. Contextual generalisation test
        b. Contextual discrimination test

4. Discussion
Introduction

The International Study on Post-Operative Cognitive Dysfunction (ISPOCD) identified several risk factors for the development of POCD (Moller et al, 1998). These included advanced age, duration of surgery, and post-operative infection. We have recently shown that peripheral orthopaedic surgery itself may result in post-operative cognitive deficits and that pre-operative sub-clinical infection may exacerbate this impairment (Cibelli et al, 2010; Fidalgo et al, 2011a). Here, we continue to explore the effects of peripheral orthopaedic surgery and infection on cognitive function.

Peripheral orthopaedic surgery induces a massive inflammatory response, including a substantial increase in the level of interleukin(IL)-1β in the hippocampus, which has been shown to impair hippocampal-dependent memory in mouse (Cibelli et al, 2010). Spatial and contextual memory initially involves the hippocampus and, with time, the neocortex, in a process which can take from days to weeks (Frankland et al, 2004; Teixeira et al, 2006; Wiltgen and Silva, 2007; Wang et al, 2009). The hippocampus plays a specific, but limited, role in memory storage (Wang et al, 2009). Older, “remote”, memories are stored in the neocortex and are not affected by hippocampal damage as such, but only by lesion to the neocortex (Kim and Fanselow, 1992; Morgan-Zola and Squire, 1990). We have investigated whether inflammation induced by peripheral orthopaedic surgery and lipopolysaccharide (LPS) injection, respectively, also adversely impacts on such remote memory, using the contextual generalisation test (Frankland et al, 2004, 2006; Wiltgen and Silva, 2007) and the contextual discrimination test (Wang et al, 2009) for this purpose.

Peripheral LPS injection has previously been shown to elevate IL-1β levels in the hippocampus but not in the pre-frontal cortex (PFC), and also to reduce brain-derived neurotrophic factor (BDNF) levels in the hippocampus (Barrientos et al, 2009; Csölle and Sperlágh, 2010; Schnydrig et al, 2007). We have investigated whether peripheral orthopaedic surgery induces these same effects.
Methods

Subjects

In all, 68 C57/BL6 male mice were employed in these experiments. These animals were aged from 12 to 14 week-old (30–35 grams; Harlan, Oxon, UK). They were group housed, maintained on a 14:10 hours light/dark cycle, given ad libitum access to food and water, and handled for 3-5 days before surgery or behavioural experiments. All the experiments were performed under Home Office License (PPL N.º 70/6104; PIL N.º 70/20834) and in compliance with the requirements of the Animals (Scientific Procedures) Act, 1986. Every effort was made to limit the number of animals employed in these experiments and to ensure that no unnecessary suffering was occasioned to the animals employed.

Surgery

Mice were anaesthetised by isoflurane 1.5 MAC, corresponding to an air concentration of 2.1% and buprenorphine 0.1 mg/kg intra-peritoneally (i.p.) were given and maintained during 10 to 15 minutes of deep-anaesthesia and postoperative analgesia. The surgical procedure essentially involved opening the animal’s left hind paw by an incision, which, exposed the tibia and allowed the insertion of an intramedullary pin in the tibia. The tibia was then fractured at a single point. However, the pin, previously inserted, prevented collapse of the tibia and enabled the wound to be sutured. This procedure is described at length elsewhere (Cibelli et al, 2010).

Lipopolysaccharide (LPS) injection

LPS (Escherichia Coli endotoxin; 0111:B4, InvivoGen, USA) was dissolved in normal saline and injected i.p. at a dose of 100 µg/kg, which has been previously shown to provoke an immune response which impairs hippocampal memory consolidation (Fidalgo et al, 2011).

IL-1β measurement by ELISA

In all, 10 animals were employed in this experiment. The animals were divided into a Control group and a Surgery group. Mice in the Surgery group were sacrificed 6 hours after surgery for hippocampal and PFC harvesting. This time-point was
previously identified as that at which IL-1β levels are maximally elevated in the hippocampus after peripheral orthopaedic surgery (Cibelli et al, 2010). Animals were rapidly decapitated after being perfused with a saline solution and brains quickly removed. Hippocampal dissections were performed on an ice-cold frosted glass plate and tissues quickly frozen in liquid nitrogen. Brain samples were aliquotted in Eppendorf tubes and stored at -80°C until analysis. IL-1β in the hippocampus and PFC was measured as previously described (Nguyen et al, 1998) using ELISA (Bender MedSystems, Austria) and normalised to total protein levels.

**BDNF measurement by western blot**

In all, 10 animals were employed in this experiment. The animals were divided between a Control group and a Surgery group. The animals in the Surgery group were sacrificed 24 hours after peripheral orthopaedic surgery. Animals were rapidly decapitated after being perfused with a saline solution and brains quickly removed. Hippocampal dissections were performed on an ice-cold frosted glass plate and tissues quickly frozen in liquid nitrogen. Hippocampal samples were stored at -80°C until the time of sonication. Protein extracts (15 µg per sample) and a biotinylated molecular weight marker (New England Biolab) were denaturated in Laemmli sample loading buffer at 90°C for 5 minutes, separated by a polyacrylamide gel electrophoresis (12%), and electrotransferred to a nitrocellulose membrane (Invitrogen, United Kingdom). The membrane was pretreated with blocking solution (5% non-fat dry milk) in Tween-containing Tris-buffered saline (10 nM Tris, 150 nM NaCl, 0.1% Tween, pH 8.0). The membranes were incubated with BDNF (rabbit anti-mouse, 1:200, Santa Cruz, USA) overnight at 4°C. Horseradish peroxidase-conjugated mouse antibody to rabbit IgG (1:200, New England Biolab) was used to detect the primary antibody. The bands were visualised with an enhanced chemiluminescence system (ECL, Amersham Biosciences, United Kingdom). Results were normalized with alpha-tubulin (1:10000, Sigma, United Kingdom).
Contextual generalisation protocol

In all, 24 animals were employed in this experiment. At day 1, all mice were placed in the conditioning context for 3 minutes after which 5 foot-shocks were delivered. The shocks were separated by a 3-min interval. Each shock was of 2 seconds duration and was 0.85 mA in strength. One minute after administration of the last shock, the mice were removed and returned to their home cage. At day 3, mice were assigned randomly to either a Control group (saline injection), an LPS group or a Surgery group. At day 32, all mice were placed in a counter-balanced manner in the acquisition environment (Context A) and in the novel environment (Context B) and their freezing responses were recorded for 4 minutes.

