The Effects of Oestrogen and Progesterone on Outcome Following Experimental Traumatic Brain Injury in Rats

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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Statement of Access

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Christine O'Connor

Dedication

To my parents, Ursula and John O'Connor, who instilled in me an inexhaustible curiosity for life and encouraged me to believe I could achieve whatever I set my mind to.

And

To Pat MacGinley, who is my abiding companion and loving partner and who has always encouraged me to believe in myself.

Published Papers

The following articles have been published or accepted for publication during the period of PhD candidature, and sections of these articles have been included in the present thesis.

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Cernak I, **O'Connor CA**, Vink R (2001) Activation of cycloxygenase-2 contributes to motor and cognitive dysfunction following diffuse TBI in rats. Clin. Exp. Pharmacol. Physiol. 28, 922–925.

Cernak I, **O'Connor CA**, Vink R (2002) Inhibition of cycloxygenase-2 improves cognitive outcome more than motor outcome following diffuse TBI in rats. Exp. Brain Res. 147, 193–199.

Vink R, **O'Connor CA**, Nimmo AJ, Heath DL (2003) Magnesium attenuates persistent functional deficits following diffuse TBI in rats. Neurosci. Lett. 336, 41–44.

O'Connor CA, Cernak I, Vink R (2003) Interaction between anaesthesia, gender and functional outcome task following diffuse TBI in rats. J. Neurotrauma 20, 533–541.

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Vink R, Young A, Bennett CJ, Hu X, **O'Connor CA**, Cernak I, Nimmo AJ (2003) Neuropeptide release influences brain oedema formation after diffuse TBI. Acta Neurochir. (Suppl) 86, 257–260.

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O'Connor CA, Cernak I, Vink R (2004) Both oestrogen and progesterone improve functional outcome after diffuse TBI in female and male animals. J. Neurotrauma, in press.

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In conclusion I respectfully acknowledge that the sacrifice of rats was central to this body of research.

Abbreviations

AIS	Abbreviated Injury Scale
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
APP	Amyloid Precursor Protein
ATP	Adenosine Triphosphate
BBB	Blood–Brain Barrier
CBF	Cerebral Blood Flow
CBV	Cerebral Blood Volume
CCI	Controlled Cortical Impact
CNS	Central Nervous System
COX	Cyclo-oxygenase
CPP	Cerebral Perfusion Pressure
CSF	Cerebrospinal Fluid
DAI	Diffuse Axonal Injury
DNA	Deoxyribonucleic Acid
DVI	Diffuse Vascular Injury

EAA	Excitatory Amino Acid
EB	Evans Blue
EC	Endothelial Cell
ER	Oestrogen Receptor
FSH	Follicle-Stimulating Hormone
FP	Fluid Percussion
FPI	Fluid Percussion Injury
$GABA_A$	γ-Aminobutyric Acid Type A
GCS	Glasgow Coma Scale
GnRH	Gonadotropin-Releasing Hormone
GOS	Glasgow Outcome Scale
ICD	International Classification of Diseases
ICP	Intracranial Pressure
i.p.	Intra Peritoneal
LH	Luteinizing Hormone
LOC	Loss of Consciousness
MABP	Mean Arterial Blood Pressure

MAC	Minimum Alveolar Concentration
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MTBI	Mild Traumatic Brain Injured
NMDA	N-Methyl-D-Aspartate
PCD	Programmed Cell Death
PTA	Post-traumatic Amnesia
PR	Progesterone Receptors
P450scc	P450 Side-chain Cleavage
ROS	Reactive Oxygen Species
rpm	Revolutions per Minute
SD	Standard Deviation
SEM	Standard Error of Measurement
TAI	Traumatic Axonal Injury
TBI	Traumatic Brain Injury
Tukey's HSD	Tukey's Honestly Significant Difference
WHO	World Health Organisation

TABLE OF CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

ABSTRACT

A number of previous studies have suggested that female outcome following Traumatic Brain Injury (TBI) differs from male outcome, possibly because of the effects of the female gonadal hormones. How the hormones affect outcome is unclear; some reports support a protective role for the gonadal hormones whereas others suggest that there is no protective effect, and at times, even a deleterious effect. In the present study, we have used a standardised model of diffuse TBI in rats to characterise the effects of the female gonadal hormones on both female and male outcome.

Initial standardization of the impact–acceleration injury model involved characterizing the effects of injury on the different functional outcome tasks, and subsequently characterizing the effects of anaesthesia on these tasks in both male and female animals. Rate of functional improvement was generally independent of pre-injury training, with a significant effect only observed with daily testing of motor function. While weekly testing of functional outcome detected persistent deficits, daily assessment allowed for the early identification of functional deficits and the more rapid characterization of functional recovery. A differential pattern of functional recovery was apparent that was dependent upon the choice of anaesthesia for each gender, and the functional assessment task used. With respect to female outcome, isoflurane was protective, pentobarbital deleterious, while halothane had no effects on female outcome relative to males.

Ovariectomy in female animals reduced their performance on functional tests both prior to and after injury to a level similar to that observed in males. Administration of a physiological dose of either oestrogen or progesterone on a daily basis after injury

generally restored the performance of the ovariectomised females back to that of their intact female counterparts. Oestrogen and progesterone also improved motor and cognitive outcome in male animals after TBI. The female gonadal hormones had a profound effect on oedema formation after TBI in both male and female animals. In female animals, the endogenous levels of the hormones altered the temporal profile of oedema formation, with a transient delayed peak in oedema noted relative to the biphasic and sustained oedema observed in males. Exogenous administration of oestrogen or progesterone after TBI in either ovariectomised females or males attenuated the oedema formation and reduced blood–brain barrier (BBB) permeability.

At the morphological level, injured intact females demonstrated less haematoxylin and eosin dark cell change and less caspase-3 immunoreactivity in the hippocampus and cortex than injured males. When administered to either male or ovariectomised female animals, progesterone was identified as being more effective than oestrogen at reducing neuronal cell death, as identified by dark cell change and caspase-3 immunopositive staining, as well as axonal injury in white matter tracts using amyloid precursor protein.

We conclude that physiological levels of both oestrogen and progesterone improve outcome in female and male animals after TBI. This improvement may be associated with the ability of the female hormones to suppress BBB opening and oedema formation after trauma, perhaps through suppression of inflammatory pathways.

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1

Traumatic Brain Injury (TBI) is the biggest killer of individuals under 33 years of age in industrialised countries. Despite the dramatic improvements in its prevention and clinical management during the last twenty years, it remains one of the most significant public health problems (Blumbergs 1997), and has even been termed the young persons 'silent epidemic' (Goldstein 1990). Victims often report serious and enduring physical, emotional, cognitive, social, vocational and financial difficulties following TBI (Sallee et al. 2000). The financial difficulties affect not only the individual and his/her family, but also the community during the treatment and rehabilitation phases.

In addition to the more obvious physical disabilities that result from moderate to severe TBI, numerous studies indicate a significant number of TBI victims have on-going post-concussional problems (Rimel et al. 1981; Leininger et al. 1990; Bohnen et al. 1992). Symptoms of post-concussion syndrome include cognitive deficits (Binder 1997), emotional difficulties including mood swings (Hanks et al. 1999), and behavioural changes such as increased levels of stress and maladaptive coping in stressful situations (Bohnen et al. 1995). The reported cognitive deficits include difficulties with memory, attention, mental slowing and concentration for considerable periods of time (MacFlynn et al. 1984; Gentilini et al. 1985; Parasuraman et al. 1991). Patients and their families may also report out-of-character bursts of anger, increases in expressed emotion such as crying, and increased levels of anxiety and depression. Furthermore, individuals often report "...this accident has changed everyone and everything" (Griffiths 1997).

Chapter 1

Given the often devastating, immediate and life-changing consequences of TBI, it is essential to understand the factors that can influence the injury and recovery patterns. One such factor, gender, has to date received only minor attention in the TBI literature (Farace et al. 2000). Indeed, Farace and Alves (2000) in an extensive review of the clinical literature, found only eight studies that considered gender as a variable. It has been suggested that this lack of studies analysing female-specific features of pathophysiology, psychological and behavioural deficits induced by TBI, as well as patterns of social integration following brain injury, has significantly hindered neurotrauma research as a whole (Bell et al. 2001).

Of the limited reports that have been published in the clinical area, the results appear conflicting, equivocal and controversial. This is partly due to the extensive design and methodological variability between studies, including a broad variety of functional outcome measures (Groswasser et al. 1998), differing schedules for post-traumatic assessment (Spettel et al. 1991; Dikmen et al. 1995), the inclusion of patients with varying levels of TBI severity (Mazaux et al. 2002), variable lengths of hospitalisation, small sample sizes (Farace & Alves 2000) and a variety of premorbid factors (Glenn et al. 2001). As an example, a meta-analysis on eight studies considering gender as a variable by Farace and Alves (2000) concluded that men demonstrate better outcomes than women after TBI as assessed by injury severity, post-concussive complaints, the enhanced ability to return to work and fewer psychiatric symptoms. In contrast, Groswasser et al (1998) found that women had better predicted outcomes at the time of discharge from in-patient rehabilitative

care on the basis of their capacity to work. In a more recent study, Steadman-Pare et al (2001) found that women rate quality of life more rigorously than men following TBI, and this affects their perceived level of recovery.

Analysis of the experimental animal literature concerning sex differences following TBI also demonstrates similar controversies. A dearth of gender-focused research (Finklestein et al. 2001), conflicting and equivocal results (Emerson et al. 1992; Emerson et al. 1993; Roof et al. 2000b), limited use of female animals (Bramlett et al. 2001) and great variation in research design parameters (Chen et al. 1999; Roof et al. 2000b; Wright et al. 2001) all contribute to the complexity of this research area.

Despite these difficulties, accumulating clinical and experimental TBI data suggests that female sex hormones may have beneficial neuroprotective and neuroregenerative effects following TBI (Groswasser et al. 1998; Finklestein et al. 2001; Melton 2001). Stein posits that although little is known about how and why females differ in their responses to TBI from males, one of the potential explanations may be the difference in sex steroids. If sex hormones do influence outcome following TBI, then further research of these factors is urgently warranted. This is particularly true if the female sex hormones provide neuroprotection since there is currently no effective therapeutic treatment for the management and care of individuals after TBI (Roberts 1998; Clifton et al. 2001).

At this point, well designed experimental research is necessary to investigate the potential neuroprotective effects of the female sex hormones (oestrogen and progesterone) on

outcome following TBI. The aim of this thesis is to characterise the effects of the female gonadal hormones on outcome following diffuse TBI in both female and male rats. Before outlining the present study, consideration will be given to (a) characteristics of TBI including epidemiology, definition and classification, (b) neuropsychological consequences, (c) neuropathology and pathophysiology, (d) an overview of female sex hormones, oestrogen and progesterone, including their biochemistry, physiological control processes and effects in the brain, (e) a brief review of recent pertinent experimental hormonal studies, and (f) an overview of experimental models of TBI.

1.1 Epidemiology

1.1.1 Incidence and Outcome

Little epidemiological information is available, especially data concerning mild brain injury, which accounts for the vast majority of head injuries throughout the world (Kraus et al. 1988; Lyle et al. 1990; Asbury et al. 1998). In addition, estimates of the number of injuries, especially mild, are not only difficult to determine but may also be underestimated or missed (Binder 1997; Asbury et al. 1998; Weight 1998). Nonetheless, data are available that have been acquired both internationally and in Australia.

International

Although the number of traumatic head injuries has declined in Western countries during the last twenty years, incidence rates remain unacceptably high. A survey of available data indicates annual incidence rates between 105 and 392 per 100,000 population. For example, a Norwegian study, conducted in a defined hospital population, reported an

annual rate of 229 per 100,000 (Mortensen et al. 1999). Similarly, a study based on national hospital estimates in France reported an annual incidence rate of 150–300 per 100,000 (Masson 2000). A national survey in the United States (Kraus et al. 1996) found two million new cases of TBI, resulting in approximately 56,000 deaths. The annual incidence rate at this level is 175–200 per 100,000 population. Two recent studies from America demonstrate variation in rates depending on the type of study undertaken. For example, Sallee et al (2000), performing a study of hospitalised cases in Alaska, reported an annual rate of 105.2 per 100,000. In contrast, Guerrero (Guerrero et al. 2000) reported a much higher national rate of 392 per 100,000 population per year for all head injured patients who visited emergency departments but were not hospitalised.

Despite the associated difficulties, all studies concur that the majority of head injuries are mild, with estimates of 75% or more of all injuries falling into this category (Kraus et al. 1988; Asbury et al. 1998; Weight 1998). King (1997) reports that as few as 8% of head injuries are classified as severe, while other studies indicate even lower rates (Masson 2000). In contrast, an Australian study reported much higher severity rates with 13.6% classified as severe and 20.3% as moderate injuries (Asbury et al. 1998).

Australia

Little epidemiological data is available concerning TBI in Australia. However, of that which is available, the rates are comparable to those of other Western countries. For example, Lyle and colleagues (1990), in a critique of previous epidemiological studies, estimated the revised brain injury incidence rate in New South Wales to be 180 per 100,000

population, rather than 392 per 100,000 as previously reported, with 3.8% out of these injuries expected to be severe. The variation in these rates distinguished between head injuries and actual brain injuries. In a New South Wales regional community study of hospitalised patients, an annual incidence rate of 106 per 100,000 population was reported (Asbury et al. 1998). This study excluded head injury patients. This research also reported higher severity rates compared to other international studies (Hillier et al. 1997; Masson 2000). The study classified 13.6% of injuries as severe, 20.3% as moderate, and the remaining majority of 62.2% as mild injuries (Asbury et al. 1998).

A recent South Australian, hospital-based study (Hillier et al. 1997) estimated an injury rate of 322 per 100,000 population per annum, with the majority (75%) being mild brain injuries. Moderate injuries accounted for 9% of the total while severe injuries totalled approximately 16%.

1.1.2 Demography of Victims and Risk Factors

TBI affects predominantly young male adults in the age range 15–24 years (Lyle et al. 1990; Kraus et al. 1996; Hillier et al. 1997; Asbury et al. 1998). High incidence rates are still present in males up to 30 years of age (Weight 1998). To further illustrate the stark risk for males in this age group, Tate et al. (1998) report that males in this group only accounted for 6.8% of the resident population, but were significantly over-represented with 26.2% of all head injuries. Moreover, males have been shown to outnumber females in a ratio of approximately two to one (Kraus et al. 1988; Hillier et al. 1997; Jennett 1998), although

some studies report lower ratio figures (Diamond 1996).

Children recorded the next highest incidence rates with falls accounting for most of their injuries (Diamond 1996). Adolescents are mostly injured playing sport (Kraus et al. 1988), while falls again account for the majority of injuries for those over 75 years of age (Asbury et al. 1998). Middle age between 34 years and 75 years is the safest period for avoiding injury, but the incidence significantly increases after 75 years of age (Asbury et al. 1998).

1.1.3 Causes

Most studies are unanimous in their findings that motor vehicle accidents, falls, sport and recreational accidents, assaults, and work-related incidents account for the majority of head injuries, with traffic accidents and falls recording the highest incidence rates (Kraus et al. 1996; Jennett 1998; Mortensen et al. 1999; Guerrero et al. 2000). However, differences were found depending on the location of the study. For example, Hillier et al. (1997) found that motor vehicle accident incidence (40%) in rural South Australia was higher than other categories, while Mortensen and colleagues (1999) in their study of a metropolitan hospitalised population found that falls accounted for 62% of head injuries and traffic accidents for 21%. In addition, causal patterns have been shown to influence injury severity. Road accidents account for most severe injuries (66%), but for only 33% of moderate and mild injury (Hillier et al. 1997). In contrast, sport and recreational accidents occur most often in the mild and moderate groups but only account for 12.5% of severe injuries.

1.2 Definitions and Classification of Head Injury

1.2.1 Definitions

Currently there is no universal and agreed definition for head injury (Fearnside et al. 1997; Bigler 2001). Controversy exists about the number of terms which are used interchangeably to describe head/brain injury, especially the terms mild head injury and concussion (Rutherford 1989; Packard et al. 1993; Bennett et al. 1997). Fearnside et al (1997) recommends the term 'head injury' as a broad category, which takes into account even the most minor head injuries, and 'brain injury' as a subgroup of 'head injury'. At the same time, he posits the importance of clinicians remaining cognisant of those individuals who appear to sustain only a minor injury in the first instance, but later may develop more serious complications.

Descriptively, head injury refers to any trauma to the head inflicted by mechanical force. It may be classified as open or closed, where open head injury refers to those injuries where penetration of the skull occurs (for example, gunshot wounds) (Richardson 1990). Brain damage caused by penetrating force tends to be more localised, following the path of the missile (Stratton et al. 1994). In comparison, closed head injuries as those involving blunt impact without penetration of the skull (Richardson 1990). Closed/blunt head injuries are more common than penetrating head injuries, and usually occur without an impact or blow to the head, such as in whiplash type injuries.

1.2.2 Classification

Meaningful classification of TBI is crucial for several levels of clinical management. To this end, numerous brain injury classification systems have been developed in an attempt to systematise management of brain injuries. The classification systems used in clinics at present are based primarily on severity indices or anatomical location of lesion site (Jiang et al. 1996).

1.2.3 Severity Indices

One of the most widely used classification indices is the Glasgow Coma Scale (GCS) developed by Teasdale and Jennett (1974). This outcome measure is based on motor and verbal responses, as well as eye opening, and was initially developed to measure depth of coma. Individuals who have sustained head injuries are categorised on this scale according to the following measurements: On admission to hospital, a score of 8 or less indicates severe injury, 9–12 moderate injury, and 13–15 mild injury (Teasdale et al. 1974). However, concerns about the sensitivity of the GCS as a measurement tool for mild brain injury have been raised (Kraus et al. 1988; Gentilini et al. 1989), while others have suggested a need for additional classification criteria that would be used in conjunction with the GCS (Williams et al. 1990).

A second severity index is loss of consciousness (LOC). It refers to any stage of altered consciousness, from being dazed to comatose (Alexander 1995). As a measure of severity, LOC of less than 20 minutes is usually considered mild injury, although this does vary in some studies (Barth et al. 1989; Gentilini et al. 1989; Packard et al. 1993). No clear time

limits exist for moderate or severe brain injury (Fearnside et al. 1998). Several scales including the Clinical Neurological Assessment Tool (CAN) (Crosby et al. 1989) and the Coma Recovery Scale (CRS) (Giacino et al. 1991) are used to rate LOC.

In 1932, Russell (1932, cited in Berker 1996) developed the concept of post-traumatic amnesia (PTA), which has been used in many studies to help define severity levels (Alexander 1995). PTA refers to memory disturbances manifesting immediately after head injury, and is defined as the period of time measured from the moment of head injury to the moment when the individual regains continuous memory for what is happening (Ruff et al. 1989; Packard et al. 1993). In some studies, PTA for mild brain injury has been set at less than one hour, while others have used less than 24 hours as their criteria (Alexander 1995; Jacobson 1995; Binder 1997).

The Glasgow Outcome Scale (GOS) (Jennett 1997) is widely used to measure outcome following head injury. This scale has five categories of which four pertain to survival: 1 good recovery; 2 moderate disability; 3 severe disability; 4 vegetative state, and 5 dead. Individuals who regain some degree of awareness are grouped into one of the first three categories, according to the degree of their dependence on others, and their ability to function sufficiently including return to work and social interactions. It should be noted that even those who are considered making good recovery might continue to experience residual and ongoing difficulties of a quite debilitating nature. Individuals classified as being in a 'vegetative state', category four of the GOS, demonstrate no sign of "psychologically meaningful activity" (Jennett 1997 p. 440). In this state, individuals

manifest periods of wakefulness and sleep, some reflex actions and normal breathing, but without other evidence that they are aware of what is going on around them (Griffiths 1997).

1.2.4 Anatomical Scales

The Abbreviated Injury Scale (AIS) was primarily developed to assess severity of impact injury (Jiang et al. 1996). This numerical scale rates severity of injury based only on anatomical injury, without reference to outcome or resulting disability. The AIS 90 (Association for the Advancement of Automotive Medicine 1990) is a revised scale, which includes more descriptors to gauge severity of brain injury. Indeed, this version of AIS classifies brain injury using the GCS and LOC if the anatomical manifestation of the injury does not reflect the actual severity level, as indicated by clinical symptoms, or in the absence of any anatomical lesion upon imaging investigation or at post-mortem (Fearnside et al. 1997).

International Classification of Diseases (ICD) is another widely recognised anatomical brain injury system, which is published by the World Health Organisation (WHO) and provides much of the epidemiological health data gathered. Although this classification system is flawed, it does provide some uniformity for the comparison of health statistics (Blumbergs 1997). This system is based on the type and location of parenchymal and extraparenchymal lesions (Jiang et al. 1996). Currently in the newly revised ICD (ICD-10), head injuries are included under the three-digit rubric SO6 with decimal points and one number following (for example, SO6.1 Traumatic cerebral oedema) to identify the major

categories of head injury.

1.3 Neuropsychological Consequences of Head Injury

Neuropsychological consequences following head injury may involve difficulties and deficits in arousal and attention, memory, language comprehension and fluency, visual perception, executive functioning, and emotional, psychological and behavioural changes (Lezak 1995; Adams et al. 1996). The problems encountered by individuals following head injury vary as widely as the type, site and severity of injury sustained. Head trauma severity ranges from a mild bump without any consequence to the severity which leads to prolonged coma, vegetative state or death. Neuropsychological assessment in general is also vulnerable to validity threats, which can seriously confound meaningful interpretation of test scores (Hannay et al. 1996). For example, premorbid cognitive and learning ability/disability, substance use/abuse, prior medication, psychiatric history and individual differences under testing conditions all potentially influence neuropsychological test outcomes. In addition, pre-morbid factors such as age, sex, personality, coping style, and social and family support play a significant role in outcome following head injury (Griffiths 1997). This overview will present a global picture of neuropsychological deficits which do not include reference to the type or site of sustained injury.

1.3.1 Mild Traumatic Brain Injury

Attention deficits, impaired verbal retrieval, memory difficulties and emotional distress appear to be the most common neuropsychological symptoms following mild head injury (Lezak 1995). Often these problems do not become apparent or disruptive until days or

even weeks after the insult. This is especially true if individuals have sustained other injuries that have necessitated their having time off from their normal activities. It is only when people try to cope with everyday activities such as full-time work, preparing meals, shopping, and family responsibilities that the deficits become apparent. The physical ailments of dizziness, nausea, fatigue and headaches, which accompany mild head injury, can also exacerbate the neuropsychological symptoms (Packard et al. 1993).

In a recent study, mildly brain injured patients were assessed using Magnetic Resonance Imaging (MRI) to gauge brain activation triggered by working memory task, in comparison with matched controls (McAllister et al. 1999). Results indicate that while task performance did not differ significantly between the two groups, brain activation patterns varied in response to increasing processing loads of working memory. Mild traumatic brain injured (MTBI) patients showed significantly increased activation during the high load condition, in comparison with controls that showed very little increase in activation from their low load to high load condition. It is thought that this increased effort on the part of MTBI patients may help to explain their memory difficulties, while deficits in neuropsychological tests are not necessarily manifested (McAllister et al. 1999). Unfortunately, individuals who have difficulty resuming their normal daily activities because of such memory difficulties are often judged to be malingerers rather than genuinely experiencing identifiable memory and cognitive deficits.

Attention deficits take the form of slowed reaction times (Lezak 1995; Spreen 1997), poor concentration or poorly sustained attention (Gentilini et al. 1989) increased distractibility,

dual task difficulties and divided attention tasks (Batchelor et al. 1995). In the acute stage following mild head injury, individuals often demonstrate perceptual difficulties, which usually abate over time. These problems may remain as subtle deficits, which frustrate the head injured person but are not obvious to acquaintances (Lezak 1995). Slowed verbal retrieval is one of the frequent complaints identified with neuropsychological assessment. This includes difficulty in recalling names of objects, places, people (Lezak 1995); paraphasias (words said back to front, for example, 'hangercoat' for 'coathanger') (Spreen 1997) as well as misnaming objects (Williamson et al. 1996). Memory deficits based on short-term memory loss also involve difficulties in recalling routine daily activities such as appointments, phone numbers, and immediate plans for the day (Clare et al. 1997).

Emotional distress as experienced by MTBI individuals often results from the fatigue and difficulties associated with coping with everyday life following injury (Wilson et al. 1996). Cognitive deficits, which tax mental efficiency of many individuals, also affect recovery from the injury and related shock. People become distressed when activities that were automatic before injury require substantial concentration and effort. These activities, necessary for daily living, include mental calculations, monitoring performances, planning, listening to two conversations at once, screening out background noise, and interacting with other people. Often people become fatigued coping with their day, which becomes more burdensome especially if they are acutely aware of their mental inefficiency. Such an experience increases the emotional distress, and often leads to depressed mood and a sense of going crazy (Wilson et al. 1996). Individuals often respond to these difficulties badly,

because the medical staff left them without explanation about such potential consequences following TBI. Indeed, some people report a perceived offhandedness by medical staff regarding their injury. Alexander (1995, p. 1255) contends practitioners' advice that individuals "...take a few days off..." following a mild head injury is often unhelpful to recovery.

1.3.2 Moderate Traumatic Brain Injury

Similar to mild head injury, individuals who are categorised as having sustained a moderate head injury report symptoms with varying features and duration (Lezak 1995). It has been shown that the majority of injured individuals, even those demonstrating good progress, still experience significant difficulties at three months post-injury and many of them do not return to their previous employment (Williamson et al. 1996). Cognitive deficits found in this group, additional to neuropsychological problems described in mild head injury group, include serious deficits in long-term memory, reduced ability to 'chunk' information into fewer pieces, and impaired memory for contextual factors about a particular incident; for example, time, date, frequency and source of information are often not remembered (Williamson et al. 1996). Moreover, difficulties with abstract thought and conceptualisation, significantly decreased ability to plan, control or execute activities or attain a goal, disordered thought processes, difficulty with tracking thought processes or conversations, and disconnected thought processes (Banich 1997) are amongst numerous problems experienced by patients with moderate head injuries.

Individuals who are able to resume independence, return to work or resume family responsibilities tend to "...differ from intact persons and from what they were..." (Lezak 1995, p. 272). In addition to cognitive deficits, these individuals may exhibit some loss of behavioural and emotional capacity, including loss of spontaneity or the ability to initiate activities, increased impulsivity, increased angry outbursts, inability to monitor their behaviour, and decreased ability to empathise or show emotion (Lezak 1995). Moreover, the overall adaptive behaviour of this group frequently appears quite poor and their ability to function seriously compromised. As expected, such individuals also experience increased levels of emotional and psychological distress. Indeed, increased levels of serious depression are frequently noted (Williamson et al. 1996), which may increase the lack of motivation of the injured individual: a symptom that is regularly reported by family members. Psychological distress also contributes to the lack of insight exhibited by moderately injured individuals about their particular deficits, and their inappropriate emotional or behavioural responses (Mathews 1990). Personality changes are also common in this group although this may be greatly influenced by the cognitive and emotional deficits experienced (Banich 1997).

1.3.3 Severe Traumatic Brain Injury

Only a minority of people with severe brain injury survive their injuries and make a good recovery (Ponsford et al. 1995). However, deficits in survivors may range from subtle to a completely vegetative state where the person requires round-the-clock nursing care. This section will outline only those deficits not already mentioned above, which do not result in

a vegetative state where the individual can no longer adaptively function at any level.

This group of head injured people often experiences language difficulties, although classical aphasia was shown to occur in only about 2% of people with severe head injury (Williamson et al. 1996). Deficits with comprehending audio or visual incoming stimuli, difficulties with word fluency involving both the ability to produce a word/phrase spontaneously and in response to cues, and deficits in capacity for naming or labelling items or objects present are amongst frequent problems. These deficits often remain long-term, although they may become quite subtle in nature. In addition, problems with motor speech, including pronunciation and word production difficulties are also noted in severely injured individuals (Lezak 1995; Williamson et al. 1996).

TBI may also impair a number of basic sensory and motor functions that affect vision (Andrewes 2001). Recent neurophysiological reports suggest that a substantial number of closed head injury survivors may experience deficits in early feature recognition, such that they do not process small visual details as efficiently or accurately as the age-matched controls (Heinze et al. 1992). Although uncommon following closed head injury, a broad array of visuospatial-constructional deficits may occur after TBI, which include alexia (the inability to read), agnosia (inability to recognise the identity of objects), and difficulties with visuospatial concept formation (Benton et al. 1993).

Even though executive functioning, which is defined as the ability "to extract and use information from the posterior brain systems, and to anticipate, select, plan, experiment,

modify and act on such information in novel situations" (Stuss et al. 1986, p. 175), remains a controversial construct in neuropsychological literature (Stuss 1995; Taylor 1996), in clinical practice it helps identify a syndrome of difficulties that people experience following TBI, especially those with severe TBI. Personality changes, which can take many forms, belong to this range of difficulties. For example, deficits in the ability to anticipate consequences, increasing inflexibility on the part of the individual who may insist actions be performed in exactly the same way each time, disinhibited behaviour and failure to execute appropriate responses, and increased levels of anxiety, frustration and extreme irritation if deviations from the expected occur may all be experienced (Lezak 1995). Moreover, individuals may be able only to employ concrete and rigid problem-solving strategies and not be able to incorporate feedback from others. This can lead to enormous frustration for both the head injured person and those attempting to interact with them. One of the greatest difficulties resulting from these deficits is the often huge behavioural and emotional change wrought by them. Family members and friends report the extreme problems associated with managing and supporting loved ones with these kinds of neuropsychological problems (Griffiths 1997; Brain Injury Association of Queensland 2002).

1.4 Neuropathology and Pathophysiology of Head Injury

TBI is a complex injury process involving both focal and diffuse changes (Povlishock et al. 2001). Falls or blows to the head tend to result in more focal injuries such as contusions and haematomas, while motor vehicle accidents result in more diffuse injury including

traumatic axonal injury (TAI), cerebral blood flow (CBF) interruption, pathological metabolic changes and adverse neuroexcitation (Smith et al. 2000). The mechanisms of TBI are classified as either primary (mechanical mechanisms resulting in early tissue deformation) or secondary (mechanisms involved in delayed onset injuries). However, there is some overlap between these two broad categories. In addition, while it is possible to broadly predict the injury progression based on the type and severity of force applied to the head, resulting brain damage is also dependant on individual differences, such as an individual's anatomy, physiology, metabolic and vascular reactions, and premorbid factors such as physical fitness (Abou-Hamden et al. 1997; Bigler 2001). This means that the same mechanical force may produce a different pattern (outcome) of primary and secondary injury in different individuals (Abou-Hamden et al. 1997).

1.4.1 Primary Traumatic Brain Injury Mechanisms

Primary damage following TBI refers to the response of bone, blood vessels and the brain to impact forces and inertial or acceleration movements (McIntosh et al. 1996; Adams et al. 2000). Mechanical forces (rotation, acceleration/deceleration, and direct force applied to the head) at the time of impact cause damage to the neurovascular system, neuronal structures including axons and the soma, and the supporting glial cells of the brain (Abou-Hamden et al. 1997). The injuries that occur as a result of primary mechanisms usually involve some kind of immediate structural change to some part of the brain.

Biomechanical Forces

Primary mechanisms of TBI involve energy transfer to the brain via contact or acceleration/deceleration movements (Blumbergs 1997). The applied forces are responsible for the mechanical damage that occurs at the time of injury, and whether the force involves acceleration, deceleration, rotation, or direct impact will determine the amount of shear stress and strain to which the brain is subjected. For the purposes of this discussion, impact in relation to head injury refers either to a stationary head being hit, or a moving head hitting a stationary object, as there is no physical difference between the forces involved in each situation (Abou-Hamden et al. 1997). In any impact to the brain the velocity of movement is imparted to the brain, along with whatever rotational forces are also present. The impact usually involves rapid deceleration forces as the brain comes to an abrupt halt. As a result of these forces, the brain may be stretched and rotated within the skull. The consequences of this type of impact involves the brain's direct striking of its bony encasement, the skull, and the less obvious shear strain that results in the pulling apart of axons and disruption of cell bodies that occurs as a result of the momentary distortion of the brain's shape and density (rotational forces) (Williamson et al. 1996).