Contextual discrimination protocol

In all, 24 animals were employed in this experiment. All mice were placed in a conditioning-box and received 1 unsignalled foot-shock, lasting 2 seconds, and having a strength of 0.85 mAmp (Context A). Five hours later all mice were placed in a completely new environment where they were not shocked (Context B). This procedure was repeated with all mice, once daily, for 3 days. At day 6, mice were assigned randomly to either a Control group (saline injection), an LPS group or a Surgery group. At day 32, all mice were placed in a counter-balanced manner in the acquisition environment (Context A) and in the novel environment (Context B) and their freezing responses were recorded for 4 minutes.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis for IL-1β, BDNF and freezing levels was performed with analysis of variance (ANOVA) followed by the Bonferroni post-hoc test or the Student-Newman-Keuls post-hoc test for multiple comparisons. A p < 0.05 was considered to be of statistical significance.
Results

i. IL-1β levels in the hippocampus and PFC after peripheral surgery

a. Hippocampus

Orthopaedic surgery significantly increased the level of IL-1β present in the hippocampus of mice measured at 6 hours post-surgery when compared to control mice. The average level of IL-1β found in the hippocampus of the control animals was $1.90 \pm 0.48 \text{ pg/µg}$, while that found in the animals which underwent surgery was $7.94 \pm 0.50 \text{ pg/µg}$. The difference between averages of the two groups was significant (Figure 1, $p < 0.05$).

Figure 1. IL-1β levels in the hippocampus measured in control and, at 6 hours after surgery, respectively. Surgery significantly increased the levels of IL-1β found in the hippocampus 6 hours later. Results are mean ± SEM (n = 5); * $p < 0.05$ vs. control.
b. Prefrontal Cortex (PFC)

Orthopaedic surgery failed to significantly increase the level of IL-1β present in the PFC of mice measured at 6 hours post-surgery when compared to control mice. The average level of IL1-β found in the PFC of the control animals was $1.27 \pm 0.11$ pg/µg, while that found in the animals which underwent surgery was $1.12 \pm 0.05$ pg/µg. The difference between averages of the two groups was not significant (Figure 2).

![Graph showing IL-1β levels in control and surgery groups.](image)

**Figure 2.** *IL-1β levels in the PFC measured in control and, at 6 hours after surgery, respectively.* Surgery fails to significantly increase the levels of IL-1β found in the PFC 6 hours later. Results are mean ± SEM (n = 5).
ii. *BDNF levels in the hippocampus*

Hippocampal BDNF levels measured at 24 hours after surgery were consistently reduced in the animals subjected to surgery when compared to control animals (*p* < 0.05). Comparison of the total quantum of BDNF in animals of the surgery group with that in animals of the control group showed a reduction of BDNF levels in the surgery group of 35.43% (Figure 3).

![Figure 3. Surgery decreases BDNF levels in the hippocampus.](image)

Surgery decreases BDNF levels 24 hours after tibial fracture when compared to control (*p* < 0.05).
iii. Assessment of remote memory after peripheral inflammation

a. Contextual generalisation test

The inflammatory response to LPS injection and orthopaedic surgery, respectively, each impaired neocortical memory when assessed using the neocortical memory contextual generalisation protocol (Fig. 4). LPS-induced inflammation resulted in reduced freezing to Context B (74.63% ± 16.09), when compared to Context A (88.88% ± 6.10). This difference was significant. Surgery-induced inflammation resulted in reduced freezing to context A (73.88 % ± 17.73), and a further reduction in freezing to Context B (52.00 % ± 17.20). Again, this difference was significant. The freezing responses of control animals to Context A (88.88% ± 8.90 ) and B (81.75 % ± 9.22) was the same. In all cases p < 0.05.

![Figure 4. Contextual fear conditioning responses in a neocortical contextual generalisation test. LPS (100 µg/kg) injection induced 72 hours after the aversive event resulted in a significant reduction of freezing time in Context B when compared to Control. Tibia surgery performed 72 hours after the contextual fear-conditioning resulted in a significant reduction of freezing time to both Context A and Context B when compared to Control. Control did not differentiate between Context A and Context B. (In all cases * p < 0.05).](image-url)
b. Contextual differentiation test

The inflammatory response to LPS injection and orthopaedic surgery, respectively, in each case impaired neocortical memory when assessed using the memory contextual generalisation protocol (Fig. 5). LPS-induced inflammation impaired the ability to distinguish between contexts (40.75% ± 8.86 (Context A); 28.00% ± 5.90 (Context B) \( p > 0.05 \)). Likewise, surgery-induced inflammation impaired the ability to distinguish between Context A (74.88% ± 6.72) and Context B (56.13% ± 5.28) \( p > 0.05 \)). Surgery-induced inflammation also resulted in higher freezing levels to both contexts when compared to Control animals \( (p < 0.05) \). Control animals were able to distinguish between contexts (63.25% ± 6.56 (Context A); 35.50% ± 3.18 (Context B) \( p < 0.05 \)).

![Figure 5. Contextual fear conditioning responses in a neocortical contextual differentiation test](image)

Figure 5. Contextual fear conditioning responses in a neocortical contextual differentiation test. Only Control animals were able to discriminate between Context A and Context B. LPS injection administered 72 hours after the aversive event affected the discrimination ability. Tibia surgery performed 72 hours after the aversive event resulted in a significantly higher freezing levels and also impaired contextual discrimination. (In all cases * \( p < 0.05 \) for within groups, + \( p < 0.05 \) for between groups).
Discussion

Remote memory is memory for events long past. It is to be distinguished from hippocampal long-term memory. Memories of everyday life events depend initially on the hippocampus. However, as these memories mature, they are thought to become increasingly dependent on other brain regions such as the neocortex (Frankland and Bontempi, 2005). Remote memory is believed to be laid down in the medial prefrontal cortex (Quinn et al, 2008). Two tests have been proposed to determine whether there has been an impairment of remote memory. The first test was developed by Frankland and colleagues (2004) and involves training mice in a contextual fear-conditioning paradigm which results in a generalisation of the learnt aversive environment as the fear-memory gets older (Frankland et al, 2004; Wiltgen and Silva, 2007). The second test was developed by Wang and co-workers (2009) and involves repeated exposure to both a shock-associated context and a different, harmless, context, creating a clear discrimination of the hazardous environment even when recalling a remote memory (Wang et al, 2009). We have shown that inflammation affects contextual-fear memory in a generalisation protocol (81.75% for Control vs. 74.625% for LPS vs. 52% for Surgery) and in a discrimination protocol (35.5% for Control vs. 28.00% for LPS vs. 56.125 for Surgery). This leads us to offer the conclusion that remote memory in mouse is impaired by severe peripheral inflammation whether that inflammation is induced by peripheral LPS injection or by peripheral orthopaedic surgery.

High levels of IL-1β are known to impair long-term potentiation (LTP) and neurogenesis (Monje et al, 2003; Koo and Duman, 2008). IL-1β is the component of the inflammatory response which we have previously identified as being involved in impairment of hippocampal-dependent memory using the same model of peripheral orthopaedic surgery as that employed in the present experiment (Cibelli et al, 2010). Our results also demonstrate that peripheral orthopaedic surgery which results in impairment of remote memory in mouse also result in an increase in the level of IL-1β in the mouse hippocampus but fails to induce an increase of the same cytokine in the PFC. The failure to find an increase in the level of this cytokine in the PFC may appear, at first sight, anomalous, when the PFC has been identified has the site of remote memory impairment in our experiments. However, this finding is consistent
with the similarly limited effect of LPS injection on IL-1β increase in the hippocampus (Barrientos et al, 2009; Csölle and Sperlágh, 2010). Moreover, hippocampal increases in IL-1β levels appear to be a common response to peripheral inflammation of different contexts of origin.

The precise mechanisms whereby this surgery-induced inflammatory response results in these disease conditions have yet to be identified. However, BDNF has previously been identified with memory function (Barrientos et al, 2004). Direct injection BDNF into the hippocampus of mice enhances synaptic strength (Kang and Schuman, 1995). Moreover, contextual learning induces an increase in the number of hippocampal CA1 neurones expressing high levels of BDNF (Chen et al, 2007). Our findings in this study of reduced levels of BDNF in the hippocampus of mice subjected to surgery may account – at least in part – for how surgery-induced inflammation adversely impacts on hippocampal-dependent memory function in this mouse model of peripheral orthopaedic surgery.

In summary, we have demonstrated that peripheral orthopaedic surgery-induced inflammation results in impairment of remote memory as measured by two tests of contextual remote memory. Such impairment is not accompanied by an increase in IL-1β in the PFC. However, peripheral orthopaedic surgery reduces the level of BDNF in the hippocampus and this may contribute, in part, to the impairment in contextual-fear memory which may result from peripheral orthopaedic surgery.
Chapter 6

ORTHOPAEDIC SURGERY DOES NOT
INCREASE ANXIETY-LIKE BEHAVIOUR IN MOUSE

1. Introduction

2. Methods

3. Results
   
   i. Social interaction test

4. Discussion
Introduction

The validity of mouse models of human psychological conditions has been the subject of considerable discussion (Bućan and Abel, 2002; Bearden et al, 2009). Extreme caution has to be used in employing murine behavioural responses as predictors of human behaviour. We have investigated whether a mouse model of peripheral orthopaedic surgery, with that surgery’s concomitant inflammatory responses, is predictive of human anxiety responses to such surgery. Previous studies have shown that in humans anxiety tends to occur prior to surgery, but diminishes rapidly in the period following surgery (Stroobant and Vingerhoets, 2008; Rörtgen et al, 2010). Anxiety is known to affect cognitive performance and decision-making (Hindmarch, 1988; LeBlanc, 2009) and has been reported as a sequela of surgery in studies on post-operative cognitive dysfunction (POCD) (Moller et al, 1998; Rasmussen et al, 2001).

Here, we have employed the social interaction test (File and Seth, 2003) to investigate the murine anxiety response to peripheral orthopaedic surgery. The social interaction test involves the assessment of the animal’s post-stimulus exploratory behaviour by placing a foreign male mouse at one end of an arena and the subject mouse at the other end of the arena. The behavioural interaction of the post-stimulus subject animal with the foreign animal is measured and compared with the same interaction of a naïve subject animal (File and Hyde, 1978; File and Seth, 2003).

In this study, we demonstrate that peripheral orthopaedic surgery and its associated inflammatory responses fail to increase post-operative anxiety-like behaviour in mouse as assessed using this social interaction test.
Materials and Methods

Animals

C57BL/6 male mice, aged between 12-14 weeks (Harlan and Charles River, UK), were employed in these experiments. Upon arrival, the animals were housed in groups of four and allowed to acclimatise to their new environment for 7 days before being employed in experiments. Their environment was maintained in a 14:10 light-dark cycle, under constant temperature and humidity, with free access to food and water.

All the experiments were performed under Home Office License (PPL N.º 70/6104; PIL N.º 70/20834) and in compliance with the requirements of the Animals (Scientific Procedures) Act, 1986. Every effort was made to limit the number of animals employed in these experiments and to ensure that no unnecessary suffering was occasioned to the animals employed.

Social interaction test

The social interaction test arena comprised a plastic box 50×40 cm, with 15 cm walls, which was lit by low light (10 lux). A camera was mounted vertically above the arena. The time spent in social interaction (sniffing, following, grooming the partner, boxing and wrestling) provided the measure of anxiety and was scored by an observer who was blind to the treatment. The first group (surgery group) comprised mice upon which the surgery described below was performed 24 hours earlier. The second group (control group) comprised animals treated in a similar manner but on whom surgery had not been performed. While undergoing familiarisation, mice were placed individually, untreated in the test arena for a 5-min familiarisation trial at least 3 hours before the social interaction test. Two mice, of the same treatment condition, were placed simultaneously into the centre of the arena for 10 minutes and the time spent in social interaction was scored as a measure of anxiety. This consisted of specific behavioural patterns such as grooming, sniffing, following the partner, crawling under and boxing.
**Surgery**

The surgical procedure was conducted under general anaesthesia in aseptic conditions. The surgical procedure involved opening the animal’s left hind-paw by an incision, exposing the tibia, allowing the insertion of an intramedullary pin in the tibia. The tibia was then fractured at a single point in the lower third. The inserted intramedullary pin prevented collapse of the tibia and enabled the wound to be sutured. Isoflurane 1.5 MAC, corresponding to an air concentration of 2.1% and buprenorphine 0.1 mg/kg intra-peritoneally were given and maintained during 10 to 20 minutes of deep-anaesthesia. This procedure is described at length in Chapter 2 (Methods).

**Data analysis**

Data are expressed as mean ± SEM. All analyses for the Social Interaction Test were carried out using non-parametric statistics as the pairs of animals were scored as a unit resulting in an “n” of eight per group. Mann-Whitney U tests were used for pair-wise comparison of surgery and control groups. A p value < 0.05 was considered to be of statistical significant.
Results

i. Social interaction test

Table 1 shows the total number of interactions of both groups of 8 animal-pairs at successive 1-minute intervals over the 10-minute duration of the social interaction test. There is a small reduction in the number of interactions from the first minute to the tenth minute in the case of both groups which may reasonably be attributed to habituation (21.0 seconds ± 2.4 vs. 20.4 seconds ± 4.5 for the control group compared to 18.9 seconds ± 4.3 vs. 15.4 seconds ± 4.1 for the surgery group). However, this reduction decrease was not statistically significant in for either group ($p > 0.05$).

<table>
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<th>3</th>
<th>4</th>
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<td>(5.63)</td>
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<tr>
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<td>15.11</td>
<td>20.22</td>
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Table 1. Amount of interaction of both groups of 8 animal-pairs at successive 1-minute intervals over the 10-minute duration of the social interaction test. There is a decrease in the amount of social interaction throughout the test duration however this decreased failed to reach statistical significance.

Over the 10 minutes’ duration of the social interaction test, there was a continuous reduction in the extent of social interaction, measured from the first half to the second half of the period of analysis, in both groups (91.78 seconds ± 12.19 vs. 80.63 seconds ± 9.31 for the control group, 96.35 seconds ± 11.97 vs. 83.85 seconds ± 7.95 for the surgery group). However, this reduction was not statistically significant ($p > 0.05$). Despite the slightly higher levels of social interaction in the surgery group throughout the overall duration of the test, this was not statistically significant when compared to control ($p > 0.05$), (Figure 1).
Figure 1. **Social Interaction Test**: Mean ± SEM of the time(s) spent by pairs of mice in social interaction after i.p. injection of vehicle (Control), or 24 hours after undergoing surgery (Surgery). There is no statistically significant difference between groups.