Deceleration forces also cause the soft brain to 'wobble' within the rigid skull after impact in response to lines of force (Blumbergs 1997; Ommaya et al. 2002). The brain is fixed within the cranial cavity by the parasagittal bridging veins, parasinusoidal granulations, cranial nerves and tentorium. The forward movement of the lobes of the brain towards the anterior cranial basal structures applies more force at the bases of the frontal lobes and the

tips of the temporal lobes. This means that surface contusions are more frequent at these sites than elsewhere in the brain (Blumbergs 1997). In addition, bruising can also happen at the point of rapid deceleration as a result of the soft brain's strong movement against the bony regions of the skull in response to translation forces generated by angular acceleration (Adams et al. 1985). This bruising is usually more prominent in the frontal and temporal lobes where the cortex normally rests on the rough surface of the skull (Mattson et al. 1990).

Shearing, Stretching and Disruption

Shear forces involve an impact to the side or across an object (axon) striking from any angle (Abou-Hamden et al. 1997). This sidewards impact may cause the axon length to bow and stretch. If the shear force impacts near the foundations, which support the axon, it may cause tearing and disruption. When a moving head comes to an abrupt stop such as in an accident, the forward-moving energy (in a motor vehicle) or accelerating energy (in a fall) is translated into rapid acceleration/deceleration, expanding and contracting wave-form movements of the brain matter, usually accompanied by fast rotational forces on the brain within the skull (Lezak 1995; Blumbergs 1997). The combination of forces results in shear strain injury (Adams et al. 2000; Ommaya et al. 2002). The movement of the brain within the skull puts shear strain on nerve fibres and blood vessels that can stretch them to the point of shearing, and potentially cause complete disruption. Strain involves either tensile (stretching) or compressive (reduction in length) stress (Abou-Hamden et al. 1997). Axons subjected to this kind of stress may either retract or stretch depending on the

magnitude of the strain applied.

The impact of shearing forces on primary brain injury is also influenced by age factors (Blumbergs 1997; Ommaya et al. 2002). For example, babies and elderly people are more vulnerable to shearing force damage to their vascular systems (Ommaya et al. 2002). For the babies, this may be related to the lack of myelination and reduced astrocyte maturity (Blumbergs 1997), whereas elderly people have reduced neuronal and astrocyte density with poorer support of vascular structures (Blumbergs 1997).

Structural damage

Structural damage following TBI can be broadly classified as either focal or diffuse (Povlishock et al. 1994). Focal injuries include neurovascular and neuronal damage such as haemorrhages and haematomas (intracerebral, subdural and extradural), contusions, lacerations, and brain stem lesions, while diffuse injuries include diffuse TAI (Povlishock et al. 2001) and diffuse vascular injury (DVI) (Abou-Hamden et al. 1997).

Haemorrhage and Haematoma

Haemorrhage (bleeding) or haematoma (blood clots) in the brain are interrelated following head injury and the terms are sometimes used interchangeably. Haemorrhages following brain injury are broadly classed as intracerebral, extradural, subdural or subarachnoid. Intracerebral haemorrhage results from stretching and rupture of small-calibre cerebral arterioles located within either the basal ganglia or the ventricles. These occur primarily in the frontal and temporal lobes (Cifu et al. 1999). Extradural haemorrhage is often

associated with skull fracture and occurs mostly above the temporal and parietal regions of the brain (Povlishock et al. 1994). Subdural haemorrhages occur commonly in the frontal regions of the brain and result from the rupture of the parasagittal bridging veins when the brain experiences deceleration forces (Blumbergs 1997). Finally, subarachnoid haemorrhages are the most common structural damage seen in head injury and may result in vasospasm and hydrocephalus (Graham 1996). These haemorrhages and haematomas may cause local vasoconstriction, increased intracranial pressure (ICP), decreased cerebral perfusion pressure (CPP) and structural herniation (Mendelow et al. 1997; Cifu et al. 1999).

Contusions

Contusions or bruising of the brain occur as a result of the skull causing damage to the brain tissue as it makes contact with the sharp edges of the skull (Griffiths 1997). Damage may occur to the capillaries, veins and arteries as well as to neuronal and glial cells of the neural parenchyma (Abou-Hamden et al. 1997). Contusions can occur either on the surface or in the deeper structures of the brain, and they often set in train secondary mechanisms leading to haemorrhage, breakdown of the blood–brain barrier (BBB) and infarction (Blumbergs 1997). They are classified into six types depending on their location: *coup* which occurs directly beneath the site of impact; *contrecoup* which occurs opposite the site of impact; *intermediate coup* which are intracerebral lesions that occur deeply within the neural parenchyma between the impact site and the opposite side of the brain; *fracture* which occurs beneath the site of a fracture; *gliding* which occurs in the parasagittal regions and are often a result of rotational acceleration forces and associated with diffuse axonal

injury (DAI); and, *herniation* contusions which involve the medial temporal lobes and the cerebellar tonsils (Povlishock et al. 1994; McIntosh et al. 1996; Griffiths 1997; Mendelow et al. 1997; Graham et al. 2000).

Lacerations

Lacerations of the brain can be defined as primary damage to the neural parenchyma which occurs at the time of injury (Cotran et al. 1999). They may be either direct or indirect depending on the type of injury sustained. Direct lacerations result from penetration of the parenchyma either by a missile or from fragments of bone, including those bone fragments following a depressed fracture of the skull (Graham 1996). In contrast, indirect lacerations occur when the parenchymal damage is secondary to other mechanical forces. The temporal lobes and/or the inferior frontal lobes are most vulnerable to this type of laceration. In addition, the lacerations may be either superficial involving the cortex and subcortical areas of the brain or penetrate into areas such as the hippocampus, temporal horn and amygdaloid nucleus (Abou-Hamden et al. 1997).

Diffuse Traumatic Axonal Injury

TAI including the concept of DAI is one of the most common causes of morbidity and mortality following TBI. It was first reported by Strich (1956) who demonstrated the presence of structural axonal injury without evidence of focal injury in severely injured TBI patients. She concluded that the axonal damage was responsible for the morbidity seen in these patients. Since then, controversy has surrounded the concept of DAI and its pathogenesis (Maxwell et al. 1997), and interested readers are referred to a comprehensive

review on the topic (Povlishock et al. 2001).

Briefly, diffuse axonal damage is a complex time dependent process beginning with axonal swellings and progressing to detachment and the development of axonal bulbs (Maxwell et al. 1997; Povlishock et al. 2001). Earlier descriptions of axonal injury (see Figure 1.1) distinguished between primary and secondary axotomy based on the hypothesis that primary axotomy resulted in tearing of the axons immediately at the time of injury. More recent experimental work, however, suggests that axonal damage is more subtle, causing changes in the axonal cytoskeleton and the eventual impairment of axoplasmic transport over a period of hours to several days (Povlishock et al. 2001). TAI results in axonal failure, interference with axoplasmic transport and the movements of electrical information along the axonal pathways from one neuron to the next and finally disconnection of the axon (Abou-Hamden et al. 1997; Povlishock et al. 2001).

Primary axotomy, now posited to result from only the most severe injuries (Adams et al. 2000), occurs when applied forces exceed a critical level and result in shearing or disintegration of the axolemma at the time of injury. Severe TBI may cause primary axotomy. At this level of injury, damage can occur almost instantly and involves complete disruption of neural tissue. Secondary or delayed axotomy (Maxwell et al. 1997) describes a delayed process of axonal damage that leads to more gradual axonal disconnection over a period of hours and is a result of damage to the axolemma rather than a function of mechanical force (Adams et al. 2000). This process has been proposed to be the result of an injury cascade involving the loss of iron homeostasis necessary for the maintenance of

electrical activity in the axon. Clinical outcome from DAI ranges from mild to severe, and results in a heterogenous pattern of injury. The level of injury depends not only on the number of damaged axons but also on the number of disrupted to non-disrupted axons (Adams et al. 2000). It is important to note that experimental models of TAI do not provide the full spectrum of TAI seen in human TBI (Povlishock et al. 2001). However, experimental work has demonstrated the delayed axonal destruction seen in clinical TBI and support the use of these models for further studies (Blumbergs et al. 1994; Blumbergs et al. 1995; Abou-Hamden et al. 1997).

Diffuse Vascular Injury

DVI is usually seen in people who die very soon after sustaining a closed head injury (Abou-Hamden et al. 1997). This type of structural damage involves numerous small haemorrhages throughout the brain that evolve over time. The white matter of the frontal and temporal lobes and the brain stem appear particularly vulnerable to DVI (Adams 1990). Examination by light microscopy reveals periarterial, perivenous and pericapillary haemorrhages and leakage of red cells into the neuropil (Abou-Hamden et al. 1997).

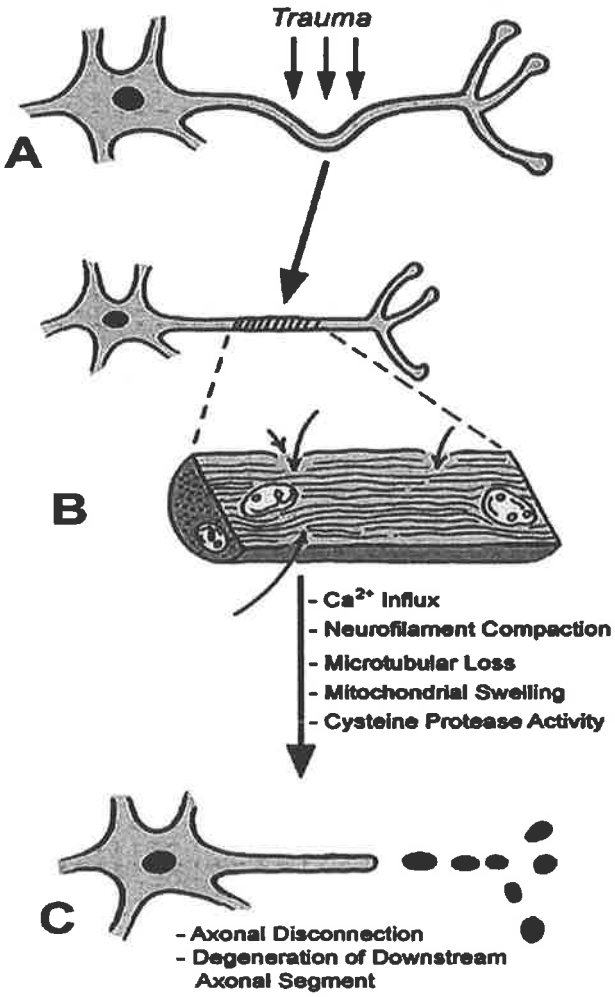


Figure 1.1 Axonal injury following severe TBI.

1.4.2 Secondary Traumatic Brain Injury Mechanisms

TBI sets in train a cascade of biochemical and physiological events that exert enormous stress on adaptive cellular processes, contribute to functional disability and influence patient outcome (Samii et al. 2001). Known as secondary injury, these events occur minutes to hours or days after the primary event. Depending on the severity of the injury,

these processes may involve, amongst others, axonal injury, metabolic changes, CBF impairment, oedema, raised ICP, ischemic-hypoxic damage, calcium influx, increased oxidative stress, free radical-mediated damage, receptor-mediator damage, inflammation, and cell death including necrosis and apoptosis (for reviews see Blumbergs 1997). At the present time the mechanisms and complex biochemical interrelationships, which underpin the secondary injury cascade following TBI are not fully understood. However, because secondary injury is time contingent following trauma, a therapeutic window exists for pharmacological treatment that may reduce the level of injury and subsequently improve patient survival and functional outcome.

Metabolic Decline in Traumatic Brain Injury

Energy demands following severe brain trauma are dramatically increased as the cerebral system strives to maintain cellular homeostasis (Vink 1994; Vagnozzi et al. 1999). According to Samii et al (2001) the metabolic disturbance involves an initial period of hyperglycolysis, followed by a persistent drop in metabolic activity which correlates with the development of behavioural deficits. These authors also propose that the disequilibrium of ionic homeostasis following TBI may contribute to the metabolic changes.

Ionic Changes Following Traumatic Brain Injury

Directly following trauma, massive ion fluxes occur across the neuronal membrane causing significantly increased levels of intracellular calcium (Ca^{++}) and sodium (Na^{+}) and a corresponding potassium (K^{+}) efflux to the extracellular spaces (Blumbergs 1997). Declines in magnesium also occur and have been related to a decrease in metabolic

activity, alterations in Na^{+}/K^{+} ATPase activity with subsequent oedema development, membrane breakdown, and excitotoxicity with intracellular Ca^{++} overload (Alexiou et al. 2000). Increased levels of intracellular Ca^{++} results in, amongst other things, neuronal organelle and membrane damage caused by calcium driven proteases and lipases and the release of nitric oxide. Impairment of the mitochondrial function as early as one hour after injury has been associated with Ca^{++} (Xiong et al. 2001), and several studies have now confirmed large intracellular increases in calcium in the acute period after experimental TBI (Fineman et al. 1993; Kawamata et al. 1995). Calcium influx is thought to occur through several mechanisms including voltage-dependent channel openings, ligand gated channel openings, and mechanical deformation of membrane and ion channels (Blumbergs 1997).

Excitatory Amino Acids

Release of excitatory amino acids (EAAs) such as glutamate and aspartate, has been implicated in an excitotoxic cascade that occurs subsequent to TBI and culminates in cell death (McIntosh 1993; Regan et al. 1994). An increase in glutamate and aspartate occurs immediately after experimental TBI with maximum increase at 10 minutes and return to normal levels by 30 minutes to one hour post-injury (Faden et al. 1989). Such release is thought to activate a number of receptors including ionotropic N-methyl-D-aspartate (NMDA) channels, which have been implicated in the development of neuronal cell death (Regan et al. 1994). Activation of these receptors results in increased intracellular free calcium concentration by initiating transmembrane calcium flux and by releasing calcium

from intracellular stores. The increase in intracellular Ca^{++} activates various proteases which may in turn lead to cell death or apoptosis. The high intracellular Ca^{++} concentration also activates lipases responsible for breaking down intracellular fatty acids, and initiating the arachidonic acid cascade (McIntosh et al. 1996; Adams et al. 2000). The subsequent deleterious arachidonic acid cascade has been associated with neuronal death and poor outcome in experimental models of TBI (McIntosh et al. 1996; McIntosh et al. 1998). Increased NMDA mediated Ca^{++} influx has also been associated with mitochondrial dysfunction, energy depletion, oxidative stress, and increased lipid peroxidation (Siesjo 1986; McIntosh 1993). The high concentration of NMDA receptors in the hippocampus is thought to be responsible for the vulnerability of CA1 neurons to apoptosis following TBI. Experimental studies have shown that NMDA receptor antagonists were beneficial to outcome following TBI, although this has not translated to the clinical arena (Maas 2001) This is perhaps not surprising given that their efficacy in experimental studies was temporally limited to administration within the first 30 minutes following TBI (McIntosh 1993).

Oxidative Stress

Oxidative stress refers to the process whereby oxygen free radicals are toxic to cells and can be defined as damage inflicted via processes involving production of reactive oxygen species (ROS) and their detrimental reactions with proteins, lipids, and Deoxyribonucleic Acid (DNA) (Cernak et al. 2000). Brain tissue is extremely vulnerable to oxidative damage because of its high rate of oxidative metabolic activity, production of reactive oxygen

metabolites, relatively low antioxidant capacity, low repair mechanism activity, non-replicating nature of its neuronal cells, and the high membrane surface to cytoplasm ratio (Shohami et al. 1997). ROS initiate tissue damage through complex mechanisms including excitotoxicity, metabolic failure and disturbance of intracellular calcium homeostasis (Marklund et al. 2001). They can be generated via arachidonic acid cascade activity, catecholamine oxidation, mitochondrial leak, oxidation of extravasated haemoglobin, and by neutrophils (Hall et al. 1992). Oxidative damage frequently involves lipid peroxidation of neuronal, glial, and vascular cell membranes and myelin (Anderson et al. 1994), resulting in the decomposition of polyunsaturated fatty acids in lipid membranes (Shohami et al. 1997), disruption of ionic gradients, and if severe enough, membrane lysis (Hall et al. 1992). However, according to Haliwell (1992) oxidative stress damage need not involve lipid peroxidation.

1.4.3 Cell death

Necrosis

Necrosis (see Figure 1.2) involves cell, organ, or tissue death, which can be localised or widespread and results from a catastrophic failure of cellular homeostasis following some form of abnormal insult such as TBI, or anoxic event (Sastry et al. 2000; Lezlinger et al. 2001). Necrotic damage involves: loss of cellular homeostasis; altered membrane permeability; changes in membrane potential involving the efflux of potassium and influx of calcium and sodium; swelling and subsequent rupturing of cells; and destruction of organelles including the mitochondria Adenosine Triphosphate (ATP) energy loss (Sastry

et al. 2000). A massive rise in intracellular calcium which is large enough to induce necrosis, activates a wide range of enzymes, particularly endonucleases, calpains, lipases, and proteases, and also leads to the production of arachidonic acid (Rubin 1998). A major intracellular event in necrosis may be free radical production within the cell because of damaged mitochondria. Necrotic damage is characterised by mitochondrial swelling, nuclear pyknosis, chromatin fragmentation, and damage to the membrane resulting in cellular destruction (Sastry et al. 2000).

Apoptosis

Apoptosis (see Figure 1.2), under normal conditions, is a type of programmed cell death (PCD) which occurs during normal cellular development and is an essential biological process that helps maintain homeostasis (Baynes et al. 1999; Kinloch et al. 1999). In contrast to necrosis, features of programmed apoptosis include active degeneration with no initial primary changes in the cell, no immediate sodium influx and potassium efflux, the organelles remain generally intact, apoptotic bodies separate and form from the cell membrane, there is a lack of cellular energy depletion, and activation of second messenger systems is necessary (Sastry et al. 2000). Apoptosis following damage to the central nervous system (CNS) may appear the same as PCD but also have some characteristics of necrosis (Wylie 1997).

When programmed apoptosis occurs other cells are not affected nor is the organism, however when apoptosis results from injury or degenerative disease the consequences can be devastating at both a cellular and functional level (Brown et al. 2001). Over-activation

of apoptotic processes to maladaptive levels following brain insult may result from several secondary mechanisms of injury including disruption of axonal transport, oxidative stress, and excitotoxicity through massive glutamate release, disrupted calcium homeostasis and mitochondrial dysfunction (Sastry et al. 2000). Once the adaptive capacity of the neuron has been exceeded ion homeostasis is lost (Adams et al. 2000), mitochondria fail and DNA damage is evident (Lezlinger et al. 2001), and calpains (Fineman et al. 1993; Nath et al. 1996) and caspases (Beer et al. 2000) are activated. Each of these mechanisms can lead to neuronal death independently or in cooperation with each other. The manner and shape of cell death is decided by the contribution of the individual mechanisms, and possibly by the set of downstream degradative mechanisms activated or inhibited in each particular case (Nicotera et al. 1999).

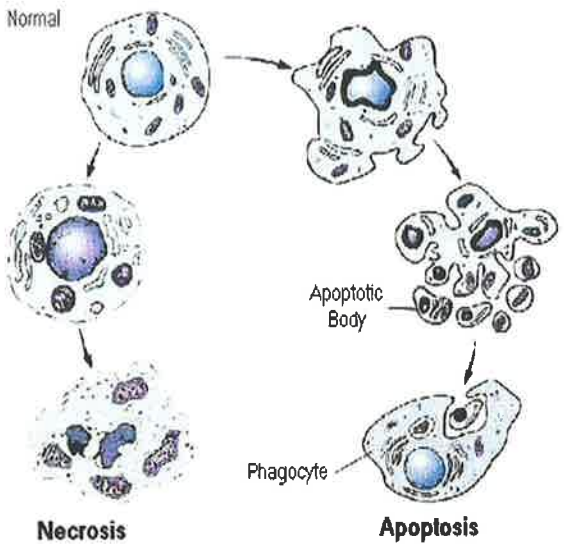


Figure 1.2 Illustration of cell death via necrosis and apoptosis.

1.5 Traumatic Brain Oedema

Cerebral oedema, a deleterious secondary injury factor, occurs following severe TBI, and can manifest either locally or diffusely in the brain. It is broadly defined as "an abnormal accumulation of fluid within the brain parenchyma associated with a volumetric enlargement of the tissue" (Klatzo 1979, p110). Klatzo posited that development of oedema requires increase in both water content and tissue volume. Harmful consequences of cerebral oedema include raised ICP (Kimelberg 1995), dangerously reduced CBF (Graham 1996), reduced cerebrospinal fluid (CSF), and deformation and shifting of brain tissue (McCance et al. 1998), all of which contribute substantially to increased morbidity and mortality following TBI.

1.5.1 Classification of Oedema

Although (Klatzo 1979) cautioned that pure types of oedema rarely exist, cerebral oedema is generally classified primarily as vasogenic or cytotoxic, based on the underlying mechanisms associated with the differing pathophysiological changes. Contemporary research and clinical practice continues to recognise these early definitions. More recently a third type of oedema, ionic oedema, has been proposed, which though corresponding to vasogenic oedema, includes different mechanistic actions (Young et al. 1994).

Vasogenic Oedema

Vasogenic oedema may result from either the immediate insult or secondary injury mechanisms associated with local cerebral ischaemia (Baskaya et al. 2000). It is defined by increased BBB permeability (discussed below) causing disruption to the balance between

oncotic and hydrostatic pressures that govern movement of fluid between blood plasma and brain interstitial fluid (Baynes et al. 1999). The compromised BBB allows water and solutes, such as protein exudates, to escape from the cerebral vasculature as a bulk flow and intrude into the interstitium of brain parenchyma, resulting in a net gain of interstitial fluid and subsequent fluid retention (Kimelberg 1995; Baskaya et al. 2000; Guyton et al. 2000). Because of the limited lymphatic system in the brain, resorption of exudate solution from the extracellular space is greatly impaired (Cotran et al. 1999). Vasogenic oedema spreads throughout extracellular spaces as a result of pressure gradients involving the least tissue resistance. This mechanism of movement explains why oedema is seen primarily in the structurally ordered cerebral white matter rather than in the more densely organised grey matter (Klatzo 1979; Kimelberg 1995).

Cytotoxic Oedema

Cytotoxic oedema is characterised as intracellular swelling in neuronal, glial and endothelial cells (ECs) in the absence of any measurable breakdown of the BBB (Kimelberg 1995; Cotran et al. 1999). As cells swell there is a concurrent reduction in extracellular space (Duvdevani et al. 1995). Stover and Unterberg (Stover et al. 2000) propose that glutamate-mediated excitotoxicity contributes to cytotoxic oedema by causing derangement of cellular metabolism through which cells lose their potassium as a result of ionic pump failure and consequently gain large amounts of sodium. Water then follows by osmosis increasing intracellular fluid volumes. Cytotoxic oedema occurs primarily in the grey matter and is usually common with ischaemia (Kimelberg 1995).

Ionic Oedema

Young and Constantini (1994) propose a third type of oedema: ionic oedema. In ionic oedema brain parenchyma also gains an increase of fluid from the vasculature similar to that observed in vasogenic oedema. However, the mechanisms of the actions involved do not include BBB breakdown; rather the net increase in oedematous fluid results from increased activation of ionic transport processes between blood vessels and brain tissue as a result of TBI.

1.5.2 Temporal Profile of Oedema

Oedema time-course findings vary widely depending on the experimental design, and especially on the type of TBI injury assessed. There are also very few studies which have used female animals. Baskaya et al (1997) determined that despite a biphasic BBB opening following controlled cortical impact (CCI), the second opening of the BBB at three days post-injury did not contribute to a further increase in oedema formation that peaked at 24h post-CCI. They suggested that the second opening of the BBB might be for resorption of oedematous fluid into the blood and emphasised the importance of vasogenic mechanisms of oedema post-CCI. Chen et al (1996), using an acceleration–impact injury model in mice, found that the time of peak disruption of the BBB preceded that of oedema (4h versus 24h), but that oedema was present as early as 4h, peaked at 24h, and significantly decreased at 7d post-TBI. They posited that oedema observed at 24h could be attributed to BBB breakdown and hence be of vasogenic origin. Similarly, Duvdevani et al (1995) noted a difference in duration of BBB opening and oedema formation following CCI. While BBB extravasation

peaked at day three and was still observed at day 10 post-CCI, oedema had significantly dissipated by day three and resolved by day seven post-CCI. They concluded that BBB repair was less rapid than oedema resolution.

Finally, a review of studies using the impact–acceleration model of diffuse TBI (Foda et al. 1994) suggests that oedema is a function of time and injury severity, with different types of oedema occurring across time and increased oedema occurring with more severe injury (Marmarou et al. 1994). Barzo et al (1997b) argue that within this model, at least three forms of oedema contribute to increased tissue fluid following TBI: vasogenic, ischaemic (cytotoxic), and neurotoxic (ionic). They demonstrate a biphasic pathophysiological response to trauma with chiefly vasogenic oedema forming in the first hour post-injury and peaking at 24h. Subsequently, a second, more extensive and slower intracellular oedema is noted, which formed within 1h post-injury and became dominant at 1–2 weeks post-trauma. In contrast to previous findings (Baskaya et al. 1997), Marmarou's laboratory proposes that the actual contribution of vasogenic oedema to the observed post-traumatic oedema may be overemphasised in quantitative terms (Barzo et al. 1996; Barzo et al. 1997b; Beaumont et al. 2000). Nonetheless, their studies do emphasise that this vasogenic component may actually be permissive for subsequent cytotoxic oedema formation.

1.5.3 Blood–Brain Barrier Permeability

The BBB is a crucial element in maintaining brain homeostasis (Chen et al. 1996). Brain capillary ECs form the BBB, which is designed to keep toxic substances out of the brain (Rubin et al. 1999). The permeability of the BBB is primarily a function of the

intercellular, occluding tight junctions between the ECs (Kimelberg 1995). Astrocytes, once considered part of the BBB, appear to have a primary role in signalling the ECs to form the tight junctions (Marieb 1998). While the BBB prevents entry from blood to brain of almost all molecules except those that are small and lipid-soluble, there are some hydrophilic molecules that can cross the BBB through active transport processes (Rowland et al. 1992). For example, a D-glucose transport system is heavily present in the BBB as constant glucose is vital for brain energy metabolism (Kimelberg 1995). Thus, the BBB is physiologically permeable. In addition, not all areas of the brain possess the BBB. The pituitary gland, the pineal gland and some parts of the hypothalamus appear to lack BBB. Indeed, it seems that this paucity allows circulating hormones to reach secretory neurons in the brain, completing the feedback regulation circuits of the neuroendocrine systems (Goldstein et al. 1986).

Temporal Profile of Blood–Brain Barrier Opening

As mentioned above, increased BBB permeability is implicated in vasogenic oedema following TBI that can lead to raised ICP (Kimelberg 1995). This pathological BBB breakdown is also associated with secondary, delayed neuronal death mediated by inflammatory processes (discussed below) (Preston et al. 2001). Several experimental studies using a variety of TBI models have outlined a temporal profile for BBB opening (Duvdevani et al. 1995; Fakuda et al. 1995; Barzo et al. 1996; Baskaya et al. 1997; Whalen et al. 1999; Baskaya et al. 2000). These studies report disparate findings primarily as a function of the TBI model used, which makes comparisons difficult. For example, Whalen

et al. (1999), using Evans Blue (EB) extravasation following CCI injury to rats, charted BBB permeability as maximal at 1h and 4h post-CCI with a subsequent 50% decrease at both 8h and 24h. Studies using the same species, injury model (CCI) and EB dye recorded a bi-phasic opening of the BBB (Baskaya et al. 1997; Baskaya et al. 2000). The first peak was noted at 4h in the injury-site cortex, adjacent cortex, ipsilateral hippocampus and contralateral cortex and then at 6h in all brain regions. Additionally, Baskaya et al (1997) reported that EB extravasation was still significant in the ipsilateral cortex of injured rats, compared to controls, at one and two days post-CCI with oedema peaking at 24h. At three days, a second BBB opening occurred, which was significantly different from the 24h post-trauma time point. In contrast, Fakuda et al (1995), using lateral fluid percussion injury (FPI) in rats, demonstrated three different time-contingent patterns: transient, prolonged, and delayed abnormal BBB opening. Duvdevani et al (1995) induced medial frontal cortex contusion TBI injury to rats and found the EB was detected in the injured area at 2h post-TBI, significantly increased by day three, and could not be detected at 10d post-TBI. Of particular interest to this project is Barzo et al.'s (1996) findings using MRI to measure the time course of BBB opening following Marmarou et al's (1994) impact–acceleration TBI model in rats. These authors reported a rapid and transient BBB opening post-TBI that is immediately apparent following injury with duration of only 30 minutes post-impact. However, delayed peaks and later time points were not assessed, as this study only covered the first 2h post-TBI.

Given that BBB permeability and oedema formation are closely linked following TBI (Kimelberg 1995), it would seem pertinent to examine the temporal relationship between BBB opening and oedema formation. In addition, such a comparison may help elucidate the complexity of vasogenic and cytotoxic oedema formation following TBI.

1.5.4 Cerebral Homeostasis

Several features of brain homeostasis and anatomical design are critically compromised as a result of oedema following TBI, which can lead to a fatal rise in ICP and subsequent ischaemia (Lezlinger et al. 2001). Firstly, the brain is encased in a bony skull, which provides protection for the brain but allows very little volume expansion of brain parenchyma (Grande et al. 1997; Cotran et al. 1999). This means that limited space is available to accommodate an increase in tissue volume.

Secondly, the volume of CSF circulating around the brain provides buoyancy support within the rigid skull, and can be reduced if, for example, blood volume enlarges (Nolte 1999). This compensatory mechanism for controlling the CSF volume in the brain assists in maintaining adaptive ICP levels within the brain vault (Zwienenberg et al. 2001).

Thirdly, a copious and constant CBF is crucial because the metabolically active brain is unable to store the essential nutrients, oxygen and glucose it requires (Baynes et al. 1999). While the mechanisms controlling CBF are not fully understood, autoregulation plays a significant role in maintaining steady cerebral perfusion pressure (CPP). CPP is defined as the mean arterial blood pressure (MABP) minus the ICP, and is vital in maintaining normal brain

function (Cifu et al. 1999).

Impaired Autoregulation

Autoregulation of CPP (60–150mm Hg MABP in humans) involves the cerebral vasculature automatically adjusting blood flow to brain tissue at any given time, depending on local conditions. Calibre changes in the cerebral vessels depend on metabolic factors (such as oxygen and carbon dioxide levels, concentrations of potassium and hydrogen ions, prostaglandins and other inflammatory mediators/modulators) and myogenic factors, i.e., muscle mechanisms, which help keep perfusion pressure constant either by constricting or dilating vasculature tissue as required (Zwienenberg et al. 2001). Impaired cerebral autoregulation following TBI leads to increased susceptibility of the brain to secondary injury. Inconsistent alterations in autoregulation has been reported in patients following TBI (Bouma et al. 1995) as well as in rats following weight–drop induced severe TBI (Engelborghs et al. 2000).

Vascular calibre changes not only affect the CPP but also alter the cerebral blood volume (CBV), which is determined by vascular diameter. CBV appears to be a significant factor determining ICP (Zwienenberg et al. 2001). The volume of intracranial contents, such as CBV, CSF and brain parenchyma, regulates ICP. Since these compounds are interdependent, alterations in any of them affects the compensatory properties of each component leading to reduced buffering effect of the CSF (Zwienenberg et al. 2001). For example, enlargement of brain parenchyma due to oedema can cause a subsequent rise in ICP, thus producing a drop in CPP followed by a decrease in CBF (Engelborghs et al.

2000). This pathological combination can lead to poor outcome following TBI (Cifu et al. 1999).

1.5.5 Treatment

Despite the often grim consequences of oedema formation, there is no especially effective treatment in current clinical practice (Finklestein et al. 2001; Melton 2001). Treatments to date, which include mannitol, corticosteroids, hyperthermia, barbiturates and drainage of CSF, have had either limited success or been completely ineffective (Roof et al. 1996; Melton 2001). While corticosteroids had limited treatment success, more recent research involving the female hormonal steroids have been encouraging, showing anti-oedematous effects of progesterone following stroke and ischaemic injury (Chen et al. 1999; Kumon et al. 2000), CCI injury (Roof et al. 1992; Roof et al. 1993; Roof et al. 1996; Galani et al. 2001) and bilateral medial frontal cortex injury (Wright et al. 2001; Shear et al. 2002). Briefly, results from this research suggest that: (a) progesterone administered after cortical contusion brain injury attenuated oedema in both female and male rats (Roof et al. 1992); (b) reduced cerebral oedema is associated with circulating progesterone without the need for oestrogen (Roof et al. 1993); (c) progesterone does not appear to attenuate BBB breakdown (Duvdevani et al. 1995); (d) progesterone treatment delayed 24h after cortical contusion injury was still effective in reducing oedema in both female and male rats (Roof et al. 1996) and (e) progesterone significantly reduced oedema in male rats after bilateral medial frontal cortex injury (Wright et al. 2001). In addition, Roof et al (2000b) found that CBF preservation underlies the oestrogen-enhanced survival following impact–acceleration

TBI, suggesting oestrogen's putative antioxidant effects as one of the most important factors reducing vasogenic oedema.