The surgery group exhibited a slightly lower level of interaction during the first minute when compared to the control group. However, this difference failed to reach statistical significance (control 21.03 seconds ± 2.38 vs. surgery 18.97 seconds ± 4.27) ($p > 0.05$), (Figure 2).

Figure 2. **Analysis of the first minute of the social interaction test**: Mean ± SEM of the time(s) spent by pairs of mice in social interaction during the first minute of contact. There is no statistically significant difference between groups.

We conclude that under the conditions presented in the social interaction test results suggest that surgery does not increase anxiety in this animal model.
**Discussion**

We have previously demonstrated that peripheral orthopaedic surgery in mouse results in an inflammatory response, with plasma interleukin(IL)-1β and IL-6 peaking at 6 hours after surgery, and remaining over basal levels at 24 hours after surgery (Cibelli et al, 2010). In addition, hippocampal levels of IL-1β and IL-6 are increased above basal levels at 6 hours post-surgery (Cibelli et al, 2010). Moreover, the neuroinflammatory response was associated with hippocampal microgliosis up to 72 hours post-surgery (Cibelli et al, 2010). When assessed behaviourally, mice subjected to surgery displayed hippocampal-dependent memory deficits in fear-conditioning tests to both context and tone tests (Cibelli et al, 2010)

Our results in the present experiment demonstrate that the same type of peripheral orthopaedic surgery fails to increase anxiety-like behaviour in mouse as assessed using the social interaction test. Anxiety is a normal reaction to a stressor of either a psychological or physical nature (Kring et al, 2007). It depends on limbic system activation of the hypothalamic–pituitary–adrenal axis and sympathetic nervous system which aims to result in a compensatory physiological change (García-Bueno et al 2008). However, a sustained activation of a stress response tends to result in physiological and psychological damage (García-Bueno et al 2008). Due to the high-order cognitive functions possessed by humans the availability of (psychological) stressors is immensely greater in humans than in mice. Post-operative anxiety in humans is well documented (Rasmussen et al, 2001; Rudolph et al, 2010). We have shown that it is not a trait possessed by mouse, no doubt because the creatures do not possess the human abilities of hindsight and foresight. This point is of importance not only because it demonstrates the inadequacy of mouse models for predicting anxiety in humans but, in addition, confirms that there are no anxiety states resulting from surgery to these animals which may confound the results of further post-surgery experiments with these animals.
Chapter 7

SURGERY-INDUCED INFLAMMATION DOES NOT AFFECT
OLFACTORY DISCRIMINATION IN MOUSE

1. Introduction

2. Methods

3. Results
   i. Olfactory habituation/dishabituation task

4. Discussion
Introduction

Establishing memory in respect of a past event is crucial for survival. Olfactory learning has been employed as an experimental model in offspring recognition (Kendrick et al, 1997); mate recognition in voles (Young et al, 2001); social recognition (Winslow and Camacho, 1995); and social transmission of food preference (Galef et al, 1994). The olfactory memory neural circuit is dependent on the olfactory bulb, hypothalamus, hippocampus and insular cortex (Kendrick et al, 1997). Olfactory learning shares the following features with human declarative memory: single trial learning; rapid encoding; long-term memory; and high storage capacity (Sternberg, 2009). Human declarative memory is the aspect of human memory that stores facts or elements that can be consciously discussed, i.e., declared.

Wilson and colleagues (2009) have demonstrated that patients with prodromal dementia have an impaired olfactory discrimination (Wilson et al, 2009). A low Brief Smell Identification Test (BSIT) score was associated with more rapid decline of episodic memory and with an increased risk of developing mild cognitive impairment.

Olfactory discrimination tests are also available for animals (Winslow and Camacho, 1995; Dluzen, 1993). Winslow and Camacho developed the habituation/dishabituation paradigm (Winslow and Camacho, 1995; Dluzen, 1993), which offers a model for examining olfactory social recognition memory. Social recognition has been inferred from a decline in olfactory investigation of conspecific (i.e., same species) intruders during repeated or protracted confrontation with a resident rat. The decline in investigation corresponds to criteria for habituation (Winslow and Camacho, 1995).

The purpose of the following experiments was to determine whether surgery induced inflammation affects olfactory social memory.
Methods

Animals

C57BL/6 male mice, aged between 12-14 weeks (Harlan and Charles River, UK), were employed in these experiments. Upon arrival, the animals were housed in groups of four and allowed to acclimatise to their new environment for 7 days before being employed in experiments. Their environment was maintained in a 14:10 light-dark cycle, under constant temperature and humidity, with free access to food and water. All the experiments were performed under Home Office License (PPL N.º 70/6104; PIL N.º 70/20834) and in compliance with the requirements of the Animals (Scientific Procedures) Act, 1986. Every effort was made to limit the number of animals employed in these experiments and to ensure that no unnecessary suffering was occasioned to the animals employed.

Habituation/Dishabituation Olfactory Task

This test consisted of 4, 2-minute-long trials, with 10-minute intervals, of the test mouse to an anaesthetised stimulus mouse in a neutral cage. At the 5th trial, a new novel (anaesthetised) mouse stimulus was then presented. After a retention interval of 24 hours, animals undergoing testing were re-exposed to the frequently presented anaesthetised mouse (Trial A) and subsequently to an additional new (anaesthetised) stimulus mouse (Trial B). A trained observer, blinded to the treatment, measured the duration of the investigatory behavior of each stimulus animal separately. This procedure is described at length elsewhere in the Methods chapter.

Surgery

Mice were anaesthetised by isoflurane 1.5 MAC, corresponding to an air concentration of 2.1%. Buprenorphine 0.1 mg/kg was given intra-peritoneally for analgesia. Both were given and maintained during 10 to 15 minutes of deep-anaesthesia. The surgical procedure essentially involved opening the animal’s left hind paw by an incision, which exposed the tibia and allowed the insertion of an intramedullary pin in the tibia. The tibia was then fractured at a single point. However, the pin, previously inserted, prevented collapse of the tibia and enabled the wound to be sutured. This procedure is described at length in Chapter 2 (Methods).
Data analysis

Data are expressed as mean ± SEM. The investigation times of the four successive habituation trials (and the fifth dishabituation trial) were compared using a 2-way ANOVA, with factors (GROUP and TRIAL). The investigation time-period for the 24-hour memory test were compared using a paired t-test. A $p < 0.05$ was considered to be of statistical significance.
Results

i. Olfactory habituation/dishabituation task

The data suggest that social recognition is intact in both naive and surgical animals in all trials within that period of assessment (see Figure 1).

Figure 1. Habituation/Dishabituation Task: Control mice (n=8) and mice which underwent surgery 1 day before (n=8) were placed in an arena with an anaesthetised stimulus mouse. Both groups were presented with a stimulus animal four consecutive times, with a new stimulus mouse being presented at Trial 5. Twenty-four hours later the frequently presented animal was reintroduced along with a new novel stimulus mouse. There was no statistical difference between mice undergoing surgery under general anaesthesia and naïve mice at Trial 4. This suggests that surgery does not impair social recognition. Data are expressed as mean ± SEM. * p <0.05

No significant statistical difference is found in exploratory behaviour between control and surgery groups at Trial 1 (37.0 sec. ± 4.12 for control group vs. 38.5 sec. ± 6.80 for the surgery group) (see Figure 1). When Trial 2 is compared with Trial 1, there is a significant reduction in the exploratory behaviour of both naïve and surgery groups across those trials (37.0 sec. ± 4.12 for control in Trial 1 vs. 22.5 sec. ± 3.92 for Trial 2; and 38.5 sec. ± 6.80 at Trial 1 vs. 20 sec. ± 3.04 for Trial 2 for the surgery group). When the results of Trial 2 and Trial 3 are compared, no statistically
significant difference is found in the naïve and surgery groups across these trials (22.5 sec. ± 3.92 for control in Trial 2 vs. 17.5 ± 3.92 for Trial 3; and 20 sec. ± 3.04 at Trial 2 vs. 9.5 sec. ± 1.20 for Trial 3 for the surgery group). When the results of Trial 3 and Trial 4 are compared, no statistically significant difference is found in the naïve and surgery groups across these trials (17.5 ± 3.92 for control in Trial 3 vs. 10.5 sec. ± 1.99 Trial 4; and 9.5 sec. ± 1.20 at Trial 3 vs. 11.5 sec. ± 2.10 for Trial 4 for the surgery group). Finally, when the results of Trials 4 and 5 are compared (see Figure 1), a statistically significant increase in exploratory behaviour is shown by the latter naïve group when compared with the same group from Trial 4 (10.5 sec. ± 1.99 in Trial 4 vs. 22.0 sec. ± 3.78 Trial 5). A similar pattern is observed in the surgery group across these trials (11.5 sec. ± 2.10 at Trial 4 vs. 26.5 sec. ± 4.47 for Trial 5). In all cases $p < 0.05$.

In the Test Trial performed 24 hours later (see Figure 1), we found that control animals exhibited an increase in exploratory behaviour in respect to the once only presented stimulus animal when compared to their behaviour to the frequently presented stimulus animal (12.0 sec. ± 1.51 in Trial A vs. 21.5 sec. ± 2.26 in Trial B). A similar pattern was observed in the surgery animals in the same context (8.5 sec. ± 2.87 in Trial A vs. 15.5 sec. ± 2.56 in Trial B). In all cases $p < 0.05$. 
Discussion

The results obtained demonstrate that there is no significant difference between the behaviour measured by the olfactory discrimination task, between control and surgery groups, at day 1 after surgery. Cibelli and colleagues (2010) reported that tibial surgery is associated with hippocampal microgliosis (Cibelli et al, 2010). IL-1β was found to be elevated in the hippocampal structure after tibial surgery (Cibelli et al, 2010). This surgery-derived neuroinflammation resulted in reduced freezing to both context and tone tests in fear-conditioning protocols (Cibelli et al, 2010).

Previous studies have demonstrated that neuroinflammation, as a result of LPS central infusion into the 4th ventricle, is associated with an increased activation of astrocytes and reactive microglia distributed throughout the brain (Hauss-Wegrzyniak et al, 2000). The greatest inflammatory response after LPS central injection has been reported to occur within the cingulate gyrus and temporal lobe, particularly the hippocampus (Hauss-Wegrzyniak et al, 2000). Again, LPS induced-neuroinflammation was associated with an increase in IL-1β and TNF-α mRNA levels, particularly within the hippocampus (Hauss-Wegrzyniak et al, 2000). Quantitative volumetric measurements by high-resolution MRI revealed a 13% and 9% decrease in the volume of the hippocampus and temporal lobe region, respectively (Hauss-Wegrzyniak et al, 2000). In the behavioural tasks performed by Hauss-Wegrzyniak and colleagues (2000) LPS induced neuroinflammation was associated with a significant deficit in spatial memory, while object recognition ability was unaffected (Hauss-Wegrzyniak et al, 2000). This indicates, that although neuroinflammation has a widespread impact in the brain, it produces distinct identifiable cognitive deficits. This may offer a plausible explanation of the differential impact of surgery-induced inflammation in cognitive processes in our mouse model.

In summary, these findings indicate that peripheral orthopaedic surgery has no effect on the neural pathways which underlie the olfactory discrimination task and, as such, it appears that inflammation derived from peripheral orthopaedic surgery affects mainly the neural circuitry underlying fear-conditioning tasks.
Chapter 8

SYSTEMIC INFLAMMATION ENHANCES SURGERY-INDUCED COGNITIVE DYSFUNCTION IN MOUSE

1. Introduction

2. Methods

3. Results
   
   i. Contextual fear conditioning responses after LPS-induced inflammation
   
   ii. Contextual fear conditioning responses after LPS-induced infection followed by surgery
   
   iii. IL-1β levels in the plasma measured at 24 hours after infection, surgery, and infection followed by surgery
   
   iv. IL-1β levels in the hippocampus measured at 6 hours after infection, surgery, and infection followed by surgery

4. Discussion
Introduction

Post-operative cognitive dysfunction (POCD) is increasingly being recognised as a complication of surgery, especially in the elderly (Bekker and Weeks, 2003). Although the mechanisms by which POCD develops remain unclear, the contribution of several risk factors, including advanced age, prolonged duration of surgery, as well as postoperative respiratory and infectious complications have been identified (Moller et al, 1998).

Recent studies reveal that surgery can trigger systemic and hippocampal inflammation resulting in cognitive decline (Wan et al, 2007; Rosczyk et al, 2008; Cibelli et al, 2010). Systemic infection increases the level of pro-inflammatory cytokines in the brain (Barrientos et al, 2009) and is capable of impairing consolidation of memory in rats (Barrientos et al, 2002; 2009). IL-1β is one of the more important of the inflammatory mediators which contribute to the development of this inflammatory response within the brain (Barrientos et al, 2002; 2009; Rosczyk et al, 2008; Cibelli et al, 2010). IL-1β contributes to the inflammatory response to both infection and injury (Barrientos et al, 2009; Cibelli et al, 2010) and exerts local, concentration-dependent, effects on hippocampal memory function (Barrientos et al, 2002; 2009). Elevated levels of IL-1β in the brain have been shown to impair hippocampal LTP (Vereker et al, 2000). LPS is known to trigger a powerful immune response (Fidalgo et al, 2011a). Sensitising the immune system with a sub-clinical dose of LPS can increase the pro-inflammatory response to a subsequent challenge with LPS (O’Dea, 2009).

We hypothesised that sub-clinical infection (induced by LPS challenge), which sensitises the immune system, may exacerbate peripheral surgery-induced impairment of memory. We further hypothesised that a sensitised immune system responding to sub-clinical infection should exhibit increased IL-1β levels which would be further increased by performing peripheral orthopaedic surgery on the subject animal. To investigate these hypotheses, we examined the extent to which pre-surgery induced LPS infection may aggravate impairment of memory function and hippocampal IL-1β levels following orthopaedic surgery.
Methods

Animals

In all, 112 male mice (C57BL/6), aged between 12-14 weeks (Harlan and Charles River, UK), were employed in these experiments. Upon arrival, the animals were housed in groups of 4 and allowed to acclimatise to their new environment for 7 days before being employed in experiments. Their environment was maintained in a 14:10 light-dark cycle, under constant temperature and humidity, with free access to food and water. All the experiments were performed in compliance with the requirements of the Animals (Scientific Procedures) Act, 1986 and under United Kingdom Home Office approved License (PPL N.º 70/6104; PIL N.º 70/20834). Every effort was made to limit the number of animals employed in these experiments and to ensure that no unnecessary suffering was occasioned to the animals employed.