The underlying mechanisms through which oestrogen and progesterone may work to reduce oedema have not been fully elucidated. However, Roof et al (1993) posit that possible neuroprotective mechanisms may include progesterone's (a) ability to inhibit active ion uptake through Na+, K+ ATPase; (b) ability to inhibit vessel growth associated with leaky BBB function after TBI; (c) actions as a free radical scavenger thus mediating lipid peroxidation and, (d) ability to modulate levels of vasopressin.

1.6 Female Sex Hormones

The sex steroid hormones, oestrogen and progesterone, are crucial to the regulation of all aspects of female reproductive activity. Together, they act in the hypothalamus, pituitary, ovary, and uterus to coordinate cyclical neuroendocrine gonadotropin production, ovulation, and uterine development in preparation for pregnancy (Joels 1997). Both hormones are also vital for postnatal mammary gland development involving oestrogen in morphological changes in the breast in puberty, and progesterone in pregnancy. Moreover, oestrogen and progesterone are involved in a broad variety of physiological functions (Inoue et al. 2002) such as male fertility (oestrogen), bone formation, as well as functions of cardiovascular, immune and central nervous systems (Graham et al. 1997; McEwen et al. 1999).

Steroid hormones form one of the four subclasses of lipids (Vander et al. 2001). The biosynthetic source of all steroids is cholesterol and the chemical structure common to all steroid hormones is the steroid nucleus (see Figure 1.3), a lipophilic tetracyclic hydrocarbon (Strobl 1994). Other examples of steroids include cortisol and aldosterone secreted by the adrenal cortex, and testosterone secreted by the testes (Guyton et al. 2000). Although only small amounts of steroid hormones are present in the body, they are crucial to homeostasis and without sex hormones reproduction would be impossible.

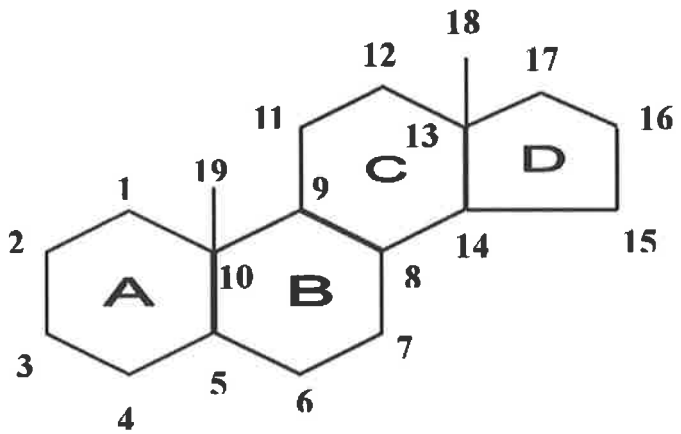


Figure 1.3 Steroid nucleus.

1.6.1 Structure

All oestrogens that occur naturally in animals have an estrane nucleus (See Figure 1.4) consisting of an aromatic A ring and one additional carbon atom at the 18 position of the steroid nucleus. Similarly, the pregnane nucleus (See Figure 1.5) is the same in all naturally occurring progestins. Progestins have four additional carbon atoms at positions 18, 19, 20, and 21 of the common steroid nucleus. The different biological activities and pharmacological properties of the oestrogens and progestins are determined by the presence of various substituents on the estrane and pregnane nucleus respectively.

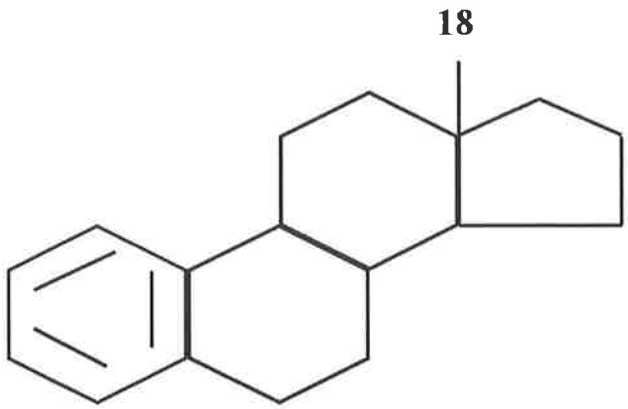


Figure 1.4 Estrane nucleus.

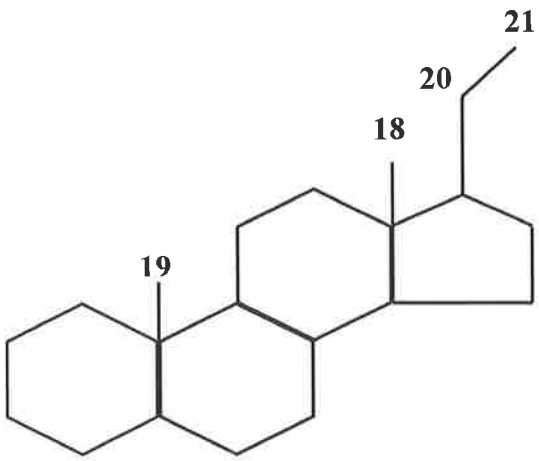


Figure 1.5 Pregnane nucleus.

In normal non-pregnant females, oestrogens are secreted in major quantities by the ovaries, although minute amounts are also secreted by the adrenal cortices. In pregnancy, the placenta also secretes vast quantities. Three main oestrogens are present in the plasma of the human female: 17β-estradiol, estrone and estriol. The principal oestrogen secreted by the ovaries is 17β-estradiol. Estrone, which is ten times less biologically active than estradiol (Strobl 1994) is mostly formed in the peripheral tissues from androgens secreted by the adrenal cortices and by the ovarian thecal cells. Estriol, which is the weakest of the oestrogens and present in high levels in the urine of pregnant women, is an oxidative product derived from estradiol and estrone (Guyton et al. 2000).

Progesterone is the most important naturally occurring progestin although another progestin, 17-alpha-hydroxyprogesterone, is also secreted in small amounts and has similar

effects. In normal non-pregnant females, progesterone is secreted in significant amounts by the corpus luteum during the latter half of each ovarian cycle. Only minute amounts of progesterone appear in the plasma during the first half of the ovarian cycle, secreted almost equally by the ovaries and the adrenal cortices (Guyton et al. 2000).

1.6.2 Synthesis

Oestrogens and progestins are steroids synthesised in the ovaries (gonads) primarily from cholesterol, which is transported through the blood bound mostly to plasma albumin and specific oestrogen- and progesterone-binding globulins (Guyton et al. 2000). A small amount of these steroids is also formed from acetyl coenzyme A. Activation of steroid hormone synthesis is a multi-step process, which involves stimulation of both hydrolysis of cholesterol esters and uptake of cholesterol into the mitochondria of cells in the target organ. Cholesterol undergoes side chain cleavage and oxidation yielding pregnenolone (Mathews 1990). These two hydroxylation reactions, as well as the subsequent hydroxylation in steroid hormone biosynthesis, involve mixed function oxidases, which utilise oxygen, nicotinamide adenine dinucleotide phosphate (reduced) (NADPH), and cytochrome P450 side-chain cleavage enzyme (P450scc). Peripheral sites of oestrogen synthesis include the liver, kidney, brain adipose tissue, skeletal muscle, and testes. The testes and adrenal gland also secrete small amounts of progesterone.

Progesterone, which is the precursor to all other steroid hormones, is synthesised directly from pregnenolone (Rupprecht et al. 1999). Dehydrogenation of pregnenolone yields progesterone, which has its own biological activity. The subsequent reactions primarily

involve hydroxylation and loss of the remaining two-carbon side chain (Mathews 1990). Following synthesis of progesterone and the male sex hormone, testosterone, almost all the testosterone and much of the progesterone are converted into oestrogens in the ovaries prior to release by the granulosa cells during the follicular phase of the ovarian cycle. During the luteal phase of the cycle, surplus progesterone is formed and is unable to be fully converted, which accounts for the large progesterone secretion at this time. The biosynthesis of oestrogen involves the conversion of pregnenolone to androstenedione and testosterone. The process involves the removal of the angular methyl group at C-19 under the action of P450c1919-aromatase. The A ring undergoes two dehydrogenations as part of the reaction, and the characteristic1,3,5(10)-estratriene nucleus results (Baynes et al. 1999).

1.6.3 Metabolism

Hormonal concentration in the blood depends on its rate of secretion by the endocrine gland and on the rate of removal from the blood, either by excretion or by metabolic transformation (Vander et al. 2001) (See Figure 1.6). The rate of excretion and metabolism of the oestrogens and progestins is slow and takes several hours. Oestrogen and progestin are primarily metabolised in the liver and less extensively in the gastrointestinal tract, brain, skin and other steroid target tissues. The main pathways of oestrogen and progestin metabolism are hydroxylation, *O*-methylation, and conjugation with either glycuronic acid or sulphate. Estrone, estriol, and 2-methoxyestrone are the most prolific oestrogen urinary metabolites while progesterone is excreted as pregnanediol or the pregnanediol conjugate (Strobl 1994).

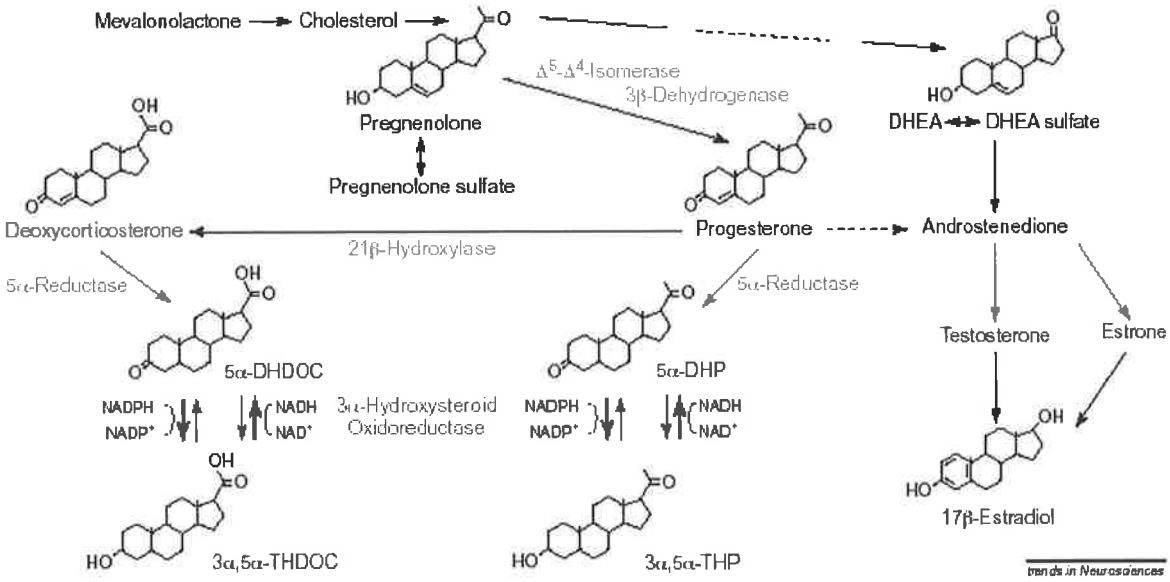


Figure 1.6 The biosynthesis and metabolism of neuroactive steroids (reproduced with kind permission of Rupprecht and Holsboer 1999).

1.6.4 Interaction with target cells

Steroid hormones impact on specific target cells through two distinct mechanisms: a) increasing the transcription of specific genes after binding to selective intracellular receptors, and b) acting directly on the cellular membrane. This section will only outline the first of the mechanisms involving intracellular receptors; a more inclusive discussion of target cell interaction is included in the section discussing the brain as a target cell (Section 1.6.9).

Steroid receptors belong to a 'super family' of related protein structures that reside within cells, including neurons, where they dimerise when bound to their specific hormone. However, tissues that express receptors for oestrogen and progesterone exhibit physiologically diverse responses to the same steroidal ligand. The structure of the

receptors influences the functional diversity demonstrated at the physiological level. These intracellular receptors are not active when there is no steroid messenger bound to them. However, once a message is received the receptors are transformed via dissociation from heat shock proteins, move to the nucleus and bind as homodimers or heterodimers to their respective response elements located in the nucleus (Rupprecht et al. 1999; Tsutsui et al. 2000; Rupprecht 2003). In this way the steroid hormone receptors act as transcription factors in the regulation of gene expression (Evans 1988). The resulting steroid-receptor complex then moves to the nuclear chromatin where the hormone binds to or activates particular portions of the DNA strands of the cell nucleus. This in turn initiates transcription of specific genes to form messenger ribonucleic acid (mRNA) (Marieb 1998; Guyton et al. 2000). The next step involves the mRNA being translated on the cytoplasmic ribosomes, producing specific protein molecules. These proteins include enzymes that promote the metabolic activities induced by that particular hormone, and in some instances, synthesis of structural proteins or proteins to be exported by the target cell. The actions involving intracellular steroid receptors are defined as genomic mechanisms meaning that they are usually delayed in onset and prolonged in duration (minutes to hours) limited by the rate of protein biosynthesis (Joels 1997; McEwen et al. 1999).

Although hormone-receptor binding is the essential first step, target cell activation by hormone-receptor interaction depends equally on three factors: 1) blood levels of the hormone, 2) the relative number of receptors for that hormone on or in the target cells, 3) the strength of the bond between the hormone and the receptor. Changes in all three factors

occur rapidly in response to various stimuli and changes within the body. For example, usually a large number of high-strength receptors produce a pronounced hormonal effect, whereas a smaller number of low-strength receptors result in reduced target cell response or endocrine dysfunction at the same blood hormone levels. In addition, receptors are dynamic structures. In some cases, the target cells form more receptors in response to rising blood levels of the specific hormones to which they respond; this is termed up-regulation. However, in other situations, prolonged exposure to high hormone concentrations desensitises the target cells, so that they respond less vigorously to hormonal stimulation. This down-regulation involves the loss of receptors and prevents the target cells from overreacting to persistently high hormone levels. As well, hormones may influence the number and affinity not only of their own receptors, but also of receptors that respond to other hormones. For example, progesterone induces a loss of oestrogen receptors (ERs) in the uterus, thus antagonizing oestrogen's actions. Conversely, oestrogen causes the same cells to produce more progesterone receptors (PRs), enhancing their ability to respond to progesterone.

1.6.5 Oestrogen Receptors

ERs exist as two structurally related subtypes, ERα and ERβ that are encoded by two distinct genes (Kuiper et al. 1996; Couse et al. 1999). Several isoforms of ERβ have been identified, the most recognised of these being ERβ1 and ERβ2 (Horlein et al. 1995; Montano et al. 1999). Both ERα and ERβ proteins share a high degree of amino acid conservation in the DNA binding domains and exhibit a lesser degree of homology in their

ligand binding domains. Two functionally distinct transcription areas have been identified in both proteins; AF1 located in the poorly conserved amino terminal domain and AF2 located in the ligand-binding domain. AF1 and AF2 can contribute both independently and together to receptor gene transcription activity in response to agonist ligands and to ligand-independent phosphorylation pathways of receptor activation, whereas their respective activities vary depending on cellular and promoter context (Tzukerman et al. 1994; Kraus et al. 1996; Endoh et al. 1999). Distribution of ERα and ERβ in the body is markedly different (McEwen et al. 1999). ERα demonstrate moderate to high expression in the pituitary, kidney, epididymis and adrenal organs, while ERβ show moderate to high expression in the prostate, lung and bladder. Both receptors are expressed greatly in the brain, ovaries, testes, and uterus (Imhof et al. 1996; Das et al. 1997; Nawaz et al. 1999).

ERs in the brain have been identified in the hippocampus, cerebral cortex, midbrain and brain stem using *in situ* hybridisation (Kuiper et al. 1998; Lee et al. 2001). High levels of ERα are expressed in the pituitary, hypothalamus, the hypothalamic preoptic area and amygdala. The sites of expression of ERβ in the brain are less certain (McEwen et al. 1999). This may be a function of the techniques used to identify ERβ. For example, in situ hybridisation data suggest widespread distribution of ERβ mRNA throughout much of the brain, whereas immunocytochemical studies show more restricted localisation of ERβ. Commercially available polyclonal antiserum to ERβ has produced a consistent pattern of strong cell nuclear label in the medial amygdala, paraventricular nucleus (PVN) and preoptic area, although in the hippocampus and cortex the results are not as consistent

(McEwen et al. 1999). In spite of the number of differences in the distributions of ERα and ERβ mRNAs, considerable overlap was seen in the preoptic area, bed nucleus of the stria terminalis and throughout the lower brainstem (Kuiper et al. 1998). Studies using double-label immunohistochemistry and *in situ* hybridisation have shown colocalisation of ERα and ERβ in cells of the preoptic area, bed nucleus of the stria terminalis and amygdala. Of particular interest for this thesis is the discovery of ERβ in the neocortex, hippocampus and nuclei of the basal forebrain, areas known to be associated with learning and memory with ERα only minimally present (Shughrue et al. 1997).

1.6.6 Progesterone Receptors

PRs have been identified as two distinct isoforms, PR-A and PR-B that arise from a single gene. The difference between PR-A and PR-B lies in an additional sequence of amino acids at its amino terminus, which is present in PR-B and not contained in PR-A. The expression of both isoforms is conserved in rodents and humans and overlap spatiotemporally in female reproductive tissues. However, the amount of the individual isoforms varies in reproductive tissues as a function of developmental and hormonal status and during carcinogenesis (Guerra-Araiza et al. 2003). For example, the ratio of mRNA expression for PR isoforms is different in various rat brain regions (Kato et al. 1994; Camacho-Arroyo et al. 1998) and also depends on the oestrus cycle sexual development (Guerra-Araiza et al. 2000).

The interaction between PRs' and the progesterone ligand induces a complex cascade of events involving various proteins. PR-B has been shown to encode a third transcription

function, AF3, which is absent from PR-A. AF3 allows binding of a group of co-activators to PR-B that is not obtained by PR-A. Therefore PR-A and PR-B show different gene transcription properties: PR-A acts as a transdominant inhibitor (Mulac-Jericevic et al. 2000; Conneely et al. 2001) whereas PR-B usually acts as a transcriptional transactivator. PR-A and PR-B are co-expressed in most tissues, and their expression is up-regulated by estradiol, while progesterone down-regulates its own receptor expression.

PR activity in the brain appears much more complex than in the reproductive system, hence results obtained from transfection experiments cannot always be extrapolated. An *in situ* hybridisation study revealed that PR-A occurs mostly in the rat hypothalamus where the PR is preferentially induced by oestrogen, while PR-B is predominantly expressed in the cerebral cortex (Kato et al. 1993). In addition, results from a recent reverse transcription polymerase chain reaction (RT PCR) study suggest the PR isoform expression in the brain is differentially regulated by oestradiol in discrete forebrain regions (Jung-Testas et al. 1999). It was found that oestradiol induces both PR-A and PR-B in the hypothalamus, with a distinct location of PR-B in the preoptic area and PR-A in the hippocampus (Camacho-Arroyo et al. 1994).

1.6.7 Control of Hormone Secretion: Oestrogen and Progestins

Hormone secretion is modulated by environmental signals that are processed by the nervous system. For example, in mice, olfactory cues from other female mice can interrupt the normal oestrous cycle and lead to pseudopregnancies or periods of prolonged diestrus (Siegel et al. 1989). It has been shown that there is a tight relationship between nervous and

endocrine systems, which significantly influences behaviour. Environmental cues or behaviour trigger neural responses, which in turn activate particular parts of the endocrine pathways, followed by subsequent changes in related target cells that may be located in another part of the body.

Oestrogen and progestin homeostasis is controlled by the hypothalamo-pituitary-gonadal-axis. Secretory control of these sex hormones begins when the hypothalamus secretes gonadotropin-releasing hormone (GnRH), firstly at the onset of puberty causing the gonads to mature to the adult state. GnRH is not secreted continuously but rather in pulses with each pulse lasting several minutes and occurring every one to three hours (Guyton et al. 2000). GnRH secretion is in response to neural activity, which takes place primarily in the mediobasal hypothalamus, especially in the arcuate nuclei. GnRH is a 10-amino acid-peptide synthesised from a 92 amino acid precursor and transported to the anterior pituitary via the portal system. Signals from the limbic system to the arcuate nuclei appear to influence the secretion of GnRH either through modifying the intensity of GnRH release or the pulse rate (Guyton et al. 2000).

In the anterior pituitary, GnRH affects the synthesis and secretion of the gonadotropic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both glycoproteins from the same gonadotrophin cell type. In both sexes, FSH stimulates gamete (sperm or egg) production, while LH promotes production of gonadal hormones. In females, LH works with FSH to cause maturation of an egg-containing ovarian follicle. It then independently triggers ovulation (expulsion of the egg from the follicle) and promotes

synthesis and release of ovarian hormones (oestrogens and progesterone). In males, LH stimulates the interstitial cells of the testes to produce the male hormone testosterone (Baynes et al. 1999). The secretion of FSH and LH is also negatively modulated by levels of FSH, LH, inhibin, estradiol, progesterone and testosterone, through a negative feedback loop. In fact, estradiol can have both negative and positive effects depending on the stage of the reproductive cycle. When progesterone is available, the inhibitory effect of oestrogen is multiplied even though progesterone has little effect (Guyton et al. 2000). From the above discussion it is clear that the concentrations of FSH, LH, estradiol and progesterone vary greatly during the reproductive cycle.

1.6.8 Systemic Physiologic Effects of Oestrogen and Progestins

Oestrogens

The primary physiologic role of oestrogen is to cause cellular proliferation and growth of the tissues related to the sex organs and other tissues related to reproduction.

Effects on Reproductive Cycle and Organs

Increased oestrogen concentration promotes oogenesis and follicle growth in the ovary, and exerts anabolic effects on the female reproductive tract. As a result, the ovaries, fallopian tubes, uterus, and vagina increase in size and begin to function. The uterine tubes and uterus begin to exhibit enhanced motility; the vaginal mucosa thickens; and the external genitalia mature (Marieb 1998). Oestrogen promotes the proliferative phase of the uterine cycle, stimulates production of crystalline mucus conducive to sperm survival and

maintains the activity of fimbriae and uterine tube cilia.

In addition, oestrogen acts to promote ovulation by stimulating formation of FSH receptors on follicle cells, and acts with FSH to induce the formation of LH receptors on follicle cells. Oestrogen also stimulates capacitation of sperm in the female reproductive tract via its effect on vaginal and uterine secretions. During pregnancy oestrogens in conjunction with relaxin, a placental hormone, induces softening and relaxation of the pelvic ligaments and pubic symphysis (Marieb 1998; Guyton et al. 2000).

Effects on Secondary Sex Characteristics and Body Function

Estradiol, acting parallel with other hormones such as insulin-like growth factor-I (IGF-I), is responsible for long-bone growth in females and feminisation of the skeleton, especially the pelvis. Estradiol also contributes to breast development and maturation of the urogenital tract and the female habitus. In addition, in adults estradiol supports breast function, influences bone turnover, promotes hydration of the skin and production of pubic and axillary hair (Marieb 1998; Baynes et al. 1999; Guyton et al. 2000).

Effects on Metabolism

Oestrogen has a number of important metabolic effects. They appear to be partially responsible for the maintenance of the normal structure of the skin and blood vessels in women. Oestrogen decreases the rate of resorption of bone by antagonising the effect of parathyroid hormone on bone but does not stimulate bone formation. Oestrogen also appears to have important effects on intestinal absorption because it reduces the motility of

the bowel. In the renal system, oestrogen also stimulates sodium and water retention by the renal tubules thereby inhibiting diuresis, however, this effect is minimal and usually not significant except in pregnancy. Oestrogen is also responsible for the increased fat deposits in females in subcutaneous tissue, breasts, buttocks and thighs.

Progestins

Effects on the Reproductive Cycle and Organs

Similar to oestrogen, progestins begin to be secreted in the body from puberty onwards. The most important function of progesterone is to promote secretory changes in the uterine endometrium during the second half of the monthly female sexual cycle (luteal phase) to enable the implantation of the fertilised ovum. The endometrium swells and its secretory activity increases greatly. In line with this effect, progesterone secreted by the corpus luteum, is responsible for the rise in basal body temperature during the luteal phase of the menstrual cycle and a drop in progesterone secretion may contribute to changes in mood as seen in premenstrual tension (Baynes et al. 1999). If no ovum implants, the corpus luteum breaks down about two days before the end of the cycle and both oestrogens and progesterone secretion declines greatly. Menstruation is caused by this rapid decline in oestrogens and progesterone, especially the progesterone.

In addition to uterine changes, progesterone aids in the development of the lobules and alveoli of the breasts, causing the alveolar cells to multiply, enlarge, and become secretory in nature. Progesterone influences secretory changes in the mucosal lining of the fallopian tubes. This is necessary for nutrition of the fertilised ovum as it moves down the fallopian

tube before implantation (Guyton et al. 2000).

Effects on Metabolism

Progesterone has little effect on protein metabolism. It stimulates lipoprotein lipase activity and seems to favour fat deposition. The effects on carbohydrate metabolism are more obvious. Progesterone increases basal insulin levels and the insulin response to glucose. In the liver, progesterone promotes glycogen storage. Progesterone increases body temperature and promotes diuresis excreting sodium and water from the kidneys.

1.6.9 The Brain as a Target Organ

The CNS is one of the main target tissues for sex steroid hormones across the life span. During early development, sex steroids influence the survival, differentiation, and connectivity of specific neurones in both the brain and spinal cord (Baulieu et al. 2000). For example, in neonatal rats progesterone morphologically shapes cerebellum (Sakamoto et al. 2001). In adults, sex steroids primarily influence synaptic transmission in the brain (Baulieu et al. 2000). Gonadal steroid hormones that are produced in various endocrine organs can reach the brain via the BBB because of their lipophilic solubility and act on brain tissue through binding to their respective intracellular receptors within target cells (Keefe 2002). The genomic mechanisms of classical steroid intracellular binding to receptors have been described previously in this review. Recent evidence has also demonstrated that certain steroids may alter neuronal excitability across the cell surface through interaction with particular neurotransmitter receptors (Lambert et al. 1995; Rupprecht et al. 1999). Steroids exhibiting these properties have been defined as

'neuroactive steroids'. In contrast to genomic mechanisms, these actions are rapid requiring only milliseconds to seconds. However, there is no absolute delineation between genomic and non-genomic effects, especially in actions that involve onset times of minutes (McEwen 1991; McEwen et al. 1999). In addition to these recognised neural steroidal actions, more recent work (Baulieu 1991; Baulieu 1998) has also identified 'neurosteroids' as steroids that are synthesised in the brain from cholesterol (*de novo*) or released as metabolic products originated from other blood-borne steroidal precursors (Jung-Testas et al. 1999; Plassart-Schiess et al. 2001). Taken together, it is apparent that gonadal steroids acting through genomic mechanisms (modulating synthesis, release and metabolism of a number of neuropeptides and neurotransmitters), non-genomic mechanisms, (influencing electrical excitability, synaptic function, and morphological features), and neurosteroidal actions impact widely on neural function and plasticity through complex mechanisms (Baulieu 1998; Mellon et al. 2002; Rupprecht 2003).

Oestrogen in the Central Nervous System

While oestrogen's crucial role in reproduction has been elucidated, the complexity and variability of its cellular and molecular actions in the CNS are poorly understood and at times research results appear contradictory (Ramirez et al. 2001). Briefly, however, research has demonstrated that oestrogen exerts multifaceted effects in the brain and produces different effects on the male and female brain (Lee et al. 2001). Oestrogen has been shown to act on a number of neuronal systems including the cholinergic, serotonergic, noradrenergic and dopaminergic systems (McEwen et al. 1999), and appears to improve

learning and memory functions (Roof et al. 2000b; Shughrue et al. 2000). Moreover, it has been located in a number of brain regions other than the hypothalamus including the hippocampus, basal forebrain, amygdala, caudate-putamen, nucleus accumbens and substantia nigra, cortex, cerebellum and spinal cord (McEwen et al. 1999; Lee et al. 2001). Oestrogens play a crucial neurotrophic role in cell proliferation and differentiation, neuronal survival and synaptogenesis during early brain development (Lee et al. 2001) and later in the adult brain through facilitating collateral axonal sprouting in the hypothalamus (Cardona-Gomez et al. 2001) and has been found to promote synaptogenesis in the hippocampus of the female rat (Woolley et al. 1990). In summary, oestrogen is posited to regulate neuronal function through (a) classical genomic processes involving its ERs, whose activation leads to delayed and prolonged effects of gene expression causing the longer term neurotrophic effects, amongst others (Woolley 1999); and (b) the more rapid non-genomic effects mediated by various signalling pathways, including G protein-coupled and ionotropic receptor pathways and their respective first messengers (Lee et al. 2001; Kelly et al. 2003). For example, oestrogen can facilitate the cellular responsiveness to glutamate, at the level of both the NMDA and non-NMDA receptors (Deb 1999) and maintain intracellular calcium homeostasis (Green et al. 2000). Non-nuclear ERs found outside of the cell nuclei in dendrites, presynaptic terminals and glial cells may also couple to second messenger systems to regulate a variety of cellular events and signal to the nucleus via transcriptional regulators such as the cAMP-response-element-binding protein (CREB) (Behl et al. 1999).

Neuroprotective Effects of Oestrogen

Research involving *in vitro*, *in vivo* and epidemiological clinical studies suggests that oestrogen facilitates brain processes including cognition and memory (Costa et al. 1999; Steffens et al. 1999; Lee et al. 2001), and fine motor skills (McEwen et al. 1999). In addition, oestrogen has been shown to protect against stroke (Simpkins et al. 1997; Alkayed et al. 1998; Rusa et al. 1999; Fukuda et al. 2000; Wise et al. 2001) and may protect against degenerative diseases such as Parkinson's disease (Saunders-Pullman et al. 1999; Strijks et al. 1999) and Alzheimer's disease (Tang et al. 1996; Waring et al. 1999; Brinton 2001), mood disorders such as depression and schizophrenia (Fink et al. 1998; Osterlund et al. 1999), and stress (Komesaroff et al. 1998; Melcangi et al. 2001; Kelly et al. 2003). Although the mechanisms involved in the neuroprotective actions of oestrogen are unclear (Cardona-Gomez et al. 2001), recent research has pointed to a number of protective actions including possible antioxidant effects (Behl et al. 1995; Behl et al. 1999; Vedder et al. 1999) anti-apoptotic properties (Jover et al. 2002b; Monroe et al. 2002), reducing the cytotoxic effects of β amyloid (Goodman et al. 1996; Green et al. 2000), reducing glutamate excitotoxicity (Moosmann et al. 1999), and mediating the effects of the mitogen-activated protein kinase (MAPK) pathways (Singer et al. 1999). Neurotoxins, amyloid β-protein, glutamate, and NMDA can cause oxidative stress and subsequent neuronal death as seen in neurodegenerative diseases. Behl et al (1999) demonstrated that 17β, its stereoisomer 17α oestradiol and ethinyl oestradiol were protective against oxidative cell death *in vitro*, and showed that the oestradiol-induced antioxidant effects depended on the presence of the hydroxyl group in the C3 position on the A ring of the steroid

molecule and were not related to oestrogen receptor activity (Behl et al. 1997). Goodman et al (1996) also demonstrated significant neuroprotection of 17β- and estriol in cultured rat hippocampus neurons against glutamate toxicity, glucose deprivation, and amyloid β-peptide (Aβ) toxicity.

Similarly, Wise et al (2001) showed that low concentrations of oestradiol (1, 10 and 30nM) significantly protected explants of rat cerebral cortex from ischaemic and metabolic damage. Additionally, these authors demonstrated that both young and middle aged rats pre-treated with physiological levels of 17β oestradiol prior to middle cerebral artery occlusion had reduced infarct damage. Other animal studies, mentioned in the previous section, also provide evidence that oestrogen is neuroprotective against cerebral ischaemia. For example, Dubal et al (1999) using ovariectomised female rats found that estradiol decreased the extent of neuronal death following ischaemia by an oestrogen β-receptor-mediated effect on Bcl-2, a proto-oncogene that can block necrotic and apoptotic cell death. Moreover, Farhat et al (1996) showed that oestrogen increased cerebral perfusion after stroke thereby improving CBF. There is ample evidence showing that oestrogen improves CBF by increasing nitric oxide (NO) production following ischaemia (Pelligrino et al. 1998; Wang et al. 1999).

Progesterone in the Central Nervous System

Besides its influence on sexual and reproductive behaviour, growing evidence regarding progesterone and its metabolites demonstrates that this hormone influences brain function via steroidal (genomic), neuroactive (non- genomic) and neurosteroidal (*de novo*

synthesis) actions (Jung-Testas et al. 1999; Rupprecht et al. 1999; Bernardi et al. 2003). The enzymes responsible for progesterone biosynthesis, cytochrome P450, which converts cholesterol to pregnenolone, and 3ß-hydroxysteroid dehydrogenase (3ß-HSD), which converts pregnenolone to progesterone, have been identified in the brain (glial cells and neurones) and spinal cord of the rat (Baulieu et al. 1990). These enzymes have also been localised in glial and Schwann cells of the rat nervous system (Baulieu et al. 1996).

Briefly, progesterone has been shown to exert numerous effects in the brain at a molecular level although the biological or physiological consequences have not yet been fully elucidated (Rupprecht 2003). Progesterone (Ramirez et al. 1996) and its three reduced metabolites including allopregnanolone (Paul et al. 1992) modulate neuronal excitability through their interaction with the inhibitory γ-aminobutyric acid type A ($GABA_A$) receptors, which form ligand-gated ion channels (Weiland et al. 1995; Rupprecht et al. 1999). Other neurotransmitter receptors modulated by progesterone's actions include 5-HT_3 (serotonin), glycine, nicotinic acetylcholine and kainite (Mellon et al. 2002; Rupprecht 2003). Progesterone's significant effects on EAA functions in cerebellar Purkinje cells have also been demonstrated using iontophoretic, extracellular single unit recording techniques in rats (Smith 1991). The eclectic modulating effects are posited to possess anaesthetic (Selye 1942; Korneyev et al. 1996), anxiolytic (Rodgers et al. 1998), and anticonvulsant properties (Frye et al. 2000), and in addition, may influence sleep patterns (Lancel et al. 1997), memory (Flood et al. 1992; Baulieu et al. 2000; Rupprecht 2003) and depression (Molina-Hernandez et al. 2001). In the peripheral nervous system, progesterone

has been shown to modulate myelin protein synthesis in Schwann cells of the rat sciatic nerve (Koenig et al. 1995), possibly through autocrine actions by stimulating the synthesis of specific myelin proteins or lipids (Baulieu et al. 2000). These results suggest potential neuroprotective effects in multiple sclerosis (Gruber et al. 2003). Taken together, it would appear that there is promising evidence of a neuroprotective role for progesterone in the CNS.

Neuroprotective Effects of Progesterone

Neurotrophic and neuroprotective effects of progesterone have been demonstrated both in *in vitro* (Ogata et al. 1993; Baulieu et al. 2000) and *in vivo* studies involving spinal cord injury (Bernardi et al. 2003; Ghoumari et al. 2003), stroke (Jiang et al. 1996; Chen et al. 1999; Kumon et al. 2000; Cervantes et al. 2002; Bernardi et al. 2003) and neurodegeneration (Vongher et al. 1999). For example, progesterone has been shown to protect spinal cord neurons from glutamate toxicity (exposure for 15 minutes) *in vitro* (Ogata et al. 1993; Baulieu et al. 2000) and may involve the modulation of inhibitory ($GABA_A$) and excitatory (EAAs) neurotransmitter receptors (Paul et al. 1992). Similarly, following *in vivo* acute spinal cord transection injury in rats, progesterone restored choline acetyltransferase (ChAT) immunoreactivity and mRNA for the α3 catalytic and β1 regulatory subunits of neuronal Na, K ATPase, and enhanced GAP-43 mRNA expression possibly through paracrine or autocrine effects involving growth factors in injured cells (Labombarda et al. 2002). Following incomplete paraplegia injury, rats treated with progesterone showed better functional activity as assessed using the Basso-Beattie-

Bresnehen locomotor rating scale, and less tissue and white matter damage at the epicentre of the injury when compared with control animals (McAllister et al. 1999). In experimental stroke involving transient middle cerebral arterial occlusion in male rats, progesterone administration before or two hours after injury reduced infarct volume and improved functional outcome as assessed using the Zea Longa scale, compared with control animals (Jiang et al. 1996). Similar improvement in post-traumatic motor outcome was also reported in a study using rotarod test, Zea Longa and adhesive-backed paper tests for motor assessment (Chen et al. 1999).

1.6.10 Sex Differences Following Experimental Traumatic Brain Injury

Recent research findings following experimental TBI indicate that sex-specific differences occur in response to TBI (Emerson et al. 1993; Roof et al. 1993; Roof et al. 1999; Roof et al. 2000b; Bramlett et al. 2001; Goss et al. 2003; Djebaili et al. 2004) and these differences may be mediated by the female sex hormones, oestrogen and progesterone (Roof et al. 1992; Roof et al. 1993; Roof et al. 1994; Roof et al. 2000b; Finklestein et al. 2001; Goss et al. 2003). Similar findings have also been noted in experimental stroke (for review see (Roof et al. 2000b) and the growing evidence of the neuroprotective effects of oestrogen and progesterone suggests that these hormones may partially explain the sex differences in outcome following TBI. Stein and Hoffman (Goss et al. 2003) however, caution that the literature is still controversial, contradictory and equivocal and that dosage of hormone, timing and duration of treatment, sex of the participants and the specific treatment hormone used all impact on results.

For example, Emerson et al (1993) found that following FPI significantly more males survived than female rats and additionally males demonstrated significantly improved motor function when compared with the female animals. These authors suggested that oestrogen given pre-injury was protective in males but deleterious to the female animals. In contrast, using a similar FPI model Bramlett and Dietrich (2001) showed that intact females had significantly smaller cortical contusions when compared with ovariectomised females and male animals following injury and that endogenous circulating female sex hormones may mediate the histopathological results. Similarly, other studies involving FPI (outlined above in the Oedema section, Section 1.5) have demonstrated that progesterone given either as pre-treatment or up to 24h post-injury significantly reduced cerebral oedema (Roof et al. 1993; Roof et al. 1996). In addition, progesterone has also been shown to improve functional (Roof et al. 1994; Asbury et al. 1998; Grossman et al. 2000; Shear et al. 2002; Goss et al. 2003; Djebaili et al. 2004) and morphological outcome (Roof et al. 1994; Asbury et al. 1998; Goss et al. 2003; Djebaili et al. 2004), and reduce lipid peroxidation and subsequently apoptosis (Roof et al. 1997; Djebaili et al. 2004) following TBI.

Few experimental studies using acceleration–impact TBI models have been undertaken to investigate sex differences following TBI. Roof and Hall (2000a) in one study found that significantly more females than males survived following impact–acceleration injury, and that cortical blood flow was significantly higher in females when compared with male animals. Oestrogen treatment significantly increased cortical blood flow in ovariectomised females compared with untreated ovariectomised female animals and also significantly

increased cortical blood in oestrogen treated male rats compared with male vehicle control animals. In this study treated animals were administered oestrogen for two weeks prior to the induction of injury. Using a similar impact–acceleration injury model modified for mice Kupina et al (2003) reported that 20% of the male animals died immediately following induced injury while all the intact female animals survived. In addition the study showed that males demonstrated significant protein degradation and neurodegeneration within three days of induced TBI but in female animals the same injury was not seen until 14 days after injury. Taken together it is clear that sex differences occur following experimental TBI and that these differences may be mediated by oestrogen and progesterone which appear to act neuroprotectively following induced TBI.

1.7 Experimental Models of Traumatic Brain Injury

Experimental studies of TBI have relied on numerous animal species including rodents, cats, pigs, sheep and non-human primates to investigate *in vivo* injury mechanisms following induced brain injury (McIntosh et al. 1996). While there has been criticism of the use of rats in experimental TBI, because of their differences from human physiology and functional responses (Povlishock et al. 1994; Cenci et al. 2002), they remain an economic (size and cost) choice for research purposes and have been shown to possess the underlying neurophysiological and psychological mechanisms necessary for associative learning and to also possess some sensory capacity and motor skills common to both humans and non-humans (MacPhail 1996). This is especially important for TBI studies which use rats and have as their goal functional outcome assessment. *In vivo* animal models also provide a

more holistic view of the complex injury response following TBI, however no one model can replicate fully the extensive pathobiology of clinical TBI (Povlishock et al. 1994).

The choice of TBI injury model depends on the aim of the particular research and it is obvious that different injury models will reflect different aspects of the biomechanical, pathophysiological and behavioural characteristics of TBI. However, regardless of the device, each model of injury must meet certain scientific criteria to be considered appropriate for TBI research (Cernak et al. 2004). For example, the injury model must produce injury that can be replicated, quantified and reflects some characteristics of human injury; the mechanical force of the device needs to be controlled in such a way that it can be reproduced and measured independently; the severity of the induced injury should predict severity of the injury outcome; and, the injury outcome as measured by certain parameters including morphological, physiological, and biochemical means is related to the mechanical force involved in the induction of the injury (Cernak et al. 2004).

The following section will provide a brief summary of the injury characteristics of the three main *in vivo* models currently used to produce experimental TBI: FP, CCI and acceleration–impact (closed skull–weight drop) devices.

1.7.1 Fluid Percussion Injury

FPI represents a direct brain deformation TBI model whereby the energy from the impact is transferred to the brain parenchyma through a skull perforated by a projectile or a craniotomy (Cernak et al. 2004) and produces open, focal brain injury. It is commonly used for the study of TBI pathology, physiology and pharmacology in a number of animal

species including rodents, cats, pigs and mice (Kline et al. 2001). FPI induced TBI involves the application of a brief fluid pressure pulse to the exposed but intact dura and depending on the desired location of maximal injury, the position of the craniotomy can be either midline (McIntosh et al. 1987), or over one hemisphere inducing a lateral injury (McIntosh et al. 1989). This injury model has been found to produce clinically relevant injury characteristics (McIntosh et al. 1989; Kline et al. 2001) including concussive injuries using midline FP (Dixon et al. 1987) and hippocampal damage and cortical contusions using lateral FP (McIntosh et al. 1987). In addition changes in CBF (Muir et al. 1992), increased BBB permeability (McIntosh et al. 1989), axonal injury (Povlishock et al. 1985), and tissue shearing (Adams et al. 2000) have also been recorded following FPI. Functionally FPI produces measurable motor and cognitive deficits (Kline et al. 2001).

1.7.2 Controlled Cortical Impact Injury

CCI, also known as rigid percussion model, provides accurate biomechanical control (Gennarelli 1994; Povlishock et al. 1994; Cernak et al. 2004). This allows for measurement and analysis of the relationship between the applied injury parameters, that is, the force, velocity and tissue deformation and the amount of tissue damage and functional outcome deficit that may occur following CCI (Kline et al. 2001). In addition the precision of the model allows for thorough standardization and therefore more reliable comparisons of data can be made from different experimental animals (Povlishock et al. 1994). The controlled impact of this device is delivered to the intact dura covering the central or lateral hemispheres of the brain and causes deformation of the underlying cortex (Cernak et al. 2004) via a compressed-air driven metal piston in contrast to the fluid pressure pulse in FP models. CCI has been used in ferrets (Lighthall 1988), rats (Dixon et al. 1991) and mice

(Smith et al. 1995) and produces clinically relevant TBI (Kline et al. 2001). The pathobiology of CCI includes DAI (Dixon et al. 1991; Gennarelli 1994), coma (Gennarelli 1994), oedema (Baskaya et al. 1997) and alterations in CBF (Batchelor et al. 1995). Functional outcomes in animals following induced CCI demonstrate deficits in motor and cognitive behaviour (Kline et al. 2001).

1.7.3 Acceleration–Impact (Closed Skull–Weight Drop)

Weight drop acceleration–impact devices can be used on either an open skull which produces primarily focal injury or a closed skull which produces diffuse injury (Kline et al. 2001) and involves either unconstrained or constrained head control (Cernak et al. 2004). One of the more recent and more commonly used weight drop models was developed by Marmarou and colleagues (1994)for use with rats. This model produces graded injury dependent on the mass and the height from which the weight is dropped (Cernak et al. 2004). The primary value of this model is that it produces widespread and DAI that is similar to that present in clinical TBI in over 50% of severely brain injured patients (Foda et al. 1994). Additional pathobiology produced via this model includes impaired cerebral autoregulation (Engelborghs et al. 1997; Engelborghs et al. 2000), oedema and BBB compromise (Foda et al. 1994; Barzo et al. 1997b), and deleterious changes in energy metabolism (Vagnozzi et al. 1999). The motor and cognitive deficits (Heath et al. 1995) apparent in rats following induced impact–acceleration are comparable to those produced after induced FP and CCI injury (Cernak et al. 2004).

1.8 Synopsis

This thesis will investigate sex differences following experimental TBI in rats induced using an acceleration–impact injury model that produces diffuse brain injury. Recent research suggests that the female gonadal hormones, oestrogen and progesterone, may mediate the differences observed. Therefore the effects of these hormones following TBI are the primary focus of this thesis. Emphasis is firstly placed on establishing an optimal assessment protocol for functional outcome especially in relation to the (a) pre-training and timing of assessment of animals after injury and (b) choice of anaesthesia. Thereafter, this thesis will examine the effects, if any, of oestrogen and progesterone following diffuse TBI on selected functional and pathophysiological outcomes. Specifically physiological doses of either 17β-estradiol or progesterone will be administered to treatment groups which comprise intact female, ovariectomised female and male rats and compared with sesame oil vehicle rats matched to each treatment group. Outcome tests to be used to determine the effects of oestrogen and progesterone include motor, cognitive, behavioural, immunocytochemical, brain water content and BBB permeability.

A brief introduction will precede each experimental investigation, along with a summary of the methodological protocol which will be fully outlined in Chapter Two. Although each chapter will report results specific to that chapter, it is expected that many of the results will have implications not only for the present investigation but also for other aspects raised in the thesis. This will result in some overlap across the chapters in interpretation and discussion. Finally, a concluding general discussion will integrate the major conclusions drawn from each chapter.

CHAPTER 2

GENERAL METHODS

2.1 Ethics

All experimental protocols were approved by the Experimental Ethics Committees of the University of Adelaide (approval numbers M-77-2001), the Institute of Medical and Veterinary Science (approval numbers 35/02; 37/02) and James Cook University of North Queensland (approval number A490) according to guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council.

2.2 Animals

Sprague-Dawley rats were group housed by gender in a conventional rodent room on a 12h light–dark cycle, and fed and watered *ad libitum*. Rats were rested for two weeks after arrival at the animal facility before inclusion in any experiment (Waynforth et al. 1992). The exact numbers of animals used in each experiment in this thesis are detailed in the pertinent chapters. All animals within the stratified sex samples were randomly assigned to their particular treatment groups (intact female, ovariectomised female and male) prior to any training or injury induction.

2.2.1 Animal Preparation

Ovariectomy

Where required, female animals were surgically ovariectomised to eliminate the effects of endogenous circulating gonadal hormones. The procedure was performed when female animals were aged seven weeks and animals were subsequently left for approximately nine

weeks before TBI was induced. As well as permitting animals to achieve the desired weight range, the nine week delay ensured that any oestrogen or progesterone present prior to ovariectomy had been metabolised.

Animals were anaesthetised with halothane (2.5% induction followed by 1% maintenance), and placed on a thermostatically controlled heating pad to ensure that rectal temperature was maintained at 37°C. A bilateral ovariectomy was then performed by ligation and dissection of the ovaries (Harkness et al. 1995). A 1cm incision was made in the midflank area, the underlying tissue and muscles separated and the peritoneum punctured immediately above the ovarian fat pad. After exposing the fat pad, the ovary was located and removed by gentle blunt dissection, followed by ligation of the fallopian tube using surgical suture. The uterus and fat pad were then gently reinserted, the abdominal wall sutured, and the outer skin closed with surgical clips (9mm Autoclip wound clips, Becton Dickinson, USA).

Smearing

In selected studies, vaginal smearing was used to determine the stage of the oestrus cycle at the time of TBI in intact females. At the time of trauma, the vaginal epithelium was scraped with a disposable inoculating loop gently inserted into the vagina. The sample of cells was subsequently spread onto a clean microscope slide and flushed with 96% ethanol. The samples were then fixed and stained according to the Papanicolaou automated staining method (Bibbo 1991). The cell types in the smear were identified (leukocytes, nucleated epithelial cells, and/or cornified epithelial cells) using an Olympus light microscope (x20)

and the stage in the oestrus cycle (metestrus, diestrus, proestrus or oestrus) at the time of lavage determined based on the presence or absence of these cell types (Maeda et al. 2000).

2.3 Surgical Procedures

2.3.1 Anaesthesia

Pentobarbital

Pentobarbital (pentobarbitone sodium, 60mg/ml, Rhone Merieux Australia Pty Ltd) was obtained as an aqueous solution from Lyppard Veterinary Supplies (Townsville and Adelaide, Australia) and stored at room temperature. Male and female animals were administered pentobarbital via intraperitoneal injection using a 25 gauge 12.5mm needle at a dose of between 40 and 60mg/kg as specified in the individual chapters. Animals were restrained by grasping the loose skin of the back and neck and the needle inserted into the left caudal area of the abdominal cavity, thus avoiding any vital organs (Van Dongen et al. 1990; Waynforth et al. 1992).

Isoflurane

Isoflurane (Isoflo^{TM}, Abbott Australasia Pty Ltd) was obtained as a volatile liquid from Lyppards Veterinary Supplies (Townsville and Adelaide, Australia) and stored below 25°C away from direct heat and sunlight. General anaesthesia with isoflurane was induced by placing the animal in a transparent, plastic induction chamber and delivering 2–3% isoflurane in oxygen via a calibrated vaporiser at a flow rate of 1.2L/minute. Maintenance of anaesthesia was achieved via a rodent nose cone covering the mouth and nose and

delivering 1–1.5% anaesthetic in oxygen at a rate 1.2L/minute.

Halothane

Halothane (halothane, Rhone Merieux Australia Pty Ltd) was obtained as a volatile liquid from Lyppards Veterinary Supplies (Townsville and Adelaide, Australia) and stored below 25°C away from direct heat and sunlight. General anaesthesia with halothane was induced by placing the animal in a transparent, plastic induction chamber and delivering 2–3% halothane in oxygen via a calibrated vaporiser at a flow rate of 1.2L/min. Maintenance of anaesthesia was achieved via a rodent nose cone covering the mouth and nose and delivering 1–2% anaesthetic in oxygen at a rate 1.2L/minute.

Lignocaine

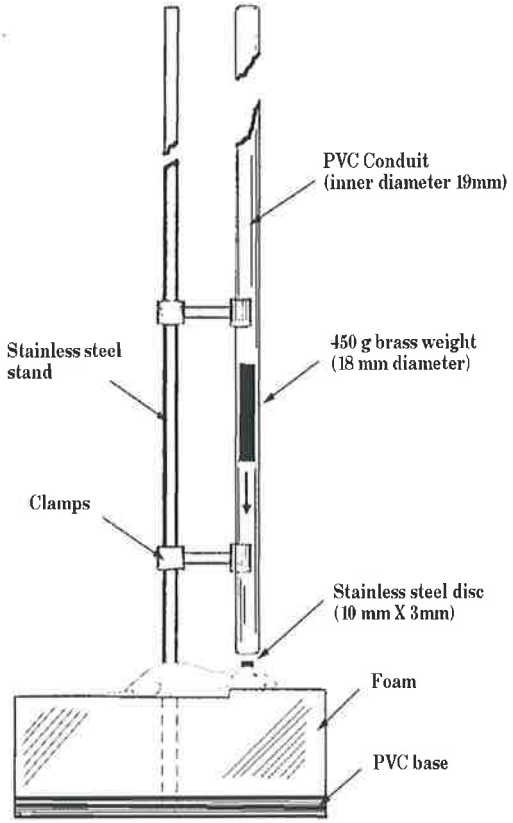
Lignocaine (lignocaine hydrochloride, 2%, Mavlab^{TB}, Australia) was supplied as an aqueous solution by Lyppards Veterinary Supplies (Townsville and Adelaide, Australia) and stored at room temperature. It was used in all animals to provide local anaesthesia prior to surgical incision. Lignocaine was administered subcutaneously via a 25 gauge, 12.5mm needle at a dose of 0.3ml to 0.5ml of 4% solution per injection site.

2.3.2 Impact–Acceleration Injury

Diffuse TBI was induced using the impact–acceleration model as developed by Marmarou and colleagues (1994). Animals were anaesthetised with pentobarbital, isoflurane or halothane as required until depth of anaesthesia was sufficient to suppress the pinched tail or pinched toe reflex. They were then placed on a thermostatically controlled heating pad

and lignocaine injected subcutaneously along the midline of the dorsal surface of the head. The skin overlaying the skull was subsequently incised at the midline and the temporalis muscles retracted to permit placement of a stainless steel disc (10mm in diameter and 3mm in depth) centrally between lambda and bregma. The disc was fixed to the skull using polyacrylamide adhesive and the adhesive allowed to set for approximately five minutes. Immediately prior to induction of injury, animals were removed from the anaesthesia and rapidly placed on a 10cm deep foam bed with the stainless steel disc placed directly beneath the weight guide tube (Figure 2.1). Injury was induced by dropping a 450g brass weight a distance of two metres down the guide tube and onto the stainless steel disc. Following injury, most animals demonstrated transient apnoea (<5min) and required manual resuscitation until respiration was stabilised. In cases of skull fracture, the animal was immediately euthanised with an overdose of sodium pentobarbital (200mg/kg) and eliminated from the study. Post-operatively, the steel disc was removed from the skull, the midline incision closed using surgical clips (9mm Autoclip wound clips, Becton Dickinson, USA) and a 5ml bolus of saline administered subcutaneously to ensure adequate hydration during the immediate recovery period. Animals were kept on the heating pad until spontaneous ambulation was commenced at which time they were returned to their cages. All aspects of the injury (weight of trauma device, height of drop, thickness and stiffness of the foam bed) were designed to reproduce the upper limit of the 'severe' level of injury as previously described (Foda et al. 1994; Heath et al. 1998).

(a)



(b)

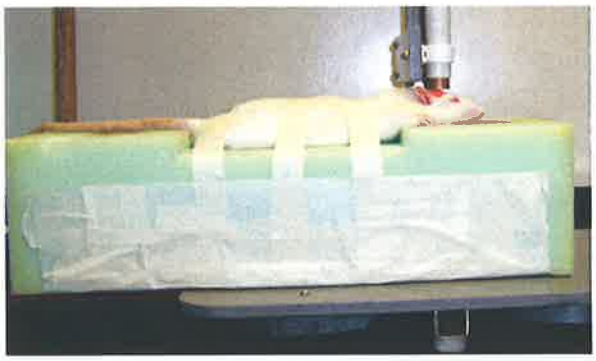


Figure 2.1 Photo and schematic representation of the (a) impact–acceleration device used to induce TBI with a significant DAI component, and (b) location of the 10mm diameter stainless steel disc.

2.3.3 Perfusion

Perfusion was performed using either 4% paraformaldehyde (tissue fixation) or saline (EB) as required. At pre-selected time points after injury, animals were anaesthetised with halothane and placed in the supine position on a wire rack. After adequate depth of anaesthesia was confirmed, a bilateral thoracotomy was performed to expose the heart, and a blunt 19 gauge, 37mm needle inserted into the apex of the left ventricle and guided into position within the ascending aorta. Heparin (5000U; David Bull Laboratories, Mulgrave, Victoria, Australia) was injected slowly and the right atrium incised to permit vascular flushing. Either 4% paraformaldehyde or saline (300–500ml as required) was then flushed through the animals to complete the perfusion process. Brains were subsequently removed for histology or EB determinations as required.

2.4 Drug Treatments

Drug treatments were administered by adding either progesterone or oestrogen to sesame oil. Control animals received sesame oil only.

2.4.1 Progesterone

Progesterone (Cat. No. p-7556, 100mg; Sigma-Aldrich, Sydney, Australia) was stored at room temperature and protected from light using aluminium foil. Progesterone was administered at a concentration of 1667µg/kg body weight dissolved in 66.7µl ethanol over low heat, and then added to 0.33ml sesame oil vehicle. This dose has been previously shown to result in physiological concentrations of serum progesterone (Gibbs 1999). The

dose was administered at 30 minutes post-trauma by subcutaneous injection to the neck area using a 23 gauge, 12.5mm needle. During assessment of functional outcome, progesterone was administered daily between 8am and 9am.

2.4.2 Oestrogen

17β-estradiol (Cat. No. E-8875; 5mg; Sigma-Aldrich, Sydney, Australia) was stored at room temperature and protected from light using aluminium foil. 17β-estradiol was administered at a concentration of 33.3µg/kg body weight dissolved in 0.33µl ethanol over low heat, and then added to 0.33ml of sesame oil vehicle. This dose has been previously shown to result in physiological concentrations of serum oestrogen (Gibbs 1999). The dose was administered at 30 minutes post-trauma by subcutaneous injection to the neck area using a 23 gauge, 12.5mm needle. During assessment of functional outcome, estradiol was administered daily between 8am and 9am.

2.4.3 Sesame Oil

Sesame oil (Cat. No. S-3547, 250 ml; Sigma Aldrich Pty Ltd, Sydney, Australia) was stored at room temperature. Thirty minutes after injury, 0.33ml of sesame oil was administered subcutaneously to the neck area of the animals using a 23 gauge, 12.5mm needle. During assessment of functional outcome, sesame oil was administered daily between 8am and 9am.

2.5 Functional Outcome

2.5.1 Rotarod

Rotarod scores were based on performance on a rotarod device described in detail by Hamm and colleagues (1994). The rotarod device consists of a motorised rotating assembly of 18 rods (1mm in diameter) upon which the animals were placed (Figure 2.2). In order to walk as the rods rotated beneath them; the animals were required to grip the rods, thus introducing a grip test component (Hall et al. 1988) to the assessment. Rotational speed of the device was increased from 0 to 30 revolutions per minute (rpm) in intervals of 3rpm for 10sec periods as detailed in Table 2.1. The duration in seconds at the point at which animals either completed the task, fell from the rods, or gripped the rods and spun for two consecutive revolutions rather than actively walking, was recorded as the rotarod score. Animals were pre-trained on the device twice per day over five days prior to injury to establish a normal, uninjured baseline. Assessment commenced 24h post-injury and was conducted each morning for 7–9 days depending on the experimental parameters of the particular study.

Figure 2.2 The rotarod device consisting of a motorised rotating assembly of 18 rods (1mm in diameter) upon which the animals are placed.

Table 2.1 Relationship between rotational speed and seconds on the rotarod device.

Rotarod speed (rpm)	Time (seconds)
0	10
3	20
6	30
9	40
12	50
15	60
18	70
21	80
24	90
27	100
30	110

2.5.2 Cognitive Outcome

The Barnes circular maze (Barnes 1979), as modified and described in detail by Fox et al (1998), was used to assess spatial reference memory following diffuse TBI (Figure 2.3). Animals were required to locate and enter a darkened escape tunnel in response to aversive

stimuli such as bright light and sound. Animals were placed under a cover in the centre of an elevated 1.2 metre diameter board containing 19 holes around the periphery. One of the holes is the entrance to a darkened escape tunnel that is not visible from the surface of the board. After activating a series a bright lights aimed at the board and switching on an aversive auditory stimulus, the cover is lifted and the latency in seconds for the animal to locate and enter the escape tunnel was recorded. Between each trail, the surface of the board and the escape tunnel was carefully wiped with distilled water to remove any olfactory cues. Animals were pre-trained on the device twice per day over five days prior to injury to establish a normal, uninjured baseline. Training on day one comprised a four minute adaptation period in the tunnel prior to the animals being exposed to the aversive light and noise stimuli. During subsequent training sessions animals were placed immediately in the start chamber for a period of 30 seconds. Assessment commenced 24h post-injury and was conducted each morning for 7–9 days depending on the experimental parameters of the study.

Figure 2.3 Barnes Circular Maze.

2.5.3 Open Field Activity

Spontaneous exploratory behaviour after diffuse TBI was assessed using the open field test (Giulian et al. 1975) (Figure 2.4). Previous studies have shown that a decrease in spontaneous exploratory activity is thought to reflect increased stress/anxiety (Vallee et al. 1997; Vallee et al. 2001; Larsson et al. 2002). No pre-training was required for this test, however, immediately prior to injury a baseline of animal activity was determined so as to compare post-injury behaviour. For both pre-injury and post-injury assessment, animals were placed in the centre of a one metre square box with 450mm high white panelled walls. The base of the box was subdivided into 100 equal 10cm squares. Over a five minute

period, the number of squares the animal entered was recorded with each square being eligible to be counted more than once should the animal leave the square and then return. Assessment commenced 24h post-injury and continued on alternate days for the duration of the particular experimental assessment parameters.

Figure 2.4 The open field test paradigm.

2.6 Oedema Measurements

Animals were re-anaesthetised at pre-selected time points with halothane and decapitated. Brains were rapidly removed from the skull, the olfactory bulbs and cerebellum discarded and the cortex and subcortex separated. The cortex and subcortex of each rat were placed

separately into pre-weighed and labelled glass vials with quick fit lids (to prevent evaporation) and weighed immediately for wet water content. The vials (glass lids removed) were then placed in an oven for at 100°C for 72hrs. Vials and brain segments were then re-weighed to obtain dry weight content. Oedema in each brain sample was calculated using the wet/dry method formula (Elliot 1949):

[(wet wt – dry wt)/wet weight] x 100].

2.7 Blood–Brain Barrier Permeability

BBB permeability after TBI was assessed by measuring extravasation of EB after intravenous injection (Kaya et al. 2001). One hour prior to the pre-selected time point, animals were anaesthetised and administered 2% EB (2 ml/kg) via the tail vein. Following 60 minutes of circulation time, animals were then re-anaesthetised and perfused with saline (see section 2.3.3) to remove intravascular EB dye. Perfusion continued until fluid from the right atrium was colourless. Immediately following perfusion animals were decapitated, the brains removed and both hemispheres weighed before being homogenised in 7.5ml of phosphate buffered saline using a Dounce glass/glass hand held homogeniser. Next 2.5ml of 60% trichloroacetic acid (Sigma, Sydney, Australia) was added and the sample mixed for two minutes on a vortex to precipitate the protein. The samples were subsequently cooled for 30 minutes and then centrifuged for 30 minutes at 1000g. The absorption of the resultant supernatant was then measured at 610nm using a UV/Vis spectrophotometer. EB was expressed in µg/mg of brain tissue using a previously obtained standard curve for EB

absorbance.

2.8 Histology and Immunohistochemistry

2.8.1 Haemotoxylin and Eosin Staining

At pre-selected time points, animals were re-anaesthetised with halothane, perfusion fixed in 4% paraformaldehyde and their brains removed following decapitation. Coronal sections were then cut within a Kopf rodent brain blocker (Kopf, USA) and the resultant 2mm sections embedded in paraffin wax. Consecutive 5μm sections were then sliced from selected wax blocks (see Figure 2.5) using a microtome and taken to water through zylene and ethanol and stained in Hemotoxylin for eight minutes. Following blueing in Scott's tap water substitute, the sections were counterstained in Young's Eosin (Eosin [yellowish] 15g, Erythrosin 5mg, Calcium Chloride 5g, Water 2L) for four minutes. The sections were then differentiated in tap water, dehydrated in ethanol, cleared in xylene and mounted in a synthetic mounting medium. Once dried and excess resin removed, the sections were viewed by light microscopy (Olympus).

2.8.2 Amyloid Precursor Protein Immunohistochemistry

At pre-selected time points, animals were re-anaesthetised with halothane, perfusion fixed in 4% paraformaldehyde and their brains removed following decapitation. Coronal sections were then cut within a Kopf rodent brain blocker (Kopf, USA) and the resultant 2mm sections embedded in paraffin wax. Consecutive 5μm sections were then sliced from selected wax blocks using a microtome and immunolabelled with amyloid precursor

protein primary antibody (1:2000; monoclonal antibody 22C11, Boehringer) by overnight incubation at 4°C. After washing the slices in PBS, slices were then incubated with IgG-HRP conjugated secondary antibody (1:400; Sigma-Aldrich) for 1h at room temperature, and the subsequent immunocomplex visualised using diaminobenzidine as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia).