Fear-Conditioning

In order to draw an LPS dose- and time-response curve, six groups of mice (n = 6 per group, experiment 1), underwent fear conditioning training and subsequently received LPS administration at different time points. Contextual fear responses were assessed at day 7.

Mice were transported to the behavioural assessment room and left in their cages for 15 minutes before starting. Training consisted of placing the subject mouse in a chamber (Med Associates Inc. St. Albans, VT, USA) and allowing exploration for 180 seconds. A single 2 second footshock (0.85 mAmp) was administered after 180 seconds. The mice were removed from the chamber 60 seconds after this footshock.

Mice received a single injection of LPS (LPS ultra pure, Sigma Chemical, St. Louis, MO, USA) administered intraperitoneally (i.p.) as follows. An 50 µg/kg injection was given to separate groups of mice at the following time-points: immediately after fear-conditioning; and 24 hours after fear-conditioning. An 100 µg/kg LPS injection was given to separate groups of mice: immediately after fear-conditioning; at 24 hours after fear-conditioning; and at 48 hours after fear-conditioning. Finally, a control group was given an injection of saline immediately
after fear-conditioning took place. These LPS doses were determined by pilot studies which assessed their dose-dependent effect on memory consolidation. At the end of the trial, each mouse was taken individually to its home cage. At day 7, mice were returned to the training environment for 240 seconds and their freezing response to the context was recorded by Video Freeze software (Med Associates Inc. St. Albans, VT, USA) at 0.5 second intervals.

In the LPS + Surgery experiment, designed to study the impact of a sub-clinical dose of LPS given prior to surgery, four groups of mice (n = 8 per group), underwent contextual fear conditioning training and 24 hours later underwent surgery, LPS injection and LPS and surgery (2 Hit). Contextual fear responses were assessed at day 4.

Contextual-fear conditioning is a well recognised and accepted behavioural paradigm for studying the effect of cytokines on memory (Barrientos et al, 2002; 2009; Cibelli et al, 2010).

Surgery

Surgery was conducted under general anaesthesia under aseptic conditions. The surgical procedure involved opening the animal’s left hind-paw by an incision, exposing the tibia, allowing the insertion of an intramedullary pin in the tibia. The tibia was then fractured at a single point in the lower third. The inserted intramedullary pin prevented collapse of the tibia and enabled the wound to be sutured. Isoflurane 1.5 MAC, corresponding to an air concentration of 2.1% and buprenorphine 0.1 mg/kg intra-peritoneal (i.p.) were given and maintained during 10 to 20 minutes of deep-anaesthesia. This procedure is described at greater detail in Chapter 2 (Methods). The surgery group and the 2 Hit groups each underwent the same surgical procedure. However, the 2 Hit group received a 50 ng/kg i.p. injection of LPS 2 hours before undergoing surgery.
Cytokine assessment

IL-1β was measured in the plasma and in the hippocampus. Four groups of mice ($n = 4$ per group) were sacrificed 6 hours after experimental manipulation for hippocampal harvesting. Then, a further 4 groups of mice ($n = 4$ per group) were sacrificed 24 hours after experimental manipulation for blood sampling. At both time-points the groups comprise: control, surgery, LPS (50 ng/kg) and LPS followed by Surgery (2 Hit). The extended period of 24 hours for measurements of IL-1β levels in the plasma (as opposed to 6 hours for the like measurement in the hippocampus) was adopted to accommodate other experimental work. Previous data in our laboratory shows that there is no significant difference in the levels of this cytokine in the plasma when measured at 6 hours and 24 hours post this peripheral orthopaedic surgery (Cibelli et al, 2010). Blood was collected through thoracotomy with cardiac blood withdrawn under deep isoflurane anaesthesia. Blood samples were collected in pre-heparinised Eppendorf tubes and centrifuged at 21,000g for 10 minutes at 4°C. For hippocampal samples, animals were rapidly decapitated after being perfused with a saline solution and brains quickly removed. All dissections were performed on an ice-cold frosted glass plate and tissues quickly frozen in liquid nitrogen. Tissue samples were stored at -80°C until the time of sonication. This procedure was performed always at the same time of day to minimize the circadian variation (between 10:00 to 12:00). Hippocampal or plasma samples were aliquotted in Eppendorf tubes and stored at -20°C until analysis. IL-1β in the hippocampus and blood was measured as previously described using ELISA (Bender MedSystems, Austria) (Nguyen et al, 1998) and normalised to total protein levels.

Data analysis

All data are expressed as mean ± SEM and analysed by analysis of variance (ANOVA) followed by Bonferroni post-hoc test. A $p$ value < 0.05 was considered to be of statistical significance.
Results

i. Contextual fear conditioning responses after LPS-induced inflammation

LPS-induced inflammation results in a dose- and time-dependent memory deficit, as evidenced by reduced freezing levels in the LPS-treated animals as compared to those in the control group (Figure 1). The control animals were found to freeze longer than LPS injected animals (47.33% ± 2.75 for control, \( p < 0.05 \)). LPS (50 and 100 \( \mu \text{g/kg} \)) injected immediately after contextual fear-conditioning, significantly reduced the animal’s recollection of the aversive event when measured 7 days later. No significant difference was found between the extent of the impairment induced by the 100 \( \mu \text{g/kg} \) dose compared to the 50 \( \mu \text{g/kg} \) dose (23% ± 3.48 for LPS 50\( \mu \text{g/kg} \) 0d \( \text{vs.} \) 24% ± 4.78, and LPS 100 \( \mu \text{g/kg} \) 0d, \( p > 0.05 \)). However, when the same doses were administered 24 hours after training, only animals injected with LPS 100 \( \mu \text{g/kg} \) showed a level of impairment in cognitive function, which was significantly different from controls (47.33% ± 2.75 for control \( \text{vs.} \) 26% ± 3.13 for LPS 100 \( \mu \text{g/kg} \) 1d, \( p < 0.05 \)). LPS injected 48 hours after the aversive event failed to affect memory (47.33% ± 2.75 for control \( \text{vs.} \) 44.33% ± 4.45 for LPS 100 \( \mu \text{g/kg} \) 2d, \( p > 0.05 \)), presumably because the consolidation process of memory is completed long before the infection occurs. Our data corroborate previous findings that inflammation within the brain impairs memory processes resulting in reduced freezing response (Barrientos et al, 2002; 2009).
Figure 1. Contextual fear conditioning responses after LPS-induced inflammation. LPS (50 and 100 µg/kg) given immediately after contextual fear conditioning resulted in a reduction of freezing time, when compared to control group. The administration of 100 µg/kg of LPS twenty-four hour after contextual fear conditioning also resulted in a marked reduction of freezing time when compared to control group. No statistically significant difference in freezing was found between the control group and either the group that received 50 µg/kg of LPS at 24 hours after contextual fear conditioning, or the group that received 100 µg/kg at the expiry of 48 hours after contextual fear conditioning. Results are mean ± SEM (n = 6). * p < 0.05 vs control.
**ii. Contextual fear conditioning responses after LPS-induced infection followed by surgery**