2.8.3 Caspase-3 Immunohistochemistry

At pre-selected time points, animals were re-anaesthetised with halothane, perfusion fixed in 4% paraformaldehyde and their brains removed following decapitation. Coronal sections were then cut within a Kopf rodent brain blocker (Kopf, USA) and the resultant 2mm sections embedded in paraffin wax. Consecutive 5µm sections were then sliced from selected wax blocks using a microtome and immunolabelled with caspase-3 primary antibody (1:1000; polyclonal antibody, PharMingen) by overnight incubation at 4°C. After washing the slices in PBS, slices were then incubated with IgG-HRP conjugated secondary antibody (1:400; Sigma-Aldrich) for 1h at room temperature, and the subsequent immunocomplex visualised using diaminobenzidine as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia).

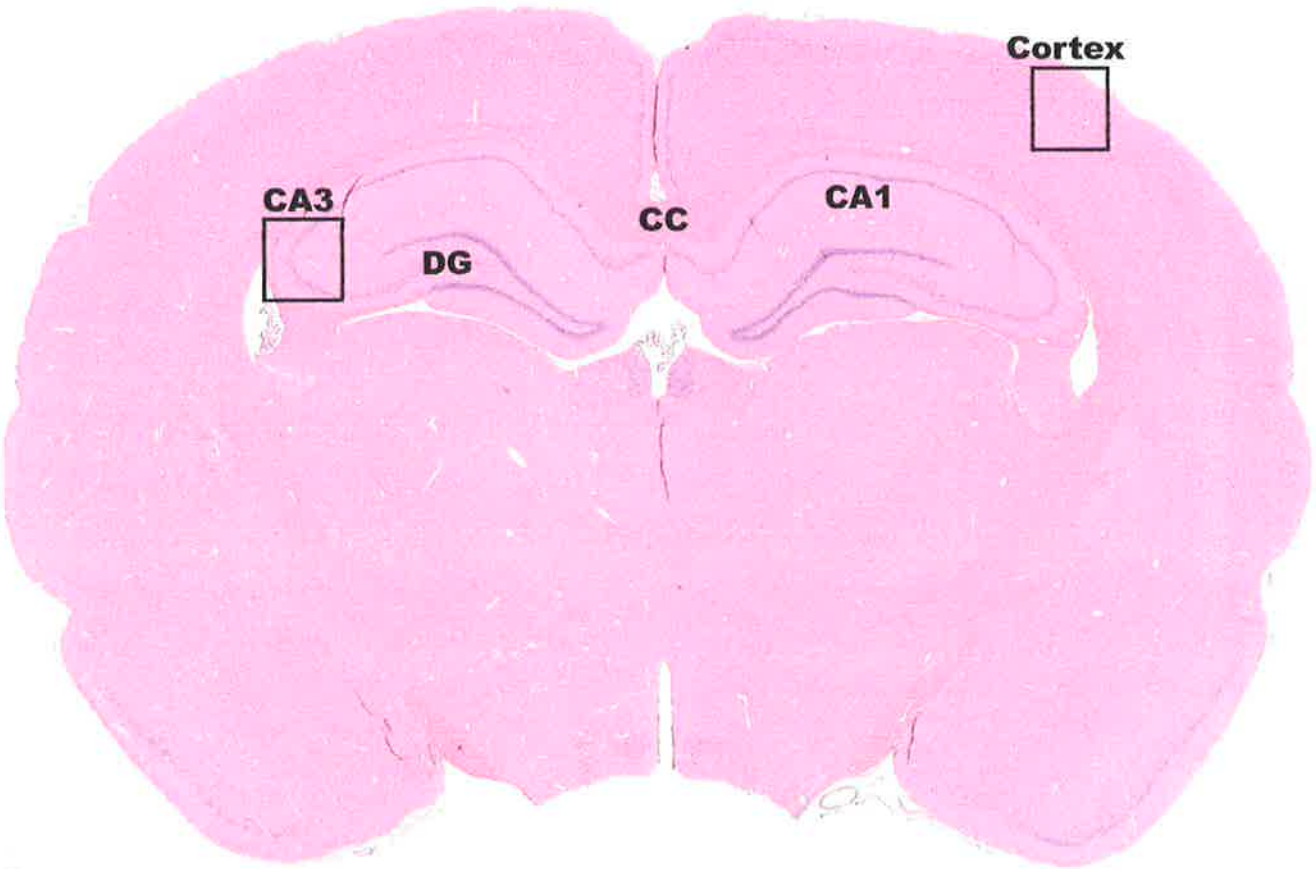


Figure 2.5 Haematoxylin and eosin stained section of rat brain showing regions of the cortex, hippocampus (CA1, CA3), corpus callosum (CC) and dentate gyrus (DG) used for morphological examination of dark cell change, caspase-3 and amyloid precursor protein immunohistochemistry.

2.9 Statistical Analysis

Data are shown as mean ± standard error of measurement (SEM). Repeated measures analysis of variance (ANOVA) followed by Tukey's HSD or Bonferroni *t*-tests were used to analyse functional outcome including Rotarod, Barnes maze and Open Field scores. One-way and two-way ANOVA followed by Tukey's HSD were used to determine brain water content, BBB permeability and COX-2 data. Significance in mortality was determined using Fisher's exact test. A p value of 0.05 was considered significant in all experiments. Prism (Graphpad^{TM} Software, SanDiego, CA) and SPSS for Windows Version 9.0 (SPSS Inc., Chicago, Illinois) statistics computer programmes were used for all analyses.

CHAPTER 3

EFFECTS OF DAILY VERSUS WEEKLY TESTING AND PRE-TRAINING ON THE ASSESSMENT OF NEUROLOGIC IMPAIRMENT FOLLOWING DIFFUSE TRAUMATIC BRAIN INJURY IN RATS

Previous studies have examined functional outcome following impact–acceleration induced diffuse TBI over an acute period (Heath and Vink 1995; 1998; 1999). What is not clear, however, is whether this injury model produces persistent functional deficits, and whether the assessment of acute outcome (<7 days post-trauma) is a valid endpoint as opposed to chronic outcome (21–30 days). This is an important point when designing studies that will examine the affects of sex related hormones on functional outcome. Furthermore, the effects of pre-training on functional outcome are unknown in this model of injury. This study therefore examines the effects of daily versus weekly pre-training and testing on the assessment of neurological impairment in male animals following diffuse TBI in rats.

3.1 Introduction

A number of experimental studies have characterised the development of functional neurological deficits following traumatic injury to the brain (for review, see Hamm 2001). It is clear that TBI results in the development of motor and cognitive deficits that may persist for a prolonged period of time after the traumatic event. Most of these studies have utilised models of injury that produce more focal damage, generally centred within a single hemisphere. However, it is becoming increasingly apparent that much of the clinical brain injury is of a more diffuse nature, with axonal injury occurring throughout the brain. Indeed, more than 80% of all cases of brain injury as a result of a motor vehicle accident show DAI (Adams et al. 1989). Even when considering all other forms of TBI, at least 60% of severe cases and 30% of moderate cases have now been shown to demonstrate significant DAI throughout the brain (Mittl et al. 1994; Blumbergs et al. 1995). Moreover,

there is a clear association between the severity of DAI and high morbidity, mortality and development of a persistent vegetative state following TBI (Foda et al. 1994; Povlishock et al. 1995). While some excellent experimental TBI models of DAI have been devised using large animals (Lewis et al. 1996; Smith et al. 1997), these models are difficult for most laboratories to implement and suffer from a lack of established functional outcome measures that are essential in the pre-clinical assessment of neuroprotective therapies. On the other hand, rodent TBI models have a well established battery of neurological tests available for assessment of functional outcome and are easy to implement in most laboratories. While a number of focal models produce some DAI, the rodent impact–acceleration model of diffuse TBI (Foda et al. 1994) has been shown to produce a significant amount of DAI, particularly in the white matter (Gennarelli, 1994; Povlishock et al. 1995), and has become widely adopted as a clinically relevant model of experimental diffuse TBI.

Initial characterisation of neurological deficits produced by diffuse TBI in rodents utilised daily tests in trained animals (Heath et al. 1995). The use of trained animals provided a baseline upon which to ascertain the recovery of function, whilst daily assessment permitted close monitoring of the recovery process. Subsequent pharmacologic studies (Heath et al. 1998; Deb 1999) used the same protocol with daily assessment in trained animals providing a rapid method to assess any positive effects of the drug intervention, while at the same time permitting correlations of neurologic outcome with early biochemical changes. With this assessment protocol, animals generally recover quickly

with a return to pre-injury function occurring within 10–14 days. As such, the ability of these models to produce persistent functional deficits has been questioned, particularly in relation to their relevance to clinical trauma. In contrast, persistent functional deficits have been demonstrated in the more focal rodent TBI studies including FPI (Pierce et al. 1998) and CCI (Dixon et al. 1999). However, these persistent functional deficits were noted using periodical assessment in untrained animals. There are therefore a number of variables that could affect the neurologic outcome following diffuse TBI, including training of animals and the use of daily versus weekly test paradigms.

Although the effects of trials and experience have been well described in focal brain injury models (Feeney et al. 1982; Schallert et al. 2000; Bland et al. 2001), such is not the case in diffuse TBI. The aims of the present study were therefore to establish whether diffuse TBI in rats produces chronic (21–30 day) functional deficits as assessed by periodical (weekly) functional tests, and thereafter, to determine whether training of animals prior to injury influences post-traumatic motor and cognitive outcome, including rate of functional recovery.

3.2 Methods and Materials

3.2.1 Experimental Design

All experimental protocols were approved by the James Cook University Experimental Ethics Committee according to the guidelines for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council. Adult male Sprague-Dawley rats (n=60; 350–400g) were randomly subdivided into two

equal groups. One group (n=30) served as an injury group while the other (n=30) was used as a sham control group (surgically prepared but not injured). Twelve animals in each group were pre-trained daily in both the motor and cognitive tasks for 10 days prior to injury while the remaining 18 animals in each group had no pre-injury exposure to the motor and cognitive assessment tasks. After injury, injured and sham animals (n=24/group) were assessed for motor and cognitive function either daily for 10 days after injury or weekly for four weeks after injury. Trained and untrained animals were equally divided between the groups (8 groups; n=6/group). The remaining 12 animals (six shams and six injured and untrained) were used for assessment of open field activity immediately prior to and after induction of injury.

3.2.2 Induction of Traumatic Brain Injury

Diffuse brain injury was induced using the impact–acceleration model as previously described (Foda et al. 1994; Heath et al. 1998). Briefly, adult male Sprague-Dawley rats (n=24; 350–400 g) were anaesthetised with sodium pentobarbital (50 mg/kg i.p.), intubated and mechanically ventilated on room air using a Harvard Rodent Ventilator. Thereafter, rectal temperature was maintained at 37°C with a thermostatically controlled heating pad. The skull was then exposed by a midline incision and a stainless steel disc (10mm in diameter and 3mm in depth) was fixed rigidly with polyacrylamide adhesive to the animal's skull centrally between lambda and bregma. The rats were subsequently placed on a 10cm foam bed and subjected to brain injury induced by dropping a 450g brass weight a distance of two metres onto the stainless steel disc. Such an injury has been previously

shown to produces severe DAI (Foda et al. 1994). Sham-operated controls (n=30) were surgically prepared but were not injured.

3.2.3 Assessment of Motor Outcome

Motor assessment was performed using the rotarod test which has been described as being one of the most sensitive tests to detect motor deficits in rodent brain injury (Hamm 1994; Hamm 2001). Briefly, animals were placed on a rotarod device consisting of a motorised rotating ensemble of 18 rods (1mm in diameter). The animal was required to walk on the rotating assembly as the rotational speed of the device was increased from 0 to 30rpm in intervals of 3rpm every 10sec. The duration in seconds at the point at which the animal completed the task (maximum of two minutes), fell from the rods, or gripped the rods and spun for two consecutive revolutions rather than actively walking, was recorded as the rotarod score.

3.2.4 Assessment of Cognitive Outcome

The Barnes circular maze (Barnes 1979) as modified and described in detail by Fox et al (1998) was used to assess spatial reference memory following diffuse TBI. In this test, animals were required to locate and enter a darkened escape tunnel in response to aversive stimuli such as bright light and a loud high-pitched auditory tone. Animals were placed under a cover in the centre of an elevated 1.2 metre diameter board containing 19 holes around the periphery. One of the holes was the entrance to a darkened escape tunnel that was not visible from the surface of the board. After activating a series a bright lights aimed at the board and switching on an aversive auditory stimulus, the cover was lifted and the

latency in seconds for the animal to locate and enter the darkened escape tunnel was recorded. Before and after each animal trial, the board and escape tunnel was carefully wiped with distilled water to remove any olfactory cues.

3.2.5 Assessment of Open Field Activity

Spontaneous exploratory behaviour after diffuse TBI was assessed using the open field test (Giulian et al. 1975). Previous studies have shown that a decrease in spontaneous exploratory activity is thought to reflect increased stress/anxiety (Vallee et al. 1997; Larsson et al. 2002). No pre-training was required for this test, however, immediately prior to injury a baseline of animal activity was determined so as to compare post-injury behaviour. For both pre-injury and post-injury assessment, animals were placed in the centre of a one metre square box with 450mm high white panelled walls. The base of the box was subdivided into 100 equal 10cm squares. Over a five minute period, the number of squares the animal entered was recorded with each square being eligible to be counted more than once should the animal leave the square and then return. Assessment commenced 24h post-injury and continued daily for seven days, then weekly for four weeks.

3.2.6 Statistical Analysis

Prism statistical software (Graphpad Software Incorporated, San Diego) was used for all statistical analyses. All data are shown as means ± SEM. A repeated measures analysis of variance (ANOVA) was used to determine group main effects. Differences between groups were analysed by two-way ANOVA followed by post-hoc Bonferroni *t*-tests. Rates of functional recovery were determined by linear regression and differences between groups analysed by analysis of covariance (ANCOVA). The level of significance was set at $p<0.05$.

3.3 Results

3.3.1 Motor Outcome

Rotarod assessed motor scores in trained animals after diffuse TBI in rats are shown in Figure 3.1A and 3.1B. In animals that were pre-trained on the rotarod for 10 days prior to injury, the pre-injury rotarod score was 112±3sec. In sham animals, there was no significant change in this score over the 10d daily observation period (Figure 3.1A). In contrast, injured animals subject to daily testing demonstrated a profound decline in rotarod score, falling to a minimum value of 40±10sec at 24h after injury ($p<0.001$ versus pre-injury and sham). Thereafter there was a gradual recovery in motor function over the 10 day assessment period, which is consistent with previously published reports (Heath et al. 1998). Using a weekly assessment schedule, pre-trained animals demonstrated persistent motor deficits after injury (Figure 3.1B). Trained animals demonstrated a pre-injury score of 108±4sec, which is similar to that observed in the trained animals used for daily

assessment. Injury in these trained animals then produced a profound decline in motor scores with no significant improvement over the four week assessment period. There was a highly significant difference between trained injured animals and trained sham animals at all time points ($p<0.001$).

In untrained animals tested for rotarod performance (Figure 3.2A and 3.2B), daily tested shams demonstrated a rotarod score of 49±7sec on day one, improving to a score of 111±3sec by 10 days (Figure 3.2A). This value is consistent with the pre-injury value after 10 days of training (Heath et al. 1998) and reflects the training curve in uninjured animals. In contrast to this normal training curve, untrained injured animals demonstrated a very slow improvement in motor function between day one and day 10, with rotarod scores over the entire post-injury period being below 40sec (Figure 3.2A). This difference between the sham and injured animals over the 10 day assessment period was highly significant at all time points ($p<0.001$) and may reflect an impaired ability of the untrained, injured animal to perform the motor task. A similar trend was observed in untrained, injured animals that were assessed weekly. Sham animals demonstrated a consistent improvement in rotarod performance with time whereas injured animals demonstrated no significant improvement in rotarod performance over the four week assessment period (Figure 3.2B). Significant differences between untrained injured animals and untrained sham animals ($0.001<p<0.05$) occurred after week two in the assessment period as the motor performance in the sham animals improved with time.

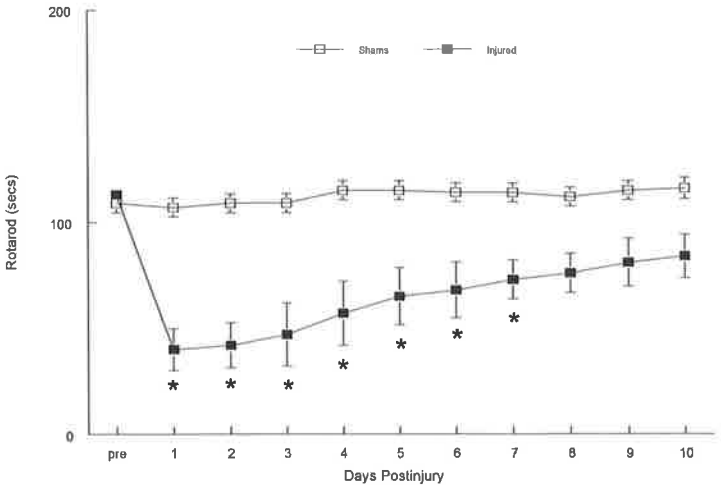


Figure 3.1A Rotarod assessed motor outcomes in pre-trained rats subject to diffuse TBI and assessed daily after injury (*p<0.05 versus shams).

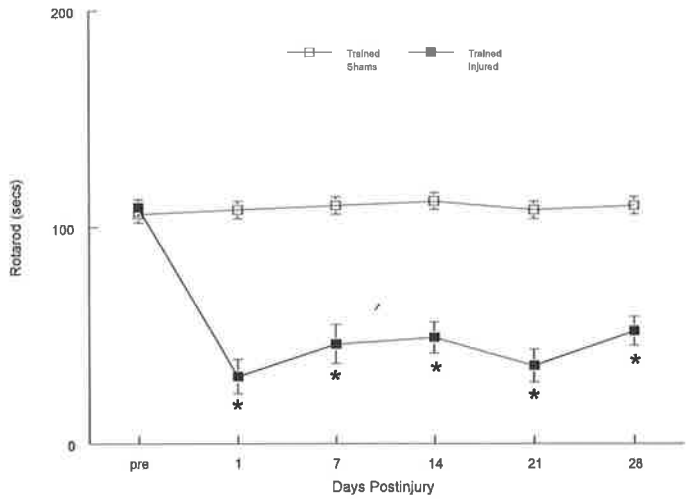


Figure 3.1B Rotarod assessed motor outcomes in pre-trained rats subject to diffuse TBI and assessed weekly after injury (*p<0.05 versus shams).

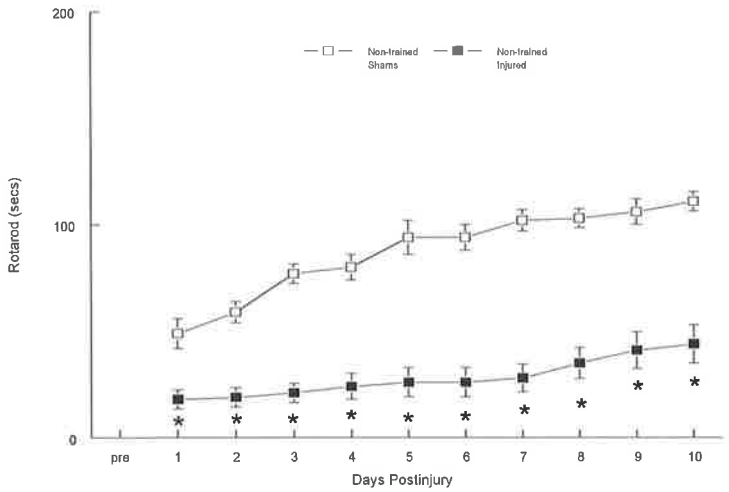


Figure 3.2A Rotarod assessed motor outcomes in untrained rats subject to diffuse TBI and assessed daily after injury. Untrained animals have their first assessment done at day one after injury (*p<0.05 versus shams).

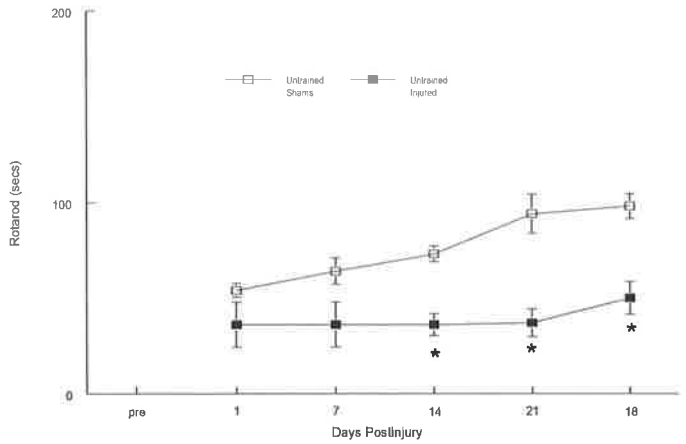


Figure 3.2B Rotarod assessed motor outcomes in untrained rats subject to diffuse TBI and assessed weekly after injury. Untrained animals have their first assessment at day one after injury (*p<0.05 versus shams).

With respect to rates of recovery in the rotarod test, data was fitted by linear regression and analysed for differences using analysis of covariance (Table 3.1). The rate of functional improvement in untrained injured animals tested daily was 2.8sec/day which was significantly less than the 5.2sec/day rate of improvement in trained animals tested daily ($p<0.001$). However, in weekly tested animals, the rate of recovery was between 3.0 and 3.2sec/wk irrespective of whether the animals were trained or otherwise. It was also noted that rotarod scores on day one in all groups were not significantly different (Figures 3.1A, 3.1B, 3.2A and 3.2B) indicating that the level of injury was comparable amongst all groups, and that pre-injury training did not affect animal performance on day one after injury.

3.3.2 Cognitive Outcome

Cognitive performance in trained animals following diffuse TBI are shown in Figure 3.3A and 3.3B. The 10 day pre-injury training program reduced the Barnes maze latency to 19±6sec. Injury in these trained animals produced a significant increase in Barnes maze latency at 24h (77±14sec; $p<0.001$) suggesting an impaired ability to escape the aversive stimuli (Figure 3.3A). Previous studies have shown that this impairment is not associated with any motor dysfunction in injured animals and that the increased latency represents a cognitive impairment (Fox et al. 1998). Thereafter, the performance in the Barnes maze improved back to pre-injury levels over the 10 day assessment period. As expected, trained sham animals did not demonstrate any increase in latency over time, and in fact, improved their performance with continued daily testing (Figure 3.3A). Significant differences

between sham and injured animals were observed on the first two days after injury ($0.001<p<0.01$), but disappeared as the injured group improved with time. With weekly assessment of cognitive function, pre-injury latency was 22±3sec which was similar to that observed in the trained animals used in the daily assessment. Injury in these animals again produced a significant increase in Barnes maze latency ($p<0.05$), however there was no subsequent improvement noted in these animals over the four week assessment period (Figure 3.3B). Trained sham animals maintained a pre-injury latency time over the four week assessment period which was significantly better than that observed in the injured animals ($p<0.001$).

In untrained animals assessed daily by the Barnes maze (Figure 3.4A), the latency at day one after injury was 124±30sec, with no significant difference between the injured and sham animals. Over the 10 day assessment period, both sham and injured animals improved their Barnes maze performance with time. Although sham animals improved their latency more than the injured animals over the 10 day period, there was not a significant difference between the two groups. In the weekly assessment (Figure 3.4B), untrained sham animals demonstrated a first day latency of the order of 120sec, which is similar to that observed in the daily tested groups. The sham animals then significantly improved their performance in the Barnes maze task over the four week assessment period such that a latency of only 23±5sec was recorded on day 28 ($p<0.05$). In contrast, injured animals did not show any significant improvement in their latency times over the assessment period. Although the differences between sham and injured animals was not

statistically significant at any individual time point, the lack of improvement with time in injured animals suggested the existence of persistent cognitive deficits following diffuse TBI in rats. This was confirmed by analysis of the rates of recovery in the Barnes maze data (Table 3.1), where significant differences were observed in rates of recovery between injured animals and sham animals. There were no significant differences in rates of recovery between trained and untrained animals within the daily or weekly tested groups suggesting that training did not affect rate of recovery. However, the degree of cognitive impairment on day one after injury was generally less in pre-trained animals than in untrained animals, with the untrained animals showing a significantly greater variance at each time point.

Thus, cognitive pre-training reduced measurement variability, and in contrast to motor outcome, resulted in improved performance immediately after TBI.

Table 3.1 Rate of functional improvement in trained and untrained rats following TBI.

	Trained	**Untrained**	**Shams (untrained)**
Daily Rotarod	5.2 sec/day	2.8 sec/day*	6.6 sec/day
Weekly Rotarod	3.2 sec/wk	3.0 sec/wk	12.2 sec/wk
Daily Barnes Maze	5.3 sec/day	6.6 sec/day	10.4 sec/day
Weekly Barnes Maze	3.9 sec/wk	3.3 sec/wk	23.2 sec/wk

* $p<0.001$ versus trained animals by ANCOVA.

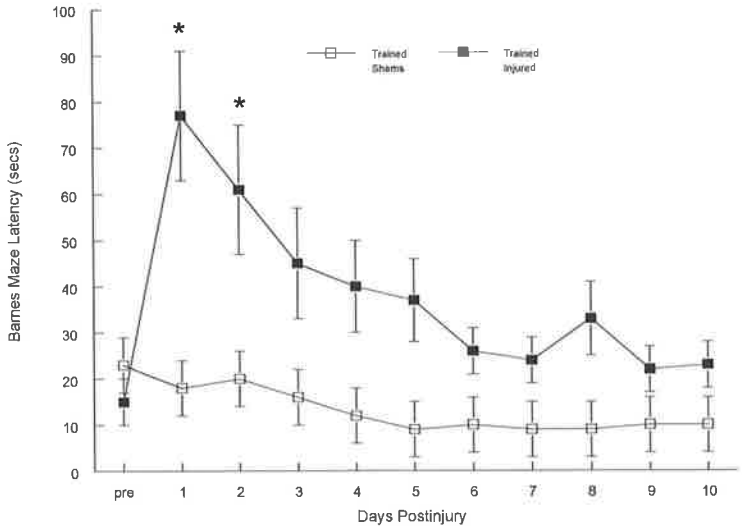


Figure 3.3A Barnes maze assessed cognitive outcomes in pre-trained rats subject to diffuse TBI and assessed daily after injury (*p<0.05 versus shams).

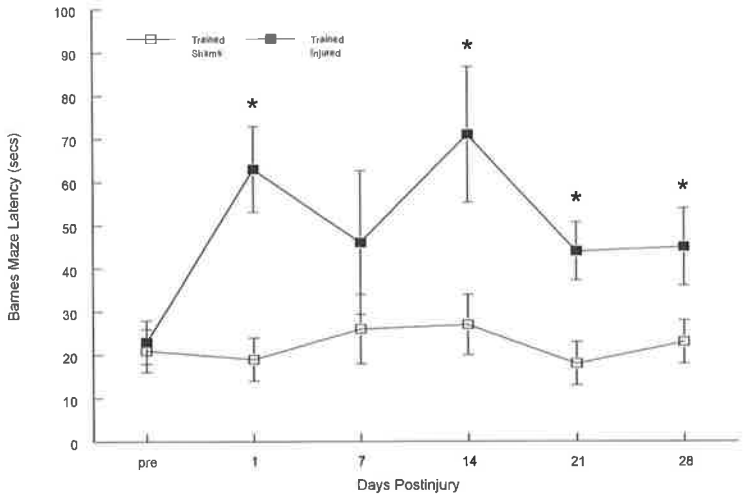


Figure 3.3B Barnes maze assessed cognitive outcomes in pre-trained rats subject to diffuse TBI and assessed weekly after injury (*p<0.05 versus shams).

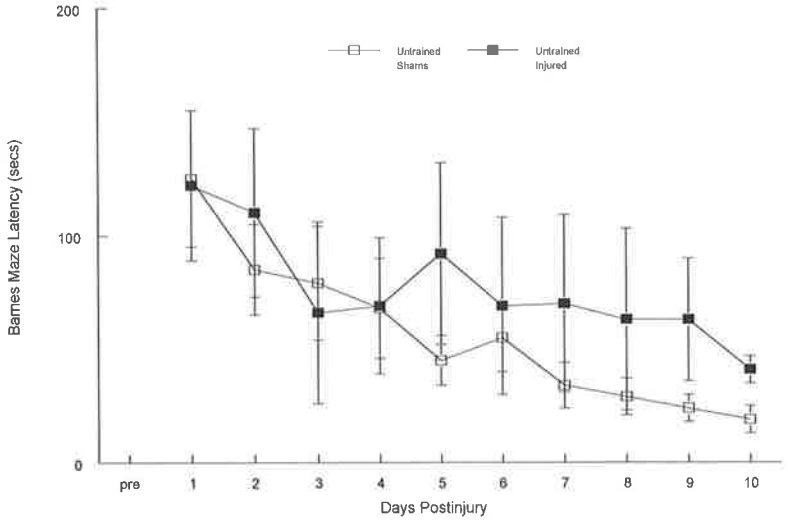


Figure 3.4A Barnes maze assessed cognitive outcomes in untrained rats subject to diffuse TBI and assessed daily after injury (*p<0.05 versus shams).

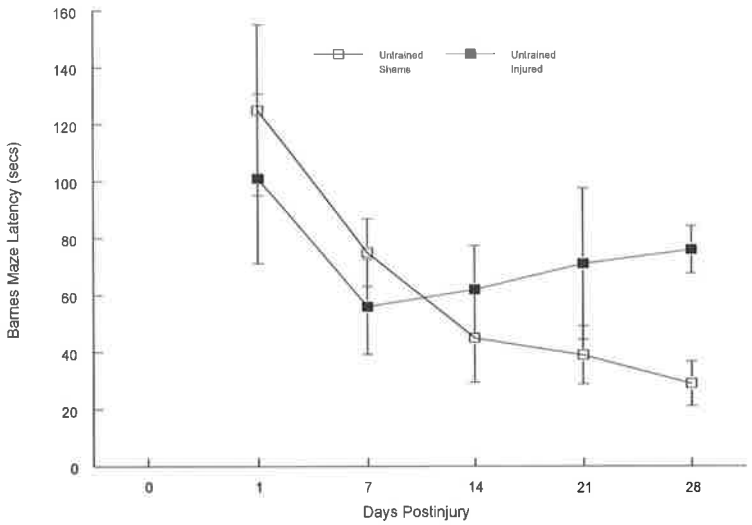


Figure 3.4B Barnes maze assessed cognitive outcomes in untrained rats subject to diffuse TBI and assessed weekly after injury.

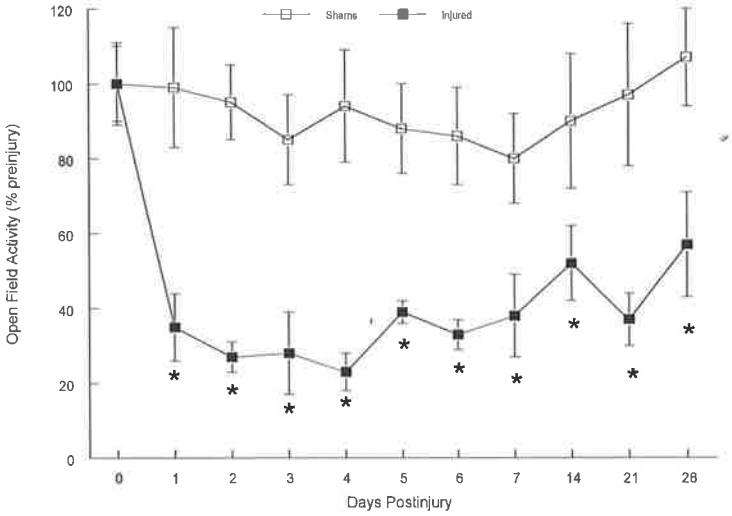


Figure 3.5 Open field activity in rats subject to diffuse TBI. All data is expressed as a percentage of pre-injury activity shown at point zero (*p<0.05 versus shams).

3.3.3 Spontaneous Activity

In the open field, spontaneous activity test, injury resulted in a significant decline in activity (Figure 3.5). Specifically, injured rats demonstrated only 32±11% (p<0.001) of their pre-injury activity over the first seven days post-trauma. This reduced spontaneous exploratory activity was associated with increased freezing behaviour where animals proceeded to a position in the open field and did not further explore their environment. Such behaviour is consistent with increased stress/anxiety and is not associated with decreased sensory or motor deficits (Vallee et al. 1997; Larsson et al. 2002). Spontaneous exploratory activity did not increase in these animals over the remainder of the four week assessment period indicating a persistent deficit in spontaneous exploratory activity after TBI. In contrast, sham animals averaged 92% of their pre-surgery activity over the entire

four week assessment period. There was no effect of daily or weekly testing on these values.

3.4 Discussion

It is well established that clinical trials are dependent on the use of functional outcome measures to determine the success of interventional pharmacologies following TBI. In the pre-clinical assessment of such pharmacotherapies, it is therefore essential to use appropriate functional tests in experimental TBI to monitor both acute (days 1–14) and chronic (days 21–30) outcome (Schallert et al. 2000). Most brain injury studies to date have examined focal injuries and generally report spontaneous recovery within 30 days, with recovery being affected by task experience and repeated testing (Feeney et al. 1982; Schallert et al. 2000; Bland et al. 2001). Similar findings have been reported with a focal axonal injury model in which cognitive function as assessed by the Morris water maze was similar to controls within a few weeks of injury (Skelton 1998). In those studies that were able to demonstrate persistent functional deficits after injury, periodic testing was used in untrained animals (Pierce et al. 1998; Dixon et al. 1999). However, one of these studies (Pierce et al. 1998) used multiple trials per day to reduce measurement variance in cognitive studies, and the popular angleboard, hind limb flexion and lateral pulsion motor tests were unable to demonstrate prolonged differences from shams. Accordingly, the selection of appropriate tests, and knowledge of the influence of experience and task exposure, is essential for future studies using diffuse TBI models.

We have used the rotarod test and the Barnes maze as the preferred motor and cognitive

assessment tasks in our diffuse brain injury studies, and have incorporated an open field, spontaneous exploration test for assessment of stress/anxiety. The rotarod test has been previously described as the most sensitive test for the detection of motor deficits following FP induced rodent brain injury (Hamm 1994; Hamm 2001) and has been successfully used by our laboratory in a number of diffuse TBI studies to assess the effects of pharmacological agents on motor deficits (Heath et al. 1998; Deb 1999). In addition to requiring the animals to increase the speed of their co-ordinated walk with time, the diameter of the rods introduces a grip component to the test which has previously been used as a motor test in its own right (Hall et al. 1988). Thus, the rotarod test in the present study is a combination of two independent motor tests, and unlike many tests that have been designed to detect left side versus right side functional differences, can be applied to diffuse TBI models that do not produce lateral injury. The decision to use the Barnes circular maze to assess cognitive function was based on work done previously by Fox and colleagues (1998) in TBI in the mouse. The Barnes maze was originally designed for use in rats (Barnes 1979) and the protocol used for the present studies was adapted from those original investigations with modifications for brain trauma (Fox et al. 1998). The technique is a simple and inexpensive method that is less affected than other maze tests by the presence of gross motor deficits. Moreover, when pre-training is required, the training period is relatively short (7–10 days). Unlike Fox and colleagues (1998), however, we found that a more consistent response was generated with the addition of an aversive sound (a loud, high-pitch auditory tone) as opposed to sole use of a bright light stimulus. Finally, the open field test is a simple test of stress/anxiety previously shown to be effective in the

detection of post-traumatic deficits in both FP induced (McIntosh et al. 1989) and diffuse TBI (Vink et al. 2003).

In the present study, the weekly rotarod test demonstrated that diffuse TBI resulted in a persistent motor impairment for at least four weeks post-trauma. The presence of a persistent motor deficit is consistent with previous studies using the beam balance test, the beam walk test and the inclined plane test to demonstrate functional motor deficits after severe diffuse TBI (Beaumont et al. 1999). The weekly rate of recovery suggested that any deficit would resolve within five months, a period similar for the resolution of composite motor deficits seen following FPI (Pierce et al. 1989).

Exposure to pre-injury training did not influence this weekly rate of functional recovery, nor did it affect the magnitude of the deficit on the first day after TBI, suggesting that pre-training is of no benefit when periodical testing is utilised to assess motor deficits. In contrast, daily testing of trained animals resulted in a rapid improvement in motor performance, and the rate of functional improvement was significantly enhanced with pre-injury training. This rapid improvement in motor performance significantly reduces the time necessary for functional tests, and has been exploited in previous pharmacologic studies (Heath and Vink 1999). Moreover, the significantly improved rate of recovery in pre-trained animals tested daily suggests that rapid and frequent exposure to familiar motor tasks may facilitate motor recovery following TBI.

The Barnes maze testing in the present experiments demonstrated that diffuse TBI in rats produces persistent cognitive deficits, which is consistent with previous observations using the Morris water maze in this model of rodent injury (Beaumont et al. 1999). As with the motor assessment outcomes, daily cognitive testing facilitated improvement in the task. Indeed, daily testing resulted in a return to 'normal' levels by 10 days post-trauma whereas weekly testing still revealed cognitive deficits as long as four weeks post-injury. Exposure to a pre-training regimen did not significantly affect the rate of cognitive recovery in injured animals, suggesting that pre-training is of no benefit in the assessment of cognitive outcome. However, the lack of pre-injury training significantly increased measurement variance in the period immediately following trauma. This increase in measurement variance would prove an obstacle to achieving statistical significance between treatment groups, although this could be overcome by increasing the number of trials per day and averaging the data (as per Pierce et al. 1998). Alternatively, one could expose the animals to repeated trials on consecutive days at a point when maximum differences between shams and injured animals would be present (3–4 weeks after injury; Figure 3.4B), a practice commonly pursued in FP TBI studies. In contrast to motor performance, it would appear that familiarity with the cognitive task does not improve rate of recovery after trauma.

Finally, the open field assessment does not involve any training or learning task, and it was therefore not surprising that the assessment was unaffected by prior or repeated exposure. Animals tested daily did not show any increase in spontaneous exploratory activity, and no significant increase was observed for the entire four week assessment period. The decrease

in activity was associated with an increased incidence of freezing behaviour where animals went into a corner of the open field and did not further explore their environment. Such freezing has been previously attributed to increased stress (Vallee et al. 1997; Larsson et al. 2002). This freezing behaviour was not observed in the sham animals who continued to explore their environment, even with repeated exposure. As such, the open field trial was considered a useful tool for the assessment of stress after TBI, and may be useful for the assessment of pharmacological interventions.

In conclusion, we have demonstrated that the rapid recovery of motor and cognitive function in rats after diffuse TBI as assessed using a daily testing paradigm does not exclude the possibility that the injury produces persistent motor and cognitive deficits. Daily testing of motor and cognitive function promotes rapid functional recovery, while weekly testing of motor and cognitive function did not enhance recovery. Rate of functional improvement was generally independent of pre-injury training, with a significant effect only observed with daily testing of motor function. Moreover, pre-injury training did not influence the degree of motor deficit immediately after trauma, although it reduced measurement variance in cognitive tests. Spontaneous exploratory activity was depressed for at least four weeks in injured animals, with this activity being unaffected by exposure to the task on either a daily or weekly basis.

CHAPTER 4

INTERACTION BETWEEN ANAESTHESIA, SEX AND FUNCTIONAL OUTCOME TASKS FOLLOWING DIFFUSE TRAUMATIC BRAIN INJURY IN RATS

The previous chapter has demonstrated that the impact–acceleration model of diffuse TBI used in this thesis produces chronic functional deficits. However, it was also clear that functional testing in trained animals saves experimental time, an important factor when assessing the effects of gonadal hormones on functional outcome. However, more factors other than pre-training or assessment intervals are important when comparing functional outcome in male versus female animals, particularly the anaesthesia and whether both sexes perform equally in any given functional task. In the present study, we have therefore examined the interaction between anaesthesia, gender and functional outcome task following diffuse TBI in rats.

4.1 Introduction

TBI causes deficits in functional outcome through both primary and secondary mechanisms of injury. Primary processes cause damage via the actual mechanical force applied to the brain tissue while secondary injury involves multifaceted neurochemical and neurophysiologic pathways that are initiated at the time of trauma but manifest over the subsequent hours, days or months after the initial event (Chesnut et al. 1993; Graham et al. 1993; McIntosh 1994; Cormio et al. 1997). Both mechanisms contribute to neuronal cell death that ultimately is responsible for the observed post-traumatic deficits in functional outcome. However, functional outcome following TBI may also be influenced by several factors other than the primary and secondary mechanisms. For example, recent studies have suggested that outcome after TBI may be dependent on gender (Stein 2001). In support of this, Groswasser and Kerin (1998) found that females fare better than males in their ability

to return to work. In contrast, a comprehensive meta-analysis of clinical studies that report on sex differences has demonstrated that men have a better outcome (injury severity, post-concussive complaints, return to work, new psychiatric symptoms) than women (Farace et al. 2000). In experimental studies, early reports suggest that female rats had a significantly higher mortality than male rats (Emerson et al. 1992), even though oestrogen was protective in male animals (Emerson et al. 1993). As with clinical studies, however, contrasting studies have reported that both oestrogen and progesterone are neuroprotective, resulting in less histopathological damage (Bramlett and Dietrich 2001) and better outcome in female rats than male rats (Roof et al. 1993; Roof et al. 2000b).

The effects of gender on outcome may not simply be a function of gonadal hormone concentration. Sex may either directly influence the dependent variable/s or interact with test variables such as the anaesthesia that is used as part of the study. For example, some forms of anaesthesia have been found to improve outcome following TBI. Indeed, isoflurane has been shown to improve functional performance on motor and Morris water maze tasks following CCI (Statler et al. 2000). Isoflurane and halothane also reduce the effects of global and focal ischemic brain injury (Wise-Faberowski et al. 2001) while pentobarbital is thought to be neuroprotective following decapitation ischemia (Hattori et al. 1986). In addition, anaesthesia may also involve sex-specific reactions including differences in metabolic rates (Hoffman et al. 1989; Torbati et al. 1999). There is also the question of whether the functional outcome tests are appropriate for cross-gender comparisons. For example, it is assumed in experimental studies that male and female

animals will perform equally well on all functional outcome tasks although no-one has determined if this is actually the case.

Thus, there are three main variables in experimental TBI studies that may influence outcome including sex, the specific tests chosen to measure outcome, and the type of anaesthesia. The purpose of this study was to investigate the interaction between these three factors in influencing functional outcome following TBI. We have chosen to examine the effects of isoflurane, halothane and pentobarbital given their common use in experimental TBI. Furthermore, to ensure this study captures the best array of recovery patterns, four separate measures — mortality, motor, cognition, and activity — will be used in both male and female animals to define functional outcome.

4.2 Methods and Materials

4.2.1 Animals

Age-matched, adult male (n=18; 350–400g) and female (n=18; 280–340g) Sprague-Dawley out-bred rats were fed and watered *ad libitum* and maintained on a 12h light/dark cycle before being subjected to TBI. All experimental protocols were approved and conducted according to the guidelines for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council.

4.2.2 Induction of Traumatic Brain Injury

Rats were randomly subdivided into six equal groups made up of six male or six female animals and prepared for injury induction. Each group was anaesthetised either with

sodium pentobarbital (60mg/kg i.p. for male rats and 40mg/kg i.p. for female rats), halothane (2% induction followed by 1% maintenance) or isoflurane (2.5% induction followed by 1.5% maintenance). During the procedure rectal temperature in all animals was maintained at 37°C with a thermostatically controlled heating pad. Following anaesthetic induction of the animals, TBI was induced using the impact–acceleration model of diffuse TBI as previously described (Marmarou et al. 1994; Heath et al. 1995). Briefly, the skull was exposed by a midline incision and a stainless steel disc (10mm in diameter and 3mm in depth) was fixed rigidly with polyacrylamide adhesive to the animal's skull centrally between lambda and bregma. The rats were subsequently placed on a 10cm foam bed and subjected to brain injury induced by dropping a 450g brass weight a distance of two metres onto the stainless steel disc. Such an injury has been previously shown to produce DAI of moderate severity (Foda et al. 1994). Subsequent to injury, all wounds were sutured, anaesthesia was terminated and after stabilising, animals were returned to their cages. Surviving animals at 24h post-trauma were assessed daily over seven days for motor, cognitive and open field outcome.

4.2.3 Assessment of Motor Outcome

Motor assessment was performed using the rotarod test which has been extensively applied to the assessment of detecting motor deficits in rodent brain injury (Hamm 1994; Heath et al. 1995), and has been described in detail in Chapter Two. Briefly, animals were placed on a rotarod device consisting of a motorised rotating ensemble of 18 rods (1mm in diameter). The animal was required to walk on the rotating assembly as the rotational speed of the

device was increased from 0 to 30rpm in intervals of 3rpm every 10 seconds. The point at which the animal either fell from the rods or gripped the rods and spun for two consecutive revolutions rather than actively walking, was recorded in seconds as the rotarod score. In previous studies in male animals, the maximum achievable score was 120 seconds because the task was terminated after two minutes. In the current study, there was no preset limit on completing the task. Animals were pre-trained on the device twice per day over five days prior to injury to establish a normal, uninjured baseline. Assessment commenced 24h post-injury and was conducted each morning for seven days.

4.2.4 Assessment of Cognitive Outcome

The Barnes circular maze (Barnes 1979) as modified and described in detail by Fox et al (1998) was used to assess spatial reference memory following diffuse TBI. Animals were placed under a cover in the centre of an elevated 1.2 metre diameter board containing 19 holes around the periphery. One of the holes was the entrance to a darkened escape tunnel which was not visible from the surface of the board. After activating a series of bright lights aimed at the board and switching on an aversive auditory stimulus, the cover was lifted and the latency in seconds for the animal to locate and enter the darkened escape tunnel was recorded. Before and after each animal trial, the board and escape tunnel was carefully wiped with distilled water to remove any olfactory cues. Animals were pre-trained on the device twice per day during five consecutive days prior to injury to establish a normal, uninjured baseline. Daily assessment commenced 24h post-injury and was continued for a period of seven days.

4.2.5 Assessment of Open Field Behaviour

Spontaneous exploratory behaviour after diffuse TBI was assessed using the open field test (Giulian et al. 1975) as described in detail in Chapter Two. Previous studies have shown that a decrease in spontaneous exploratory activity is thought to reflect increased stress/anxiety (Vallee et al. 1997; Larsson et al. 2002). No pre-training is required for this test, however, immediately prior to injury a baseline of animal activity was determined so as to compare post-injury behaviour. For both pre-injury and post-injury assessment, animals were placed in the centre of a one metre square box with 450mm high white panelled walls. The base of the box was subdivided into 100 equal 10cm squares. Over a five minute period, the number of squares the animal entered was recorded with each square being eligible to be counted more than once should the animal leave the square and then return. Assessment commenced 24h post-injury and continued daily for seven days.

4.2.6 Statistical Analysis

Data are shown as means ± SEM. A repeated measure analysis of variance was used to determine group main effects versus time. A two-way analysis of variance was used to determine effects of sex and anaesthesia on outcome. Student-Newman-Keuls post-hoc tests were used to determine group differences on specific days after injury. Significance in mortality was determined using Fisher's exact test. A p value of 0.05 was considered significant.

4.3 Results

4.3.1 Mortality

In animals that were anaesthetised with pentobarbital, significantly more males survived than females (Figure 4.1). Indeed, 80% of female animals died within 24h after trauma while no male animals died. This is consistent with previous reports (Emerson et al. 1992; Alexiou et al. 2000). In contrast, females demonstrated a 100% survival with isoflurane anaesthesia versus an 83% survival in male animals. Survival in male and female animals under halothane anaesthesia was identical. Because only one pentobarbital-anaesthetised female survived the injury, that group has not been considered in the functional outcome analysis. We have therefore only reported the pentobarbital outcomes in male animals and the halothane and isoflurane outcomes in both males and females.

4.3.2 Motor Outcome

In male animals, rotarod mean raw score prior to injury was 109±10sec (Figure 4.2). In contrast, female animals achieved significantly greater pre-injury rotarod raw scores than their male counterparts (236±10sec; $F[4,25]=39.44$; $p<0.001$). Although there were differences in weight between the groups, they were age-matched. Furthermore, there was some overlap in weight between the groups, and the significant difference in rotarod performance was still apparent in this subgroup of animals with matched weights. Therefore, the pre-injury difference in rotarod performance was unlikely to be due to weight variation. After injury, there were significant declines in rotarod scores across all of the groups, with some recovery in motor function over time as the animals were repeatedly

exposed to the task. A significant difference between males and females (F[13,196]=171.44; p<0.001) existed at all time points after injury with the exception of day one. Furthermore, the female animals improved their rotarod performance at a faster rate after injury than the male groups. There were no significant differences within the male or female animals with respect to anaesthesia.

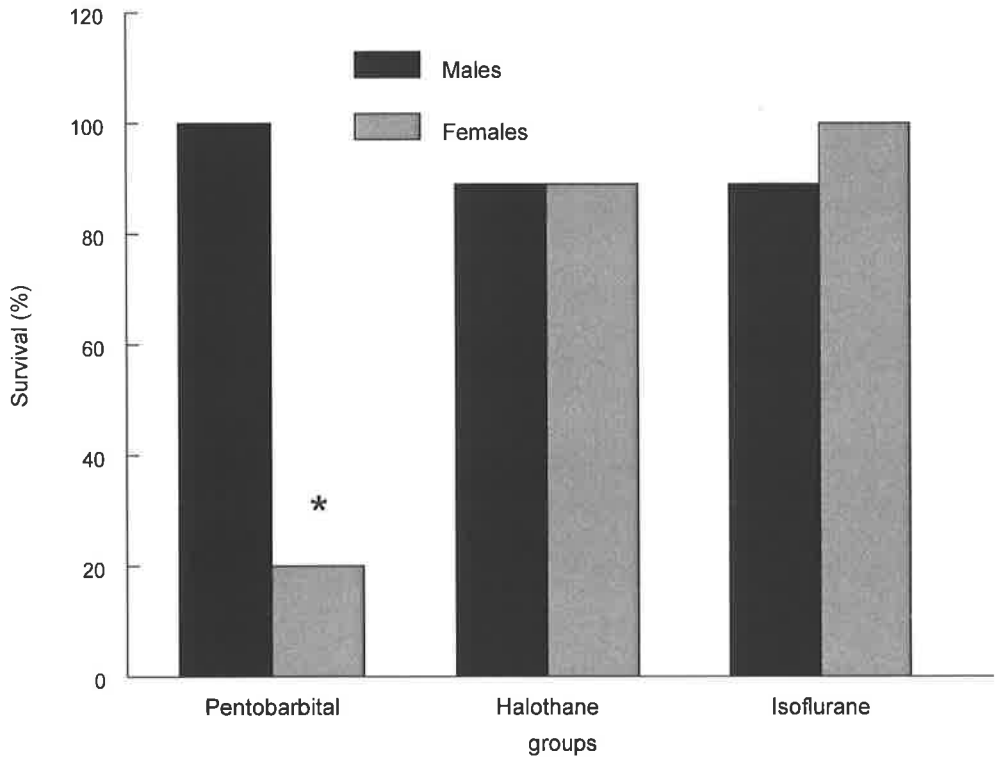


Figure 4.1 Survival following diffuse TBI in male and female rats anaesthetised either with pentobarbital, halothane, or isoflurane at the time of trauma (*p<0.05).

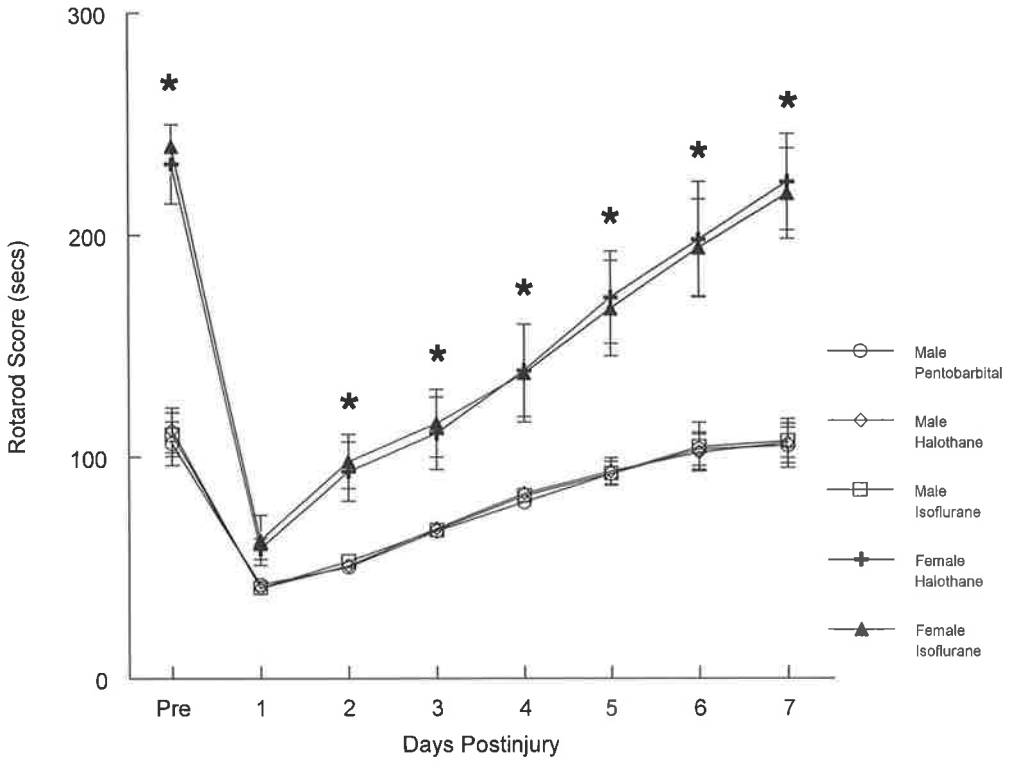


Figure 4.2 Rotarod motor score following diffuse TBI in male and female rats anaesthetised either with pentobarbital, halothane, or isoflurane at the time of trauma. Female animals had significantly better rotarod scores (*p<0.05) than the male animals.

However, when the rotarod data is expressed as a percentage of the pre-injury scores of each group, a different picture emerges (Figure 4.3). As with the raw data, there were significant differences between the male and female animals. However, with the analysis, the females demonstrated a worse performance at some time points compared with the

males after injury (F[13,196]=65.04; p<0.001). This difference was only small and transient with female animals recovering to a point equivalent to the males by day seven after trauma. There were no significant differences in the rate of motor recovery between the groups, and there were no effects of anaesthesia between the groups.

4.3.3 Cognitive Outcome

Male performance on the Barnes cognitive maze is shown in Figure 4.4. Prior to injury and after five days training, all animals were able to locate the escape tunnel within 10 seconds of exposure to the aversive stimuli. After injury, there was a significant increase (F[7,136]=388.34; p<0.001) in the latency to escape the aversive stimuli in all groups. With repeated exposure to the tasks, animals were able to improve their performance such that they had restored the Barnes maze latency to essentially pre-injury levels by day seven after injury. There were no significant differences between the male groups with respect to the effects of anaesthesia on outcome. Female animals demonstrated a similar increase (see Figure 4.5) in Barnes maze latency with gradual recovery in cognitive function with repeated exposure to the task over the seven day assessment period. When comparing the male and female cognitive performance after injury, there was a small but significant difference in male and female latency in isoflurane anaesthetised animals whereby females performed better than their male counterparts (F[1,10]=6.26; p<0.05). However, this difference was not apparent in halothane-anaesthetised animals.

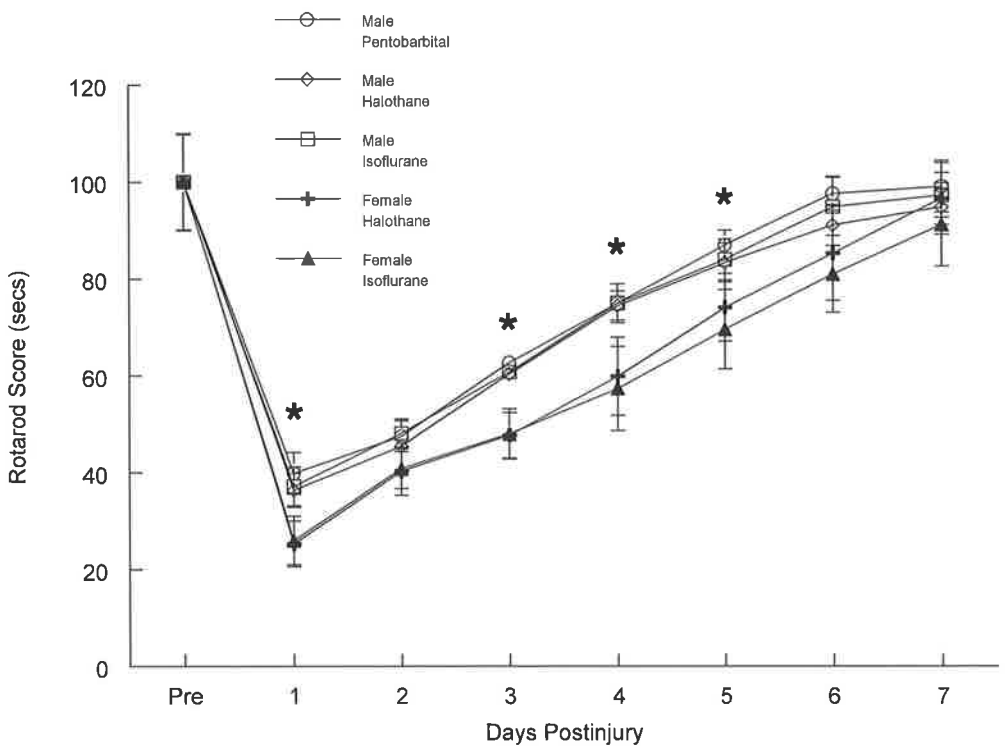


Figure 4.3 Rotarod motor scores after diffuse TBI expressed as a percentage of pre-injury baselines in each sex group and anaesthesia injury group. Female animals had significantly lower percentage of pre-injury rotarod scores than the male animals (*p<0.05).

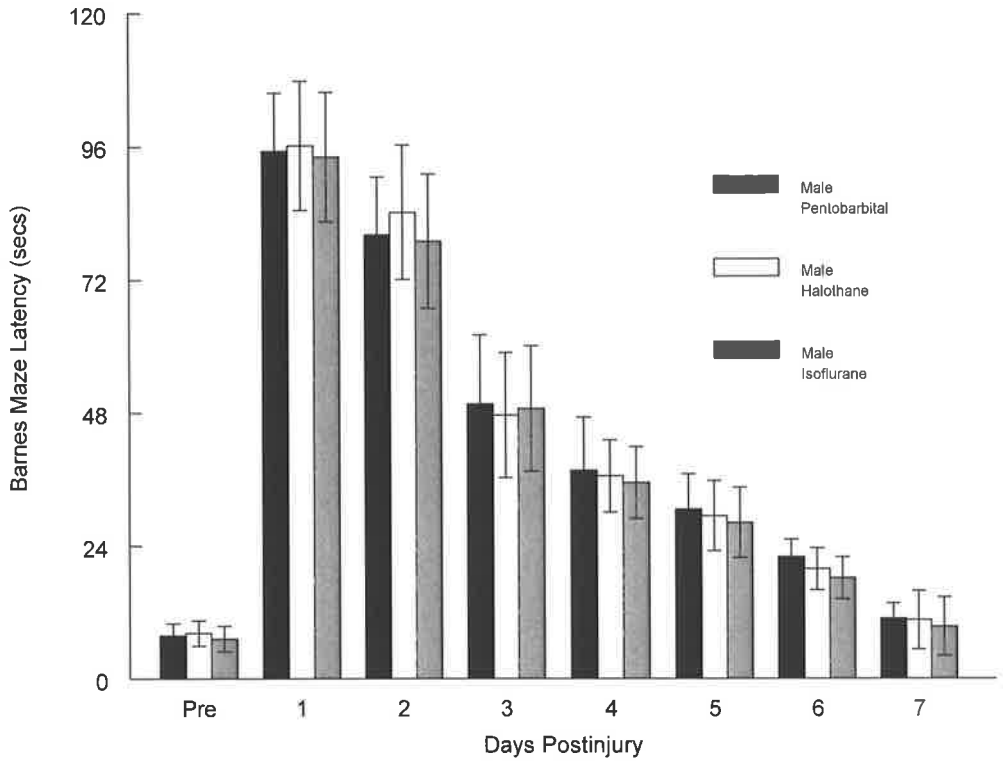


Figure 4.4 Barnes maze latency in male rats following diffuse TBI. There was a significant increase in latency in all animals after injury ($p<0.01$), although there were no significant differences across the groups.

4.3.4 Open Field Outcome

Prior to injury, male animals traversed a mean of 130±20 squares in a five minute period (Figure 4.6). After injury, the number of squares traversed was typically between 20 and 50, reflecting a significantly depressed exploratory behaviour after diffuse TBI (F[23,120]=94.41; p<0.001). This reduced exploratory activity was associated with increased freezing behaviour where animals proceeded to a corner position in the open field and did not further explore their environment. Such behaviour is consistent with increased anxiety and is not associated with decreased sensory or motor deficits (Vallee et al. 1997; Larsson et al. 2002). Female animals performed significantly better than male animals (F[15,224]=61.17; p<0.05) both before and after injury, with less freezing behaviour apparent after injury. Compared to the male pre-injury value of 130±20 squares, female animals traversed 210±18 squares prior to injury suggesting that females display an inherent higher degree of spontaneous exploratory activity than males. After injury, there was a decline in exploratory behaviour in female animals that seemed to be less than in the male animals. However, when this decline is expressed as a percentage of pre-injury values, the decline was similar to that observed in the male animals (results not shown). However, unlike the male animals, female animals anaesthetised with isoflurane always recovered to pre-injury values. Not only was this recovery significant when compared to male animals (F[15,176]=114.51; p<0.001), it was also significant when compared to female animals under halothane anaesthesia (F[1,10]=14.31; p<0.05).

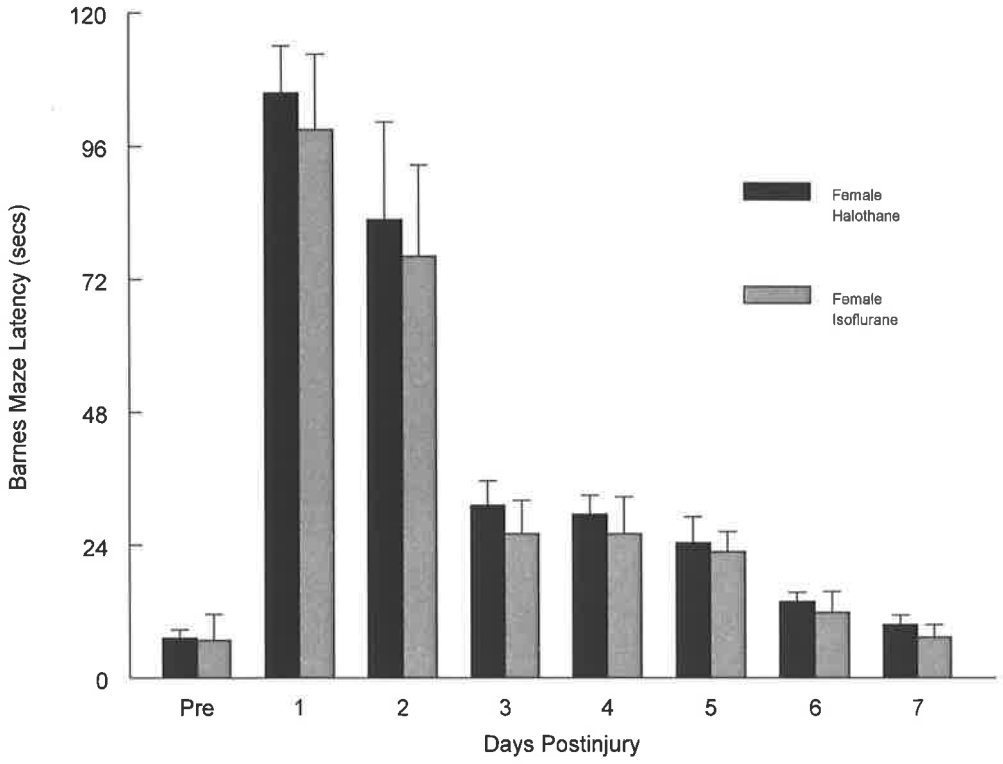


Figure 4.5 Barnes maze latency in female animals following diffuse TBI. There was a significant increase in latency in all animals after injury ($p<0.01$), reflecting a cognitive impairment following trauma.