Sub-clinical infection induced by LPS (50 ng/kg) at 24 hours after the aversive event failed to significantly affect freezing time when compared to control animals (44% ± 4.24 for control vs. 47.63% ± 3.01 for LPS 50ng/kg, \( p > 0.05 \)). Orthopaedic surgery performed 24 hours after the training also failed to significantly affect freezing time when compared to control animals (44% ± 4.24 for control vs. 46.25% ± 3.18 for surgery, \( p > 0.05 \)). However, LPS (50 ng/kg) injected 22 hours after training and followed by orthopaedic surgery 2 hours later, resulted in a significant reduction of freezing time when compared with controls (44% ± 4.24 for control vs. 31.25% ± 4.88 for 2 Hit, \( p < 0.05 \)) (Figure 2). We thus confirmed our first hypothesis as only the 2 Hit group animals displayed memory impairment.

![Figure 2](image_url)

**Figure 2. Contextual fear conditioning responses after LPS-induced infection followed by surgery.** Only LPS (50 ng/kg) infection induced at 22 hours after the aversive event, and followed by orthopaedic injury and surgery 2 hours later, resulted in a significant reduction of freezing time when compared with controls. Results are mean ± SEM (n = 8). * \( p < 0.05 \) vs control.
iii. IL-1β levels in the plasma measured at 24 hours after infection, surgery, and infection followed by surgery

Sub-clinical infection of mice with LPS (50 ng/kg) did not cause increased IL-1β levels in plasma measured at 24 hours ($p > 0.05$). Orthopaedic surgery significantly increased the level of IL-1β present in the serum of mice measured at 24 hours post-surgery (2 pg/ml ± 0.41 for control vs. 44.45 pg/ml ± 7.04 for surgery, $p < 0.05$). An additional significant increase in the level of IL-1β levels was found in plasma when orthopaedic surgery was performed in animals which were given LPS (50 ng/kg) 2 hours previously, demonstrating that sub-clinical infection exacerbated the level of IL-1β found in the serum after orthopaedic surgery (44.45 pg/ml ± 7.04 for surgery vs. 57.13 pg/ml ± 3.04 for 2 Hit, $p < 0.05$) (Figure 3).

**Figure 3.** IL-1β levels in the plasma measured at 24 hours after infection, surgery, and infection followed by surgery, respectively. Surgery significantly increased the levels of IL-1β found in the plasma 24 hours later. An additional significant increase in the level of IL-1β levels occurred when surgery was performed in mice infected with LPS (50 ng/kg) 2 hours previously. Results are mean ± SEM (n = 4). * $p < 0.05$ vs control.
iv. IL-1β levels in the hippocampi measured at 6 hours after infection, surgery, and infection followed by surgery

Sub-clinical infection of mice with LPS (50 ng/kg) failed to increase IL-1β levels in the hippocampus of injected animals measured at 6 hours ($p > 0.05$). Orthopaedic surgery significantly increased the level of IL-1β present in the hippocampus of mice measured at 6 hours post-surgery (1.26 pg/mg ± 0.11 for control vs. 6.11 pg/mg ± 0.56 for surgery, $p < 0.05$). An additional significant increase in the level of IL-1β levels was found in the hippocampus when orthopaedic surgery was performed in animals which had been given LPS (50 ng/kg) 2 hours previously (6.11 pg/mg ± 0.56 for surgery vs. 9.32 pg/mg ± 0.86 for 2 Hit, $p < 0.05$), demonstrating that sub-clinical infection increases the level of IL-1β found in the hippocampus after orthopaedic surgery (Figure 4).

We thus confirmed our second hypothesis as an LPS injection prior to surgery resulted in higher levels of IL-1β in the plasma and in the hippocampus, which exacerbated surgery-induced cognitive dysfunction at a time-point at which surgery alone did not result in a fear-conditioning memory deficit.

![Image](image_url)

**Figure 4.** IL-1β levels in the hippocampus measured at 6 hours after infection, surgery, and infection followed by surgery, respectively. Surgery significantly increased the levels of IL-1β found in the hippocampus 6 hours later. An additional significant increase in the level of IL-1β levels occurred when surgery was performed in mice infected with LPS (50 ng/kg) 2 hours previously. Results are mean ± SEM (n = 4); * $p < 0.05$ vs control.
Discussion

We have shown that sub-clinical systemic inflammation induced by injection of LPS (50 ng/kg) does not impair the consolidation of memory in mice. We have confirmed previous findings (Cibelli et al, 2010) that surgery impairs cognitive function in mice and is accompanied by a significant increase in the level of IL-1β in the plasma and in the hippocampus (Cibelli et al, 2010). We now show that pre-existing sub-clinical inflammation in animals which are subsequently subjected to surgery is capable of exacerbating the impairment of cognitive function resulting from surgery and is accompanied by a significantly increase in the levels of IL-1β found in plasma and in the hippocampus beyond the levels occasioned by surgery.

Insult to the hippocampus results in memory loss (retrograde amnesia) and an inability to form new memories (anterograde amnesia). This memory loss occurs in a time-specific manner, with more recent memory being more severely affected than older memory, which is consistent with a limited role for the hippocampus in memory storage (Barrientos et al, 2002; 2009; Cibelli et al, 2010). Barrientos and colleagues found that microinjection of IL-1β into the dorsal hippocampus impairs the consolidation processes that are required to contextualise memory storage (Barrientos et al, 2002). Hippocampal microgliosis has been described after LPS injection and tibial orthopaedic surgery (Cibelli et al, 2010). Activated microglia are known to produce reactive oxygen species, cytokines and other neurotoxic substances (Barrientos, 2009; Cibelli et al, 2010). It is also likely that microglial cells are responsible for local production of IL-1β after surgery (Cibelli et al, 2010). IL-1β and activated microglia have been demonstrated to impair LTP (Vereker et al, 2000; Griffin et al, 2006) which is associated closely with memory formation (Barrientos et al, 2002; 2009). Concurrently, inflammatory disruption of hippocampal LTP has been hypothesised as underlying the cognitive deficits seen in the aftermath of IL-1β or *E.coli* injections (Barrientos et al, 2002; 2009).

Sub-clinical injection of LPS may sensitize monocytes and microglial cells to the immunological responses trigged by the subsequent orthopaedic surgery. The International Study on Post-Operative Cognitive Dysfunction (ISPOCD) (Moller et al,
1998) identified post-operative infection as a risk factor for the development of POCD. In our study, we induced sub-clinical inflammation by administering a dose of LPS prior to surgery and found that this resulted in an exacerbated inflammatory response. This is particularly relevant because mice and rats are quite resistant to infective processes, even after splenectomy complicated by faecal contamination (Kestering et al, 2005), and it may well be that bacterial infection, when complicated by the inflammatory response induced by peripheral orthopaedic surgery, may present greater risks of an adverse outcome in humans than in mice.