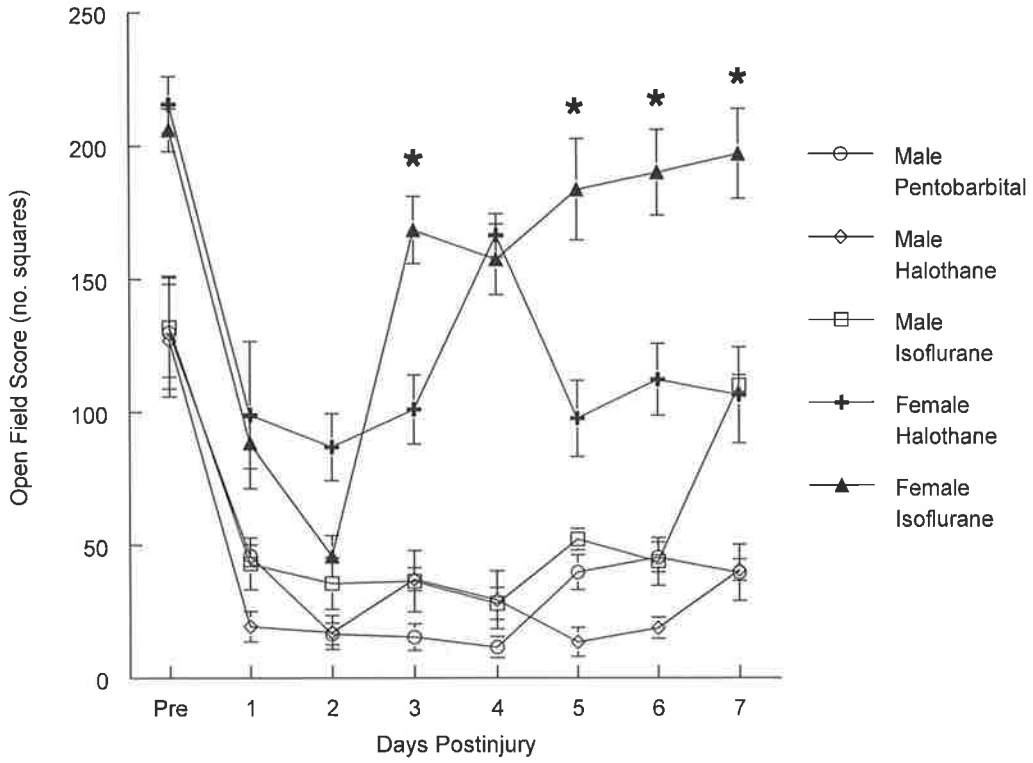


Figure 4.6 Changes in open field activity in male and female rats following diffuse TBI. Isoflurane anaesthetised female animals had a significantly higher activity level (* $0.001<p<0.05$) than all other groups.

4.4 Discussion

To the best of our knowledge, this is the first study to describe the interactions between sex and anaesthesia on different tests of functional outcome following experimental diffuse TBI. Previous studies have shown that anaesthesia does not affect functional outcome in sham-operated animals that were not subject to injury (Heath and Vink 1996; Vink et al. 2003). However, the present study shows that animals subjected to impact–acceleration

TBI demonstrate a differential pattern of functional recovery that is dependent upon the choice of anaesthesia for each sex, and the functional assessment task used. Isoflurane is particularly protective in females on cognitive tests, pentobarbital is deleterious to female mortality, while halothane anaesthesia has the least influence on sex-related outcome. With respect to both motor outcome and open field tests, females perform better than males both before and after injury, irrespective of the anaesthetic used. However, when motor outcome was normalised as a percentage deficit to allow cross-sex comparison, there was little difference between sexes after diffuse TBI.

The influence of anaesthesia on outcome following various brain insults has been extensively studied. Isoflurane has been previously reported to be protective relative to halothane with respect to motor scores, cognitive outcome and hippocampal damage following brain trauma (Statler et al. 2000), and this may be associated with its ability to either reduce post-insult glutamate release (Patel et al. 1995) or increase CBF (Murr et al. 1993). Following ischemia, isoflurane delays onset of cell depolarization, reduces cerebral metabolic rate and reduces histologic damage relative to halothane (Verhaegen et al. 1992; Nellgard et al. 2000), however this improvement is not associated with any difference in CBF (Verhaegen et al. 1992). In contrast, isoflurane was not protective relative to halothane or pentobarbital in a model of cold-induced oedema in male and female animals (Kaieda et al. 1989). In fact, halothane was shown to reduce oedema formation relative to pentobarbital and isoflurane, although there were no differences with respect to the other physiological variables measured in this study. No mortality was observed in this model

and functional outcome was not assessed. Both mortality and functional motor outcome have been assessed in brain-injured mice (Tecoult et al. 2000), and halothane used at its minimum alveolar concentration (MAC) of 1% has been shown not to affect either variable, an observation consistent with the present study. Previous studies using isoflurane in male and female animals subject to diffuse TBI have demonstrated that females have a significantly reduced mortality when compared to males (Roof et al. 2000b). Female animals had a mortality of 0% compared to the 17% observed in male animals. This is identical to the mortality observed in the present study. Moreover, the use of greater numbers of animals in the study by Roof and colleagues (2000b) suggests that our observed trend with a small number of animals would reach significance if the group size were increased.

Other studies have highlighted the deleterious effects of barbiturates on outcome. Our own earlier studies have shown that pentobarbital adversely affects survival in female animals following TBI (Emerson et al. 1992; Alexiou et al. 2000). These studies were performed at an anaesthetic dose of 60mg/kg which is slightly higher than the 50mg/kg pentobarbital shown to produce an equivalent level of anaesthesia as obtained with the inhalation anaesthetics at their individual MAC (Kaieda et al. 1989). In the present study, we reduced the female pentobarbital dose to 40mg/kg in an effort to compensate for the differences in pentobarbital metabolism between male and female animals (Torbati et al. 1999). While this was the lowest dose we were able to use and still obtain adequate anaesthesia, it did not improve female survival following TBI. This difference in survival between sexes is

unlikely to be due to any difference in body weight since we had the same difference in mortality even in the subgroup of animals that had similar body weights. Furthermore, we have previously shown that female survival after TBI can be improved in pentobarbital anaesthetised female animals with the use of magnesium sulphate (Alexiou et al. 2000), an event unlikely to be related to weight of the animal. We are left to conclude that pentobarbital is deleterious to female survival following TBI. Although the mechanisms are unclear, barbiturates have been shown to increase the frequency of hypoxic partial pressure (pO_2) values after brain injury (Murr et al. 1993), an effect that may be associated with its depressant effects on cardiovascular and respiratory systems (Steen 1991). Whether female cardio-respiratory centres are more sensitive to pentobarbital than the male centres after TBI is unknown and would require further investigation.

In the current study, we also observed that female animals do better at the rotarod and open field tests both before and after injury compared to their male counterparts. This is consistent with a number of previous reports examining task-related sex differences in animals. Specifically, female mice perform better than males in sensorimotor tasks (McDermott et al. 1994). Interestingly, in this study, oestradiol improved performance in male animals but not female animals suggesting that the better motor score was related to effects mediated at the ERs, receptors that are already occupied in the female animals. Similarly, oestrogen has also been shown to improve maze performance in mice (Heikkinen et al. 2002) supporting a positive cognitive role of the female hormone. Following brain injury, oestrogen has been shown to improve blood flow and improve

survival in female rats under isoflurane anaesthesia (Roof et al. 2000b). Since progesterone has also been shown to reduce oedema following brain insults (Roof et al. 1993), it is more likely that both oestrogen and progesterone contribute to improved functional outcome in females after TBI in different ways. However, since the pre-injury performance in females in the present study was also significantly better than in males, it is necessary to normalise the data to permit comparison of post-injury performance across sexes. When expressed as a percentage of pre-injury performance, the deficit in rotarod performance in females was virtually identical to that observed in males. Thus in terms of relative functional motor deficit, females are just as affected as males by TBI. However, because they have a better 'baseline' performance, the raw scores always reflect that females do significantly better than males.

This similarity in post-traumatic deficits following normalization was also apparent in the open field test where female animals under halothane anaesthesia performed similar to the males after injury. Isoflurane anaesthetised females, on the other hand, performed better than their male counterparts and better than the halothane anaesthetised females. Open field is a measure of anxiety or stress (Larsson et al. 2002) and reduced spontaneous exploratory activity in this test is though to reflect increased stress/anxiety in the animal (Vallee et al. 1997). Thus, our results suggest that TBI induces increased stress and anxiety and that this is, in part, relieved by isoflurane in females. This suggests some form of interaction that confers an anxiolytic effect. The improved performance in females cannot be attributed to female hormones since halothane anaesthetised females did not demonstrate an improved

performance in this test. Similarly, it cannot be solely attributed to isoflurane since male animals under isoflurane anaesthesia did not demonstrate such an effect.

One limitation of the present study is the lack of histological examination of sex differences. However, previous studies have shown that the presence of endogenous levels of female hormones reduce contusion volumes in females after TBI, irrespective of the stage of oestrous cycle the animals were in (Bramlett and Dietrich 2001). Furthermore, isoflurane has been shown to attenuate hippocampal damage after brain trauma (Statler et al. 2000). Taken together, these previous histopathological studies support the findings in the present study that females have an improved functional outcome after TBI and that this can be enhanced with isoflurane anaesthesia.

In summary, this study has highlighted a number of factors that can affect functional outcome scores following induced acceleration–impact TBI. Firstly, pre-injury functional sex differences need to be taken into account when assessing post-injury outcome following TBI. Secondly, pentobarbital is deleterious to female outcome and is an inappropriate anaesthesia choice for studies investigating sex related differences. Third, isoflurane appears to be neuroprotective in female rats following experimental TBI and therefore may confound outcome analysis. Finally, there were no effects or interactions involving halothane and sex which leads us to conclude that halothane is the anaesthesia of choice when examining gender related outcome following TBI.

CHAPTER 5

EFFECTS OF OESTROGEN AND PROGESTERONE ON NEUROLOGIC IMPAIRMENT FOLLOWING DIFFUSE TRAUMATIC BRAIN INJURY IN FEMALE RATS

The previous chapter has demonstrated that halothane had little effect on outcome when comparing males and females, while pentobarbital was deleterious to female outcome and isoflurane protective. Accordingly, all further studies in this thesis will use halothane as the anaesthetic to permit cross-sex comparisons.

5.1 Introduction

Recent research has provided evidence that in addition to the severity of the primary brain insult and secondary injury cascade, several variables may influence the functional outcome and recovery following TBI. One such factor, sex, may produce differences in functional recovery as a result of the female gonadal hormones. Previous studies have identified that these female gonadal hormones (oestrogen and progesterone) may be protective following TBI and improve survival and outcome (Roof et al. 1999; Finklestein et al. 2001). However, many of these studies used isoflurane as an anaesthetic, which we have shown in the previous chapter to be protective in females. They also have used injury models that produced a focal lesion and a significant ischaemic component, both of which are not always present in diffuse TBI. The impact–acceleration model of injury that we have chosen to use in our studies produces a DAI without significant ischaemia or focal lesions (Foda et al. 1994) and is therefore more representative of the majority of clinical head injury cases.

The aim of this experiment was to investigate the effects of oestrogen and progesterone on clinically relevant outcome measures (motor, cognitive and behavioural) following the induction of diffuse TBI in female rats. Prior to examining the possible effects of

exogenously administered gonadal hormones, it was necessary to first clarify the pre- and post-injury functional outcome status of ovariectomised females compared with that of intact females and male animals. This was especially important given the findings of Chapter Two which demonstrated significant differences between male and female functional patterns.

5.2 Methods and Materials

5.2.1 Animals

Adult intact female (n=15; 300–340g), ovariectomised female (n=48; 330–400g) and male (n=10; 400–450g) Sprague-Dawley out-bred rats were fed and watered *ad libitum* and maintained on a 12h light/dark cycle for two weeks before being subjected to TBI. Ovariectomies were performed at seven weeks of age (180–240g) under halothane anaesthesia (3% induction followed by 1% maintenance), and animals allowed to recover for approximately nine weeks, at which time TBI was induced. This nine week delay ensured that the circulating levels of the gonadal hormones were insignificant at the time of trauma and that there was some overlap in weight between male and female groups.

5.2.2 Induction of Traumatic Brain Injury

Intact female (n=10), ovariectomised females (n=10 injured; n=11/treatment group: sesame oil vehicles, progesterone and oestrogen) and male (n=10) rats were subdivided into eight groups and prepared for injury induction. An additional five intact and five ovariectomised animals served as injury shams (surgery but no induction of injury). TBI was induced under halothane anaesthesia using Marmarou's (1994) impact–acceleration model of

diffuse TBI as described in detail in Chapter Two. Surviving animals at 24h post-trauma were assessed daily over nine days for motor, cognitive and open field outcome.

5.2.3 Drug Treatment and Administration

At 30min post-trauma, ovariectomised female animals were administered a subcutaneous injection of either 33.3 μg/kg 17β-estradiol, 1667μg/kg progesterone or equal volume sesame oil vehicle (0.33 ml). These doses have been previously shown to result in physiological serum levels of the respective hormones (Gibbs 1998; Gibbs 1999). Throughout the nine day assessment period after injury, daily injections were administered to maintain these physiological levels (Gibbs 1998; Gibbs 1999).

5.2.4 Assessment of Functional Outcome

Functional outcome was assessed using the rotarod, Barnes and open field tests as described in detail in Chapter Two. Briefly, for rotarod tests, animals were pre-trained on the device twice per day over five days prior to injury to establish a normal, uninjured baseline. Similarly, on the Barnes maze, animals were pre-trained twice per day during five consecutive days prior to injury to establish a normal, uninjured baseline. After injury, daily assessment of rotarod and Barnes maze performance was conducted at 24h post-injury and was repeated daily for a period of nine days. Exploratory behaviour after diffuse TBI was assessed using the open field test (Giulian & Silverman 1975). No pre-training is required for this test, however, immediately prior to injury a five minute baseline of animal activity was determined so as to compare post-injury behaviour. Assessment was

conducted at 24h post-injury and repeated on days 3, 5, 7 and 9 of the assessment period.

5.2.5 Statistical Analysis

Data are shown as means ± SEM. A split plot repeated measures analysis of variance was used to determine within subjects and interaction effects. Multivariate Pillai's Trace was chosen as the criterion statistic as it is robust with respect to violations of assumptions (Olson 1976). A p value of 0.05 was considered significant. If an interaction effect was observed the within subjects simple effects of time were analysed using separate one-way repeated measures ANOVA followed by a Bonferroni multiple comparisons test for selected pairs. If between groups simple effects on specific days after injury were of interest, one-way analysis of variance followed by Tukey's post-hoc tests was used. Significance in mortality was assessed using Fishers exact test.

5.3 Results

5.3.1 Mortality

There was no significant difference across the treatment groups with respect to mortality following induced diffuse TBI (p>0.05). A total of six animals (n=1 out of 10 intact female animals, n=4 out of 43 ovariectomised females and n=1 male) died following trauma before any treatment was administered.

5.3.2 Motor Outcome

Gender comparisons

Rotarod scores prior to and after injury in untreated animals are shown in Figure 5.1A. Prior to injury, there was a significant difference ($p<0.05$) in baseline pre-injury motor scores (intact injured females 237±4sec; ovariectomised females 163±3.8sec; males 118.8±4sec). This finding is consistent with those in Chapter Four that reported a significant difference between intact female and male animals' pre-injury motor ability. Pre-injury motor scores between intact female shams and intact female injured animals were not significantly different (intact shams 224±10.1sec; intact injured 237±4sec).

Intact female shams showed no significant change in their motor function across the nine day assessment period confirming that the surgical preparation (without induction of injury) did not impact upon rotarod performance. This is consistent with our previous results in male animals shown in Chapter Three. After injury, there was a significant decline in motor performance in all of the injury groups. Intact females ($t[9]=74.437$, $p<0.0005$), ovariectomised females ($t[9]=32.419$, $p<0.0005$), and males ($t[9]=7.691$, $p<0.0005$) recorded significant motor deficits at 24 hour after severe TBI when compared with their respective pre-injury scores. In intact female animals this difference persisted for six days after injury before returning to pre-injury levels ($t[9]=2.126$, $p>0.05$). Ovariectomised females demonstrated significant motor deficits across the entire assessment period (up to day nine, $t[9]=7.151$, $p<0.001$), even though there was some improvement in the task with repeated exposure. Motor deficits also persisted in male

animals until day six of the assessment period, and thereafter were not significantly different from their pre-injury values (t[9]=3.266, p>0.05).

There were significant time by group interaction effects for all animals (Pillai's Trace=1.606, F[18, 38]=8.597). The interaction can be explained primarily by the intact female group's steep rate of improvement in motor scores over the nine day assessment period when compared with the other groups' recovery patterns.

Chapter Four demonstrated that there was little difference between male and female post-injury rotarod performance when the raw data is expressed as a percentage of the pre-injury score. A similar pattern is noted with the current data (Figure 5.1B). After injury, the only significant difference between male and female animals occurred at day one post-injury (males 43%, females 24.6% of their respective pre-injury scores) of the nine day assessment period. However Tukey's post-hoc tests demonstrated that while ovariectomised females were not significantly different from intact females on day one post-injury (ovariectomised females 23%, intact females 24.6% of their respective pre-injury scores) they had significantly lower rotarod scores after injury than both the intact females and the males on all other days of the assessment period (p<0.05 on day 2; p<0.0005 on days 3–9) suggesting that the female gonadal hormones do influence outcome.

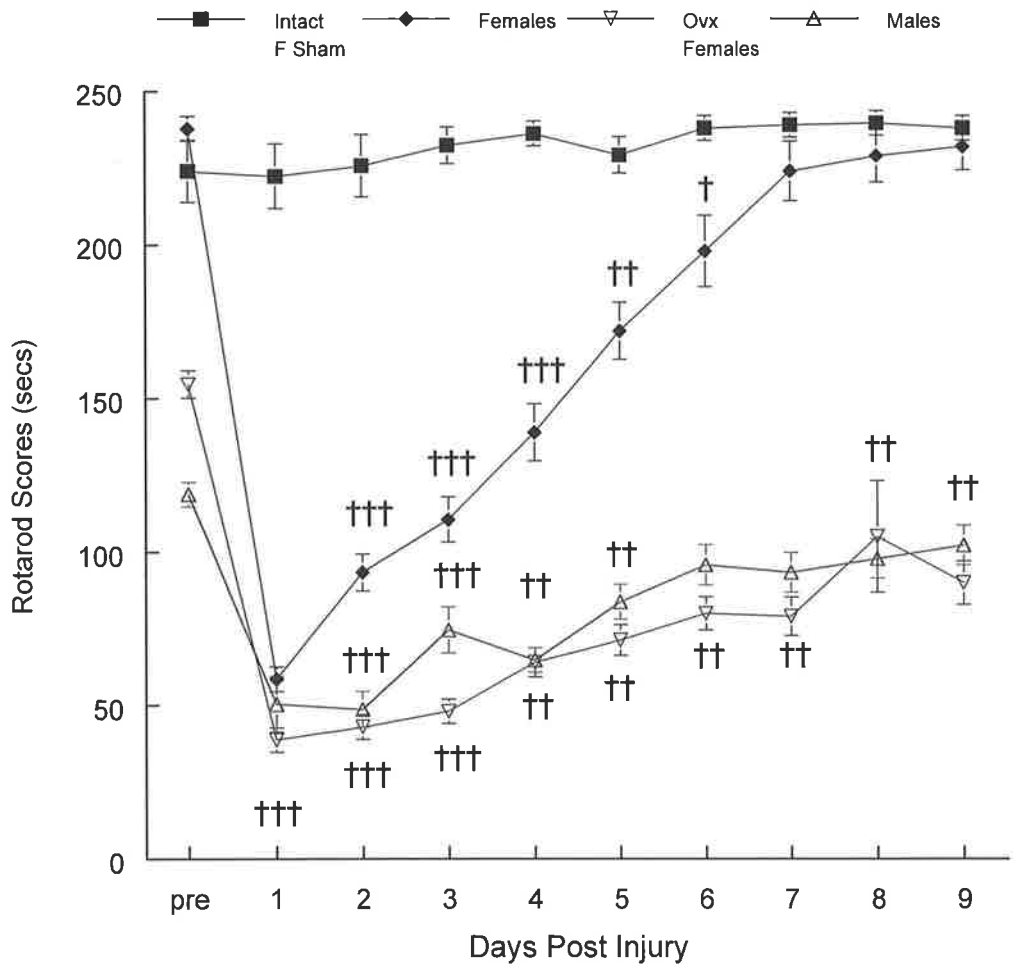


Figure 5.1A Motor (Rotarod) scores for intact female shams, intact female injured, ovariectomised injured and injured male animals following TBI. Data are the means ± SEM (n=10 per group) († $p<0.01$, †† $p<0.001$, ††† $p<0.0005$ compared with respective pre-injury scores).

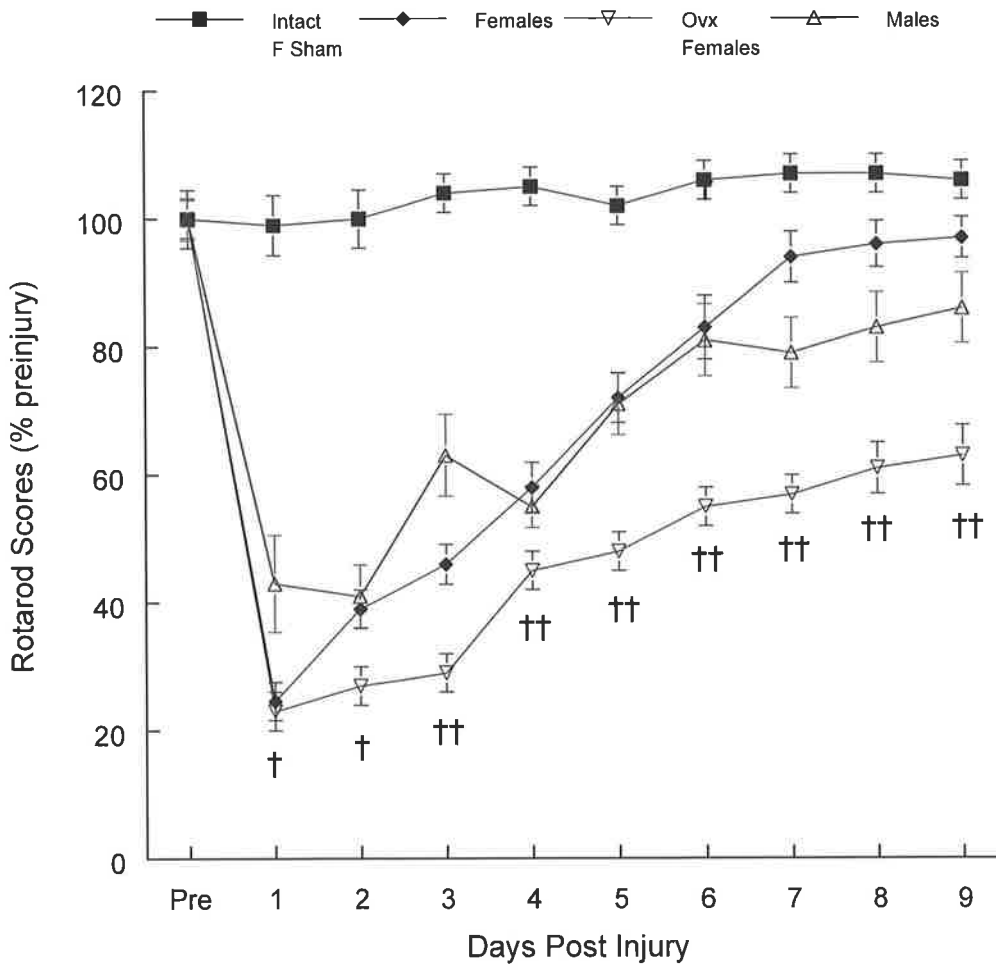


Figure 5.1B Motor (Rotarod) scores for intact female shams, intact female injured, ovariectomised injured and injured male animals following TBI expressed as a percentage of pre-injury baselines in each group. Female animals had significantly lower percentage of pre-injury rotarod scores than the male animals, † $p<0.05$. †† $p<0.0005$.

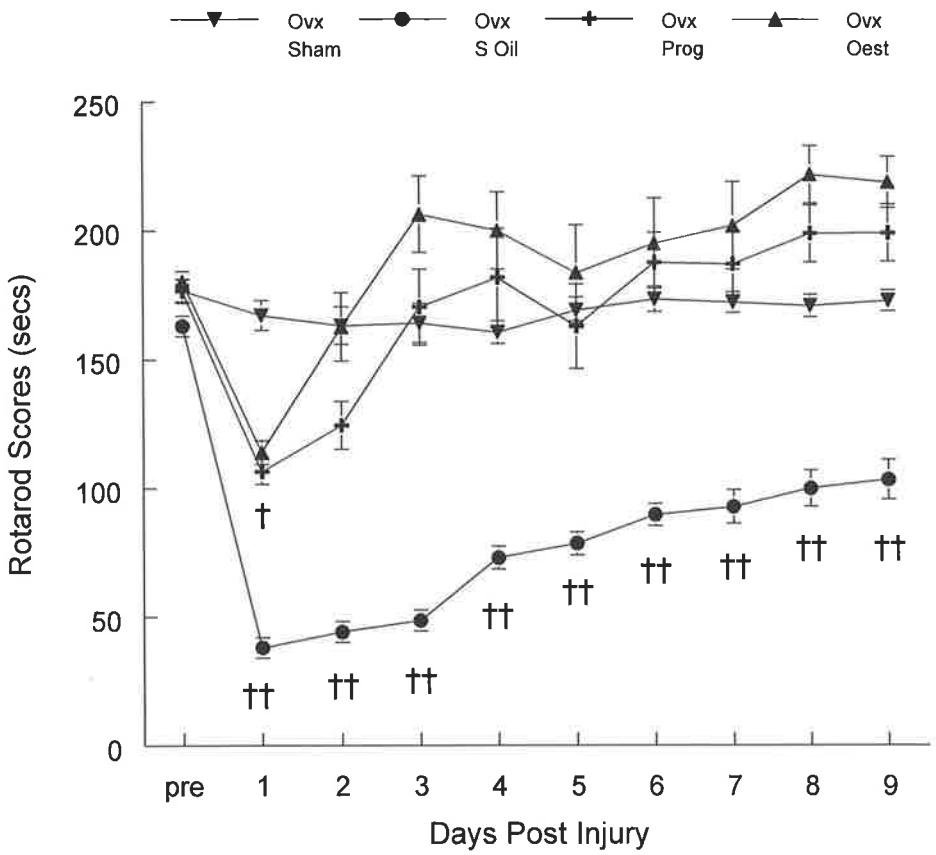


Figure 5.1C Motor (Rotarod) scores for vehicle treated ovariectomised females, progesterone treated ovariectomised females and oestrogen treated ovariectomised females following TBI. Data are the means ± SEM (n=10 per group). † $p<0.001$, †† $p<0.0005$ compared with respective pre-injury scores.

Treatment Groups

The effects of exogenous administration of the hormones on rotarod scores are shown in Figure 5.1C. Prior to injury, there was no significant difference in the pre-injury rotarod scores between the ovariectomised female animals (shams 176.80±4.90; sesame oil 163.10±3.78; progesterone 176.70±4.70; oestrogen 180.44±2.56sec). Ovariectomised female shams demonstrated no significant change in their motor scores across the nine day assessment period confirming that the surgical preparation and the ovariectomy at seven weeks did not affect motor outcome. The lack of effect of surgery on rotarod outcome is identical to that observed in male and intact female sham animals.

After trauma, vehicle treated ovariectomised females (t[10]=35.740, p<0.0005), progesterone treated ovariectomised females (t[10]=13.704, p<0.001), and oestrogen treated ovariectomised females (t[10]=11.573, p<0.001) recorded significant motor deficits at 24h compared with their respective pre-injury motor scores. Vehicle treated animals continued to demonstrate significant motor deficits across the entire assessment period (day nine of assessment, t[10]=7.580, p<0.0005). In contrast, both hormone treatment groups demonstrated significantly improved motor scores at every time point of the assessment period compared with vehicle treated ovariectomised animals (p<0.001). Progesterone treated ovariectomised females recovered to pre-injury motor ability levels by day three after injury (t[10]=0.338, p>0.05). Similarly, oestrogen treated ovariectomised females returned to pre-injury motor levels by day two of the nine day assessment period (t[10]=1.402, p>0.05). There was no significant difference between progesterone and oestrogen treated animals at any time point after injury.

There was a significant time by group interaction between vehicle treated, progesterone treated and oestrogen treated ovariectomised females, (Pillai's Trace=0.991, F[16, 40]=2.457, p<0.05]. The interaction can be explained mostly by the significantly improved recovery patterns observed in progesterone and oestrogen treated animals during the assessment period compared with vehicle treated controls.

5.3.3 Cognitive Outcome

Gender Comparisons

Barnes maze cognitive performance in untreated animals prior to and following diffuse TBI is shown in Figure 5.2A. Prior to injury, all animals were able to locate the escape tunnel within approximately 10sec of exposure to the aversive light and noise stimuli (intact females 8.2±0.44sec; ovariectomised females 11.1±1.70sec; males 10.5±1.88sec). There were no significant differences between the groups. Intact female shams showed no significant change in their latency to escape the aversive light and noise stimuli across the nine day assessment period, confirming the surgical preparation did not affect cognitive outcome. This is consistent with previous results in males (Chapter Four).

Cognitive deficits recorded at 24 hours post-injury were significant compared with respective pre-injury baseline scores; intact females (t[9]=42.327, p<0.0005), ovariectomised females, (t[9]=4.886, p<0.001), and male animals, (t[9]=3.996, p<0.001). In intact females this deficit persisted (despite the sharp improvement at day three) until day five of the assessment period, after which no significant difference from their pre-injury cognitive scores was observed, (t[9]=3.882, p>0.05). Similarly, male animals

demonstrated significant cognitive deficits until day six after injury when they recovered to pre-injury Barnes maze levels, (t[9]=2.281, p>0.05]. In contrast, ovariectomised females did not regain their pre-injury latency scores, maintaining a significant cognitive deficit across the entire assessment period, (day nine of assessment t[9]=3.658, p<0.01).

Comparisons between groups showed that at 24 hours after injury, injured intact females had a significantly greater cognitive deficit than males (p<0.05), while injured ovariectomised animals were not significantly different from either of the two groups (p>0.05). By day seven, injured intact females had recovered sufficiently to record significantly improved scores when compared with ovariectomised injured females (p<0.005) and injured male animals (p<0.05).

There was a significant time by group interaction observed in Barnes maze cognitive performance for intact and ovariectomised female, and male animals (Pillai's Trace=1.361, F[18, 40]=4.734, p<0.0005). The interaction reflects both the sharply increased latency to escape the aversive light and noise stimuli observed in female animals (injured and ovariectomised) immediately after injury compared with male animals, and the profound cognitive improvement seen in intact females between days two and three after injury compared with that of males and ovariectomised females.

In summary, these findings demonstrate that ovariectomy in female animals decreases the rate of cognitive recovery when compared to both intact female and male animals, suggesting that the female gonadal hormones may also influence cognitive outcome after

TBI.

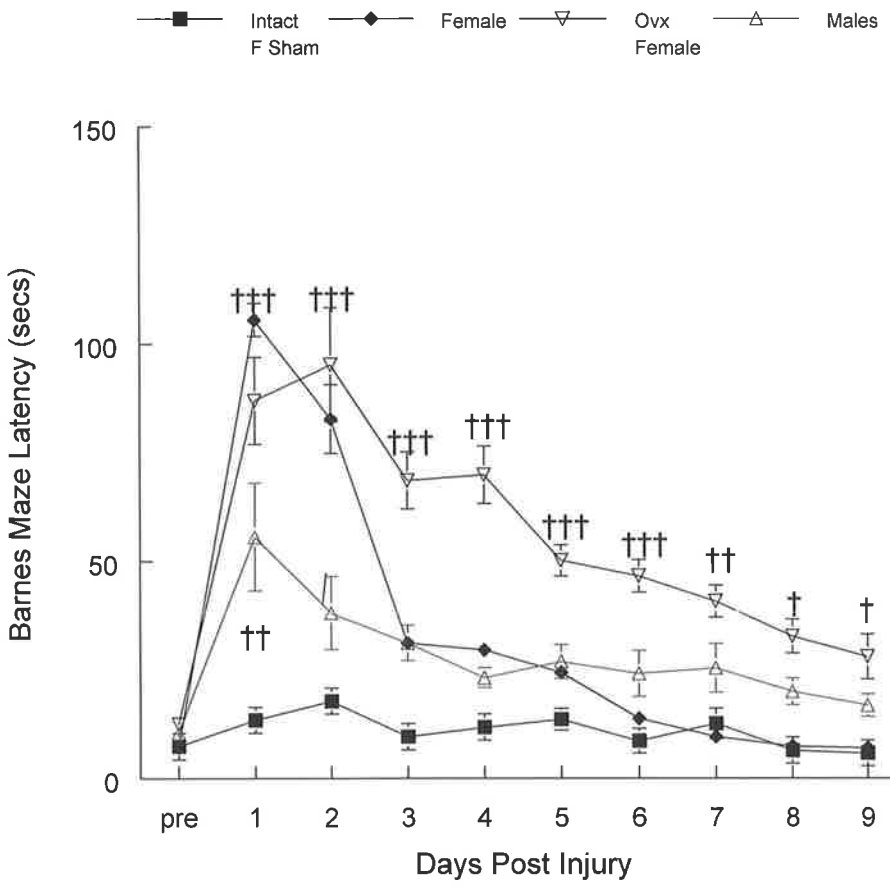


Figure 5.2A Barnes maze latency in intact female shams, intact females, ovariectomised females and male animals following diffuse TBI. Data are the means ± SEM (n=10 per group). † $p<0.01$, †† $p<0.001$, ††† $p<0.0005$ compared with respective pre-injury scores.