In summary, the current work shows that LPS-induced inflammation disrupted hippocampal memory consolidation as evidenced by reduced contextual freezing time exhibited by infected mice. Likewise, surgery caused hippocampal-dependent memory impairment, which was accompanied by increased levels of IL-1β both in plasma and hippocampus. Although, a sub-pyrogenic dose of LPS alone failed to impair memory function, still this same dose of LPS, when administered prior to surgery, exacerbated surgery-induced cognitive dysfunction as evidenced by further reduction of contextual freezing time. Also, it caused a concomitant additional increase in the levels of IL-1β in both plasma and hippocampus of those animals. Our data suggest that sub-clinical infection may sensitise the immune system augmenting the severity of post-operative cognitive dysfunction.
Chapter 9

SUMMARY OF RESULTS AND CONCLUSION
The effects of peripheral surgery-induced inflammation and the role of the pro-inflammatory cytokine interleukin 1-beta (IL-1β) on cognitive function in mouse in several different contexts have been explored in this dissertation.

Contextual fear-conditioning experiments were designed to determine the impact of isoflurane-induced anaesthesia and LPS, respectively, in the development of anterograde amnesia and retrograde amnesia. My findings indicate that LPS-induced inflammation, but not isoflurane-induced anaesthesia, results in memory impairment in mouse. This memory impairment causes a permanent retrograde amnesia. Blocking the action of IL-1β reduces the hippocampal memory deficit induced by LPS (Chapter 3). These findings are important because they suggest that inflammation, rather than anaesthesia, is responsible for surgery-induced cognitive dysfunction (Cibelli et al, 2010).

I found that there is resultant inflammation in the mouse brain and cognitive impairment in the mouse in our model of surgery-induced peripheral inflammation. Moreover, such surgery is associated with increased levels of IL-1β in the plasma and in the hippocampus. Surgery also induces hippocampal microgliosis without being associated with an increase in apoptosis. Injection of an IL1-ra results in reduced microgliosis and reduced IL-1β levels in the plasma and in the hippocampus. Although surgery did not affect the acquisition in the spatial reference memory task, nevertheless, surgery animals spent less time in the target quadrant in the probe trial. In fear conditioning tasks, mice in the surgery group displayed memory impairment, which was reduced to normal levels with the injection of an IL1-ra before surgery. Injection of an IL1-ra alone causes a memory deficit in mouse which is consistent with a role for IL-1β in POCD (Chapter 4). Consistent with this finding, the IL1-β response to peripheral orthopaedic surgery is attenuated by an IL1-ra which also reduces inflammation-induced cognitive dysfunction (Cibelli et al, 2010). Notwithstanding the fact that this finding identifies a therapeutic target, caution is advised when considering the use of an IL1-ra due to its own effect on hippocampal memory.
I employed two tests of contextual remote memory to demonstrate that the inflammatory response to surgical insult also results in impairment of remote (prefrontal cortex (PFC)) localised memory. Unlike the hippocampus, such impairment is not accompanied by an increase in IL-1β in the PFC. Peripheral orthopaedic surgery results in reductions in the level of hippocampal brain derived neurotrophic factor (BDNF) and this may contribute, in part, to the impairment of memory found after such surgery (Chapter 5). This is the first demonstration that the inflammatory response initiated by LPS and peripheral orthopaedic surgery also results in impairment of pre-frontal cortex localised remote memory (Fidalgo et al, 2011b).

Next, using the social interaction test to examine the murine anxiety response to peripheral orthopaedic surgery, I found that, under those conditions, peripheral orthopaedic surgery does not increase anxiety in our animal model (Chapter 6). In addition, using the olfactory habituation-dishabituation task to investigate murine olfactory memory after peripheral orthopaedic surgery, I found that, under the conditions presented in that task, peripheral orthopaedic surgery does not affect olfactory memory in the animal model employed (Chapter 7). Thus, I thereby provided the first evidence that surgery-induced inflammation is not associated with an increase in anxiety-like behaviour (Fidalgo et al, 2011b) or, impairment of olfactory memory in mouse which allowed us to offer the conclusion that surgery-induced inflammation affects mainly the neural networks which underlie hippocampal-dependent memory (Cibelli et al, 2010).

Finally, I studied the extent to which LPS-induced inflammation may aggravate impairment of memory function following orthopaedic surgery using fear-conditioning tasks, while IL-1β levels in plasma and hippocampus were measured using ELISA. LPS-induced inflammation disrupts hippocampal memory consolidation as evidenced by reduced contextual freezing time exhibited by treated mice. Likewise, surgery causes hippocampal-dependent memory impairment, which is associated with increased levels of IL-1β both in plasma and hippocampus. However, a sub-pyrogenic dose of LPS alone fails to impair memory function. This dose of LPS, when administered prior to surgery, exacerbates surgery-induced cognitive dysfunction as evidenced by further reduction of contextual freezing time.
Also, it causes a concomitant additional increase in the levels of IL-1β in both plasma and hippocampus of those animals. I found that sub-clinical inflammation may sensitise the immune system augmenting the severity of post-operative cognitive dysfunction (Chapter 8). The International Study on Post-Operative Cognitive Dysfunction (ISPOCD) identified post-operative infection as a risk factor for the development of POCD (Moller et al, 1998). I have provided the first demonstration that a sub-clinical dose of LPS can sensitise the immune system exacerbating surgery-induced cognitive dysfunction in mice.
REFERENCES


- de Angelis L, File SE (1979) Acute and chronic effects of three benzodiazepines in the social interaction anxiety test in mice. Psychopharmacology (Berl.) 64:127-129


- Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J. Physiol. (Lond.) 232:331-356


- Chen J, Kitanishi T, Ikeda T, Matsuki N, Yamada MK (2007) Contextual learning induces an increase in the number of hippocampal CA1 neurons expressing high levels of BDNF. *Neurobiol Learn Mem* 88:409-415


brain-derived neurotrophic factor and impairs remote memory in mouse. *Neuroscience* 190: 194-190.


- File SE, Hyde JR, MacLeod NK (1979) 5,7-dihydroxytryptamine lesions of dorsal and median raphé nuclei and performance in the social interaction test of anxiety and in a home-cage aggression test. *J Affect Disord* 1:115-122


- Niesink RJ, Van Ree JM (1982) Antidepressant drugs normalize the increased social behaviour of pairs of male rats induced by short term isolation. *Neuropsychopharmacology* 21:1343-1348

monocytes during subclinical endotoxemia predisposes the lungs toward acute injury. *J. Immunol* 182:1155-1166


- Price CC, Garvan CW, Monk TG (2008) Type and severity of cognitive decline in older adults after noncardiac surgery. *Anesthesiology* 108:8-17


- Savage GH (1887) Insanity following the Use of Anaesthetics in Operations. *BMJ* 2:1199-1200


- de Toledo L (1971) Changes in heart rate during conditioned suppression in rats as a function of US intensity and type of CS. *J Comp Physiol Psychol* 77:528-538