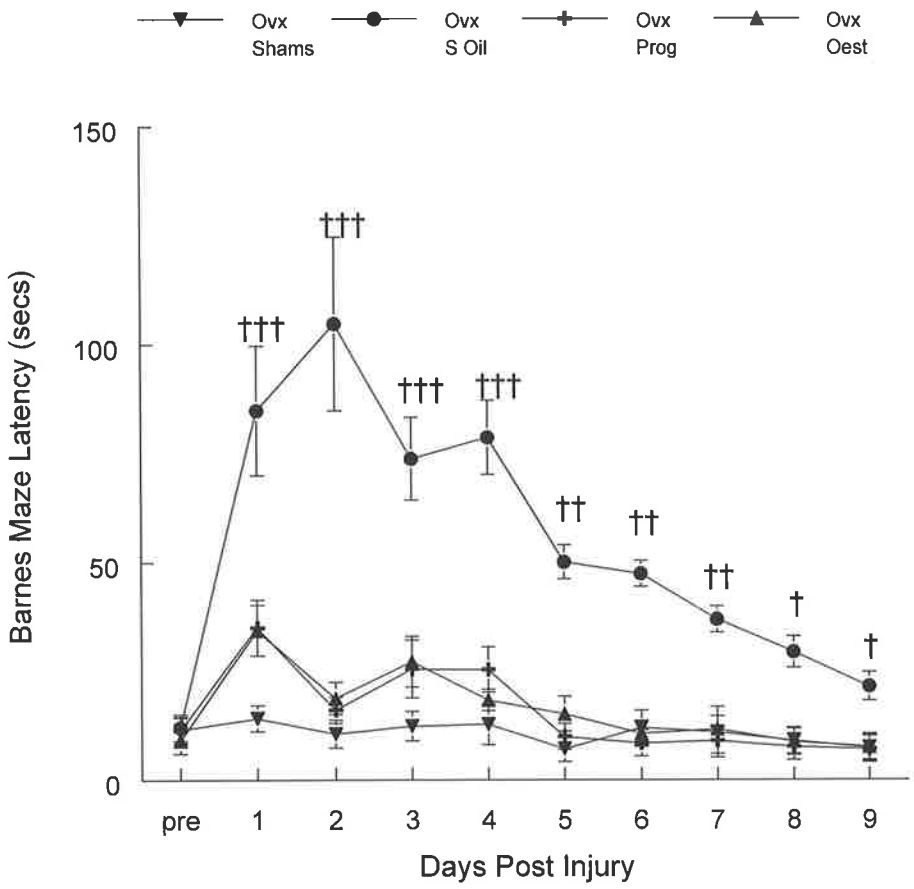


Figure 5.2B Barnes maze latency in ovariectomised female shams and ovariectomised females treated with sesame oil vehicle, progesterone or oestrogen. Data are the means ± SEM (n=10 per group). † $p<0.01$, †† $p<0.001$, ††† $p<0.0005$ compared with respective pre-injury scores.

Treatment Groups

The effects of exogenous administration of the hormones on Barnes maze performance prior to and following TBI are shown in Figure 5.2B. Prior to injury, there were no significant differences between the ovariectomised female groups with regards to latency to escape the aversive light and noise stimuli (shams 11.6±2.9sec; sesame oil 12.1±5.4sec; progesterone 11.4±4.3sec; oestrogen 9.1±4.3sec). Ovariectomised female shams recorded no change relative to their pre-injury time to locate the escape tunnel, confirming that the surgical procedures did not affect cognitive outcome. This is consistent with our previous results in both males and intact females.

After injury, vehicle treated ovariectomised animals demonstrated profound cognitive deficits 24h after trauma as measured by the significant increase in their latency to escape the aversive stimuli when compared with their respective pre-injury baseline scores (t[9]=4.886, p<0.001). This increase in latency to locate the escape tunnel persisted across the entire assessment period, even though there was improvement in the task with repeated exposure (at day nine after injury, t[9]=3.658, p<0.01). In contrast, progesterone treated ovariectomised animals only had an increased latency to locate the escape tunnel at 24h after injury (t[9]=4.002, p<0.01). At all other time points, their latency to locate the escape tunnel was not significantly different from their pre-injury time. Similarly, oestrogen treated ovariectomised animals reflected a similar pattern as the progesterone treated animals in their Barnes maze performance. Their time to locate the escape tunnel was significantly increased at 24h after injury (t[9]=4.245, p<0.01), but at no other time across

the entire assessment period.

When comparing across groups, vehicle treated ovariectomised females required significantly more time to escape the aversive light and noise stimuli after injury than either of the hormone treated groups (F[2, 26]=86.829; p<0.001). There was no significant difference between the progesterone and oestrogen treatment groups. A significant latency x group interaction was observed (Pillai's Trace=1.078, F[18, 38]=2.467, p<0.01) after injury. The primary explanation for the interaction effect was the significant pattern of cognitive deficit recorded in vehicle treated ovariectomised females compared with the relatively mild level of injury noted in both progesterone and oestrogen treated ovariectomised females.

5.3.4 Open Field Outcome

Squares Traversed and Gender Differences

Open field behaviour was assessed by observing the number of squares animals traversed in the Open Field during a five minute assessment period. During the same time period, the freezing behaviour of animals was also recorded. Figure 5.3A summarises the number of squares traversed by intact females, ovariectomised females and males following induced TBI. Prior to injury, there was no significant difference in mean number of squares traversed between intact female shams, intact female and male animals (intact shams 232±13.7, intact females 215.6±9.3, males, 226.2±16.9 squares). However, ovariectomised female animals traversed fewer squares in the five minute assessment period (161.9±9.9 squares) which was significantly less (p<0.05) than the other groups. Intact female shams

demonstrated no significant change in the number of squares traversed across the nine day assessment period confirming the surgical procedures did not affect spontaneous exploratory activity. Some non-significant habituation (Thompson 1993) was observed on day seven and nine of the assessment period. After injury, a significant decline in the number of squares traversed compared with respective pre-injury activity level was recorded in the five minute assessment period; intact females ($t[9]=17.926$, $p<0.0005$), ovariectomised females ($t[9]=13.002$, $p<0.0005$), and males ($t[9]=16.023$, $p<0.0005$). Furthermore, no animal group recovered to pre-injury activity levels across the entire nine day assessment phase which is consistent with previous observations made in this laboratory (Vink et al. 2003).

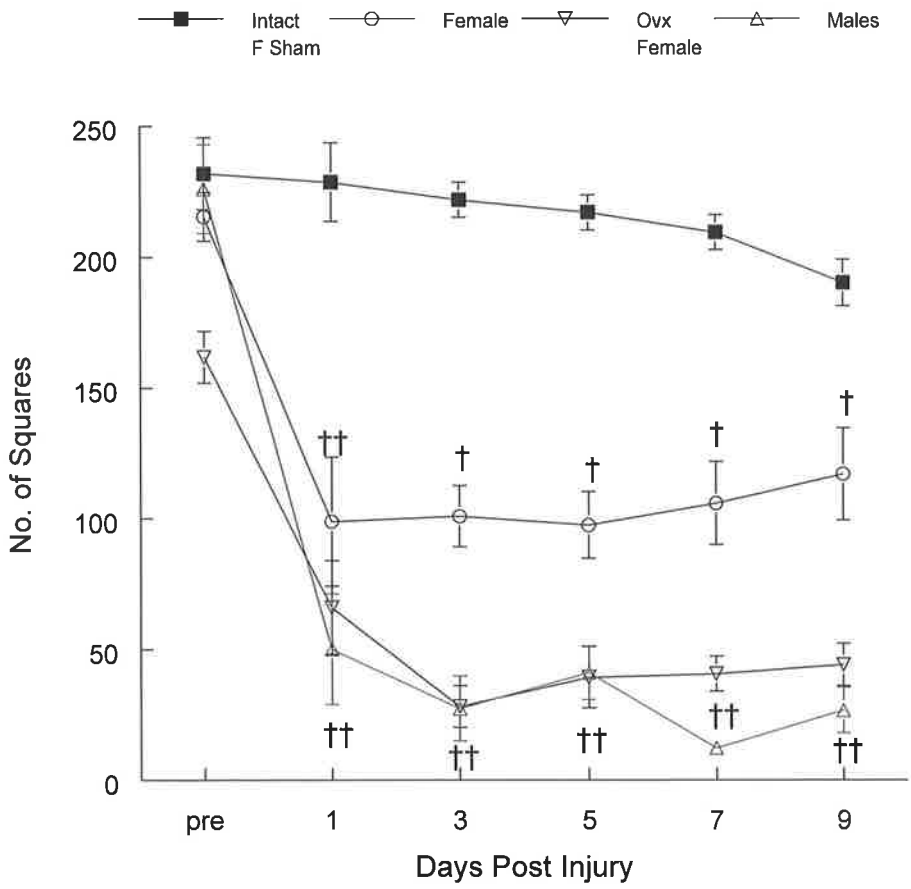


Figure 5.3A Changes in open field activity (number of squares traversed) in intact female shams, intact females, ovariectomised females and male animals following diffuse TBI. Data are the means ± SEM (n=10 per group). † $p<0.001$, †† $p<0.0005$ compared with respective pre-injury scores.

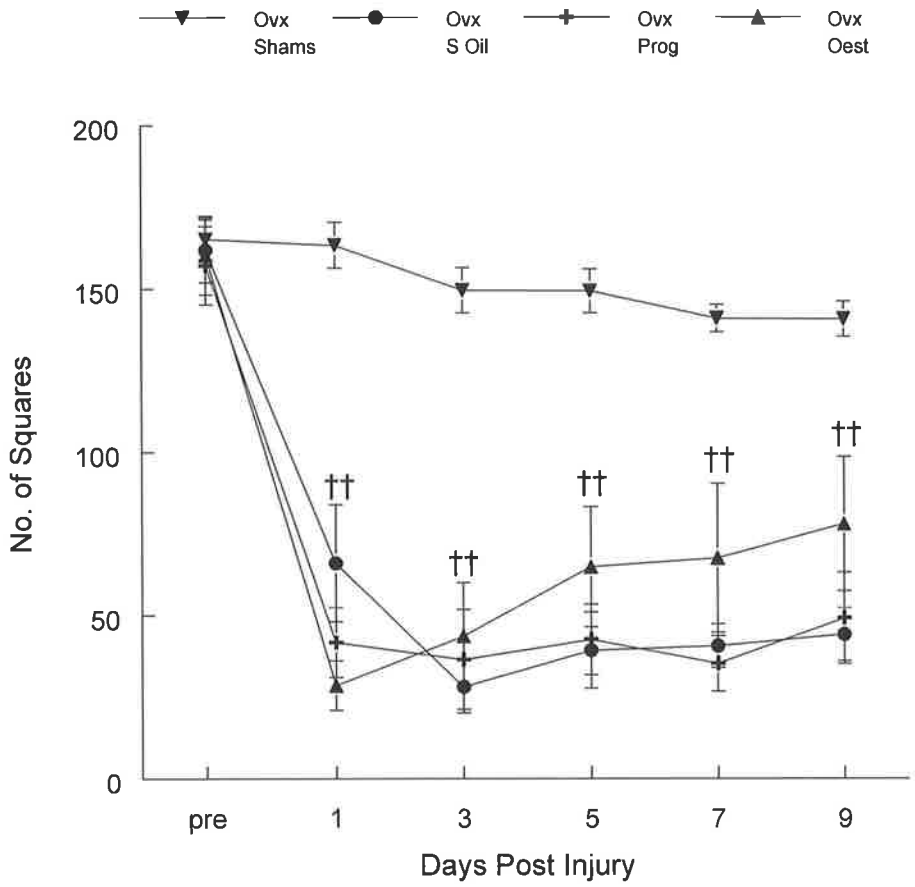


Figure 5.3B Changes in open field activity (number of squares traversed) in ovariectomised shams and ovariectomised females treated with sesame oil, progesterone or oestrogen following diffuse TBI. Data are the means ± SEM (n=10 per group). †† $p<0.0005$ compared with respective pre-injury scores.

Treatment Groups

The effects of exogenous administration of the hormones on open field spontaneous activity prior to and following TBI are shown in Figure 5.3B. Prior to injury, there were no significant differences in the mean number of squares traversed in the Open Field between the ovariectomised female animals (ovariectomised shams 165.20±13.54; sesame oil vehicle, 161.90±31.44; progesterone, 157.20±11.95; oestrogen, 160.33±12.04 squares). Ovariectomised sham animals recorded no significant change in the number of squares traversed across the nine day assessment period confirming that the surgical procedures did not affect the levels of activity in these animals. All treatment groups showed a significant decrease ($p<0.05$) in the number of squares traversed at 24h compared to their respective pre-injury levels. There was no significant improvement over the remainder of the nine day assessment period, and no significant differences between the treatment groups.

Freezing Behaviour

As part of the five minute open field test, we also measured the time that an animal remained in a stationary location without exploring their environment as a measure of anxiety (Vallee et al. 1997; Larsson et al. 2002). Prior to injury (Figure 5.4A), none of the animals demonstrated any freezing behaviour in their pre-injury five minute assessment period. Intact female shams also recorded no freezing behaviour across the nine day assessment period confirming that the surgical procedures did not affect the freezing pattern of these animals.

After injury, there was no increase in freezing behaviour of intact female animals within the first 24 hours (t[9]=1.500, p>0.05). By day three, however, freezing behaviour increased significantly (t[9]=17.926, p<0.0005) before steadily declining over the next few days such that it was not significantly different from pre-injury values by day seven (t[9]=1.380, p>0.05). In contrast, both ovariectomised females and males had significantly increased freezing behaviour at 24h after injury, (t[9]=8.045, p<0.0005 and t[9]=9.095, p<0.0005, respectively) compared with their respective pre-injury activity levels. There was no recovery in freezing behaviour in either group across the remainder of the nine day assessment period.

Intact females demonstrated a significantly different pattern of freezing behaviour compared with ovariectomised females and male animals, suggesting that the female gonadal hormones may have a significant effect on freezing behaviour after TBI. This may have accounted for the significant time x group interaction observed in the Open Field freezing behaviour scores (Pillai's Trace=1.300, F[10, 48]=8.906, p<0.001).

Treatment Groups

The effects of exogenous administration of the hormones on open field freezing behaviour prior to and following TBI are shown in Figure 5.4B. None of the animals demonstrated any freezing behaviour in their pre-injury five minute assessment period. The freezing activity levels of ovariectomised shams also did not vary across the nine day assessment period, confirming that the surgical procedures did not affect their freezing behaviour.

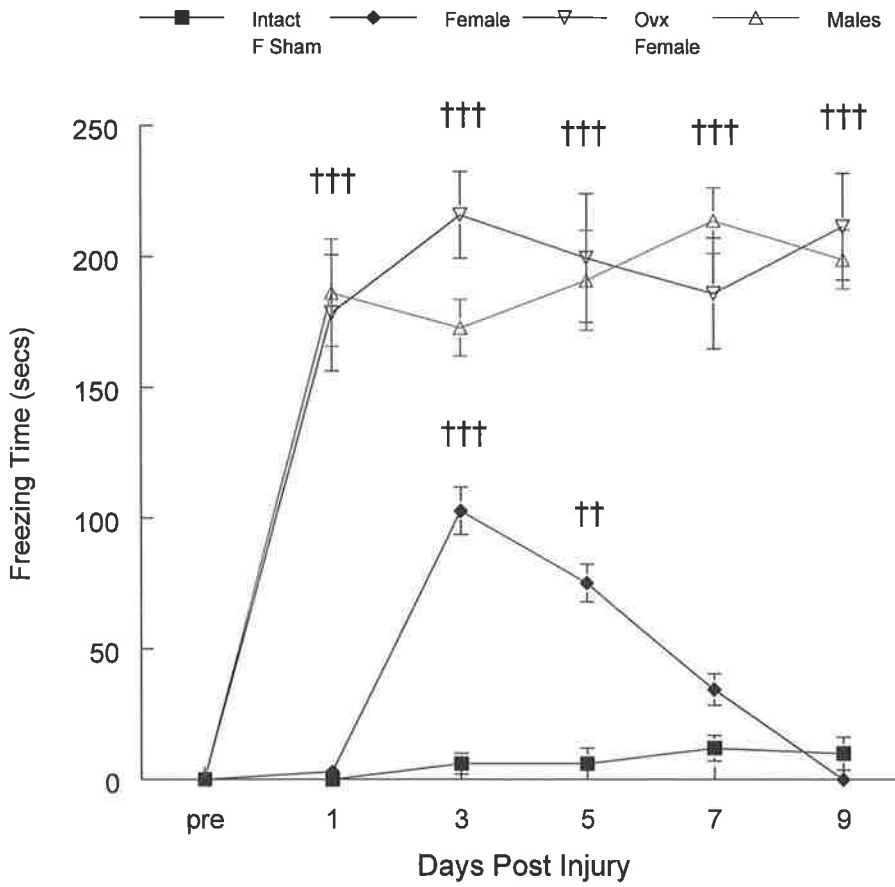


Figure 5.4A Changes in open field activity (freezing time) in intact female shams, intact females, ovariectomised females and male animals following diffuse TBI. Data are the means ± SEM (n=10 per group). † $p<0.01$, †† $p<0.001$, ††† $p<0.0005$ compared with respective pre-injury scores.

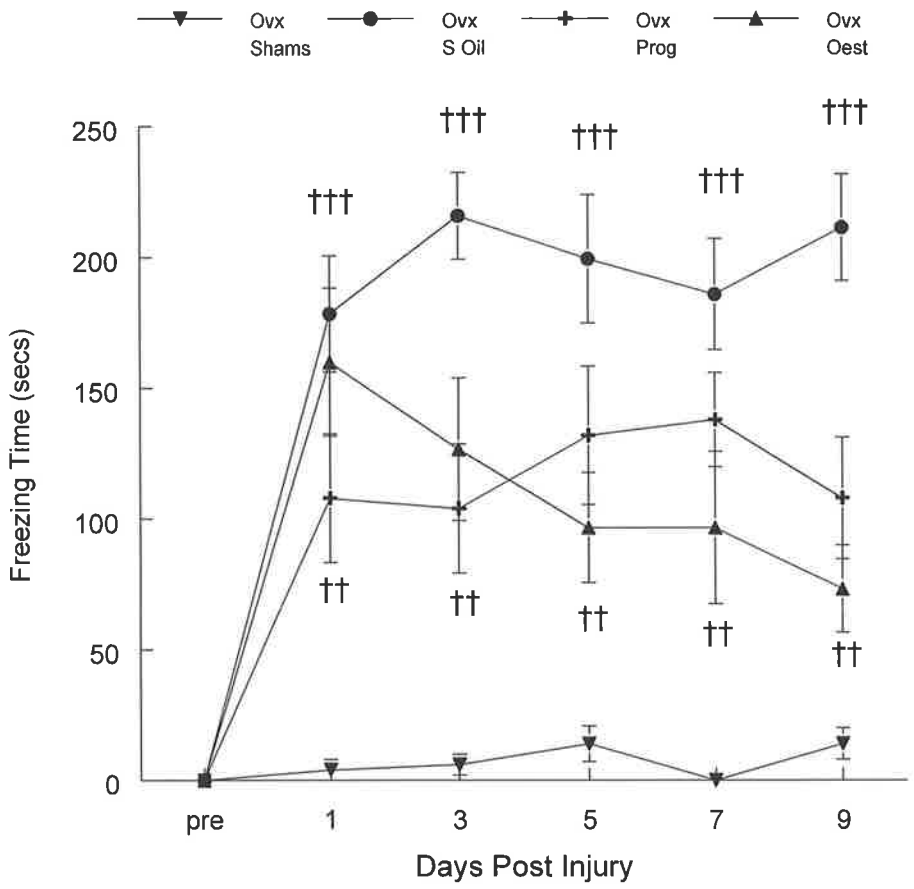


Figure 5.4B Changes in open field activity (freezing time) in ovariectomised shams and ovariectomised females treated with sesame oil, progesterone and oestrogen following diffuse TBI. Data are the means ± SEM (n=10 per group). †† $p<0.001$, ††† $p<0.0005$ compared with respective pre-injury scores.

The freezing times for all ovariectomised animals, recorded 24h post-injury, were significantly different compared with respective pre-injury baseline levels; vehicle treated ovariectomised animals, (t[9]=8.055, p<0.0005), progesterone treated ovariectomised animals (t[9]=4.943, p<0.001), and oestrogen treated ovariectomised animals, (t[9]=8.055, p<0.0005). However, both oestrogen and progesterone treated animals had significantly less freezing behaviour than vehicle treated ovariectomised females (F[2, 26]=17.571; p<0.0005). There was no significant improvement in any group after 24h over the remainder of the nine day assessment period.

5.4 Discussion

The present study has demonstrated that ovariectomy in female animals reduces their performance on functional tests following TBI. Of interest was the fact that the performance of an ovariectomised female after injury was similar to that of an injured male, and in some functional tests, worse than that of the males. In contrast, administration of a physiological dose of either oestrogen or progesterone on a daily basis after injury generally restored the performance of the ovariectomised females to that of their intact female counterparts. We can conclude that the female gonadal hormones play an important role in determining outcome in females following TBI.

While there have been a number of reports supporting a neuroprotective role for oestrogen and progesterone following various insults to the CNS (Roof et al. 1999; Finklestein et al. 2001), few have examined the effects of the hormones on functional outcome following TBI. Early reports from this laboratory (Emerson et al. 1993) have shown that oestrogen is

protective in male animals, but may be deleterious to survival outcome in female animals following focal TBI. In contrast, oestrogen has been shown to improve female survival following diffuse TBI relative to males (Roof et al. 2000b). Of note is that these contradictory studies used pentobarbital and isoflurane, respectively, as the anaesthetic and we have since shown that both of these agents in themselves may account for the differences in female survival that was observed (Chapter Four). In a cortical lesion model, Roof and colleagues (1993) were able to demonstrate that females perform better than males after injury in the Morris water maze, an observation they later attribute to the protective effects of progesterone (Roof et al. 1994). Progesterone has been shown to improve sensorimotor and cognitive outcome following TBI in a series of papers by Stein and colleagues (Shear et al. 2002; Goss et al. 2003; Djebaili et al. 2004). These studies demonstrate a marked protective effect of progesterone in a cortical contusion model that results in profound focal injury. In the present study, we demonstrate that progesterone confers a beneficial effect on motor and cognitive outcome in ovariectomised animals following diffuse TBI. However, the beneficial effect of progesterone was no more pronounced than that conferred by oestrogen.

In assessing motor function after TBI, we noted that females performed significantly better on the rotarod test than males, both before and after injury. Ovariectomised animals demonstrated a rotarod performance that was identical to the males when using the raw data. However, raw data may not be indicative of the relative deficit in each group, and the data was therefore normalised against the group pre-injury performance. In this case,

ovariectomised females recorded significantly greater deficits after injury than males, indicating that removal of the gonadal hormones from intact females significantly affects their motor recovery after trauma. Administration of either oestrogen or progesterone after injury restored function to that observed in intact female animals. Similar effects of the gonadal hormones were observed for the Barnes maze where the ovariectomised animals were again significantly worse than male animals, as opposed to the hormonally treated ovariectomised animals that subsequently performed better than both the male and the intact female animals. No effect of the individual gonadal hormones was observed on spontaneous exploratory activity, although ovariectomised females performed identically to males. Intact females also performed significantly better than all the other injury groups. This suggests that both hormones may need to be present to confer a beneficial effect on this parameter. This is supported by the results of the freezing time in the open field, where the individual hormones reduced freezing time, but not to the level of an intact female.

The significant improvement in functional outcome with post-injury administration of either oestrogen or progesterone suggests that it is not the presence of the hormone at the time of injury that is protective, but rather the presence of the hormone as the tissue attempts to recover from the injury. Moreover, we have chosen a dose of the hormones that result in physiological levels, which is less than the dose used in previous studies of hormonal neuroprotection after TBI (Finklestein et al. 2001). Serum levels of oestradiol in the rat range between 5–50pg/ml while progesterone levels range between 7–18ng/ml over the oestrus cycle. A single subcutaneous injection of oestradiol at the dosage used in the

current experiments results in a serum concentration that peaks at ~50pg/ml at 5h after injection, while the progesterone dose peaks at ~10ng/ml at 5h after administration (Gibbs 1998; Gibbs 1999).

A number of mechanisms by which the individual hormones confer their neuroprotective properties have been proposed (Roof et al. 2000b). Oestrogen has powerful antioxidant properties (Dixon et al. 1991) as well as the ability to attenuate excitotoxicity and amyloid β peptide toxicity (Goodman et al. 1996). It also reduces apoptosis by up-regulating bcl-2 expression (Cardona-Gomez et al. 2001) and has been reported to act as a neurotrophic factor (Gibbs 1999; Green et al. 2000). Progesterone has been shown to reduce membrane lipid peroxidation after TBI (Roof et al. 1997) as well attenuating oedema (Roof et al. 1992; Wright et al. 2001). It has also been shown to protect against glutamate toxicity (Ogata et al. 1993) and more recently, shown to inhibit caspase-3 activation (Djebaili et al. 2004). Thus, both hormones have a number of different properties whereby they could each contribute to neuroprotection after TBI. Whether the hormones would have a similar effect in males will be investigated in Chapter Six.

CHAPTER 6

EFFECTS OF OESTROGEN AND PROGESTERONE ON NEUROLOGIC IMPAIRMENT FOLLOWING DIFFUSE TRAUMATIC BRAIN INJURY IN MALE RATS

6.1 Introduction

As demonstrated in the previous chapter, ovariectomised female rats exogenously treated with either oestrogen or progesterone significantly improved their motor and cognitive performance following induced TBI. At this point, however, it is pertinent to consider the possible neuroprotective role, if any, that oestrogen and progesterone may play in relation to males. Two factors make this an urgent priority in TBI research. Firstly, TBI affects primarily young male adults in the age range 15–24 years (Hillier et al. 1997; Asbury et al. 1998) who sustain brain injury at a ratio of approximately two to one when compared with females (Kraus et al. 1988; Jennett 1998). Secondly, as previously mentioned, the dearth of clinically effective treatments following TBI significantly hinders recovery from these often debilitating injuries. The current study examines effects of oestrogen and progesterone on male outcome following diffuse TBI in rats. All conditions including model, anaesthesia, drug dosages and outcome tests have been kept identical to that used in the previous chapter examining female animals.

6.2 Methods and Materials

6.2.1 Animals and Induction of Traumatic Brain Injury

Adult male (n=38; 380–440g) Sprague-Dawley out-bred rats were fed and watered *ad libitum* and maintained on a 12h light/dark cycle for two weeks before being subjected to TBI. Rats were then randomly subdivided into four groups (n=5 surgical shams; n=11/treatment group: sesame oil vehicles, progesterone and oestrogen), and prepared for injury or sham surgery. In the treatment group animals, TBI was induced under halothane

anaesthesia using the impact–acceleration model of diffuse TBI as previously described in detail in Chapter Two. Sham animals were surgically prepared but not injured.

6.2.2 Drug Treatment and Administration

At 30min post-trauma, animals were administered a subcutaneous injection of either 33.3μg/kg 17β-estradiol, 1667μg/kg progesterone or equal volume (0.33 ml) sesame oil vehicle. These doses have been previously shown to result in physiological serum levels of the respective hormones (Gibbs 1998; Gibbs 1999). Throughout the nine day assessment period after injury, daily injections were administered to maintain these physiological levels (Gibbs 1998; Gibbs 1999).

6.2.3 Assessment of Functional Outcome

Functional outcome was assessed using the rotarod, Barnes and open field tests as described in detail in Chapter Two. Briefly, for rotarod tests, animals were pre-trained on the device twice per day over five days prior to injury to establish a normal, uninjured baseline. Similarly, on the Barnes maze, animals were pre-trained twice per day during five consecutive days prior to injury to establish a normal, uninjured baseline. After injury, daily assessment of rotarod and Barnes maze performance was conducted at 24h post-injury and was repeated daily for a period of nine days. Exploratory behaviour after diffuse TBI was assessed using the open field test (Giulian & Silverman 1975). No pre-training is required for this test, however, immediately prior to injury a five minute baseline of animal activity was determined so as to compare post-injury behaviour. Assessment was

conducted at 24h post-injury and repeated on days 3, 5, 7 and 9 of the assessment period.

6.2.4 Statistical Analysis

Data are shown as means ± SEM. A split plot repeated measures analysis of variance was used to determine within subjects and interaction effects. Multivariate Pillai's Trace was chosen as the criterion statistic as it is robust with respect to violations of assumptions (Olson 1976). A p value of 0.05 was considered significant. If an interaction effect was observed the within subjects simple effects of time were analysed using separate one-way repeated measures ANOVA followed by a Bonferroni multiple comparisons test for selected pairs. If between groups simple effects on specific days after injury were of interest, one-way ANOVA followed by Tukey's post-hoc tests was used. Significance in mortality was assessed using Fishers exact test.

6.3 Results

6.3.1 Mortality

There was no significant difference across the treatment groups with respect to mortality following induced diffuse TBI (p>0.05). Of the 38 animals used in the study three died following trauma before any drug treatment was administered.

6.3.2 Motor Outcome

Rotarod scores prior to and after injury are shown in Figure 6.1. Prior to injury, there was no significant difference in the rotarod score between any of the groups (male shams, 119.00±0.77sec; sesame oil vehicle, 118.78±0.81sec; oestrogen, 119.70±0.95sec;

progesterone, 119.80±0.63sec respectively). In sham animals, there was no significant change in motor function across the nine day assessment period confirming the surgical procedure did not affect motor outcome. This is consistent with our previous results (Chapter Four).

After injury, there were significant declines in rotarod scores in vehicle treated (t[9]=7.691, p<0.0005), progesterone treated (t[9]=3.969, p<0.001), and oestrogen treated (t[9]=5.741, p<0.001) animals by 24h, with the decline in the hormone treated males being significantly less than in the vehicle treated controls. Vehicle treated animals recorded a significant motor deficit until day eight of the nine day assessment period at which point no significant difference was observed compared with their pre-injury scores (t[9]=3.083, p>0.05). This spontaneous recovery with repeated exposure to the assessment task has been discussed in detail in Chapter Three. In contrast, hormone treated animals demonstrated a more rapid recovery to pre-injury levels. Progesterone treated male animals were no longer significantly different from pre-injury by day five following injury, (t[9]=1.843, p>0.05), while oestrogen treated males returned to pre-injury motor levels by day four of the nine day assessment period, (t[9]=2.747, p>0.05).

There were no significant between group differences in motor ability between oestrogen treated and progesterone treated animals at any time point after injury, (p>0.05 level at each time point). In contrast, both hormone treated animal groups demonstrated significantly improved motor scores at every time point of the assessment period compared with vehicle treated controls (alpha level varying from p<0.0005 to p>0.05). There was

also a significant time x group interaction observed for motor scores during the assessment period between vehicle treated, progesterone treated and oestrogen treated males, (Pillai's Trace=1.149, $F[18,38]=2.849$, $p<0.01$). The interaction can be explained mostly by the heterogenous pattern of recovery observed in the progesterone and oestrogen treated animals from day three of the assessment period compared with vehicle treated controls.

6.3.3 Cognitive Outcome

Figure 6.2 illustrates the cognitive recovery pattern for male treatment groups following TBI. Prior to injury, there were no significant differences between the male treatment groups with regards to latency to escape the aversive light and noise stimuli (shams 9.00±1.5sec; sesame oil 10.5±1.8sec; progesterone 11.8±1.2sec; oestrogen 12.3±1.6sec). Over the nine day assessment period, shams also recorded no change from their pre-injury time to locate the escape tunnel confirming the surgical procedure did not affect cognitive outcome.

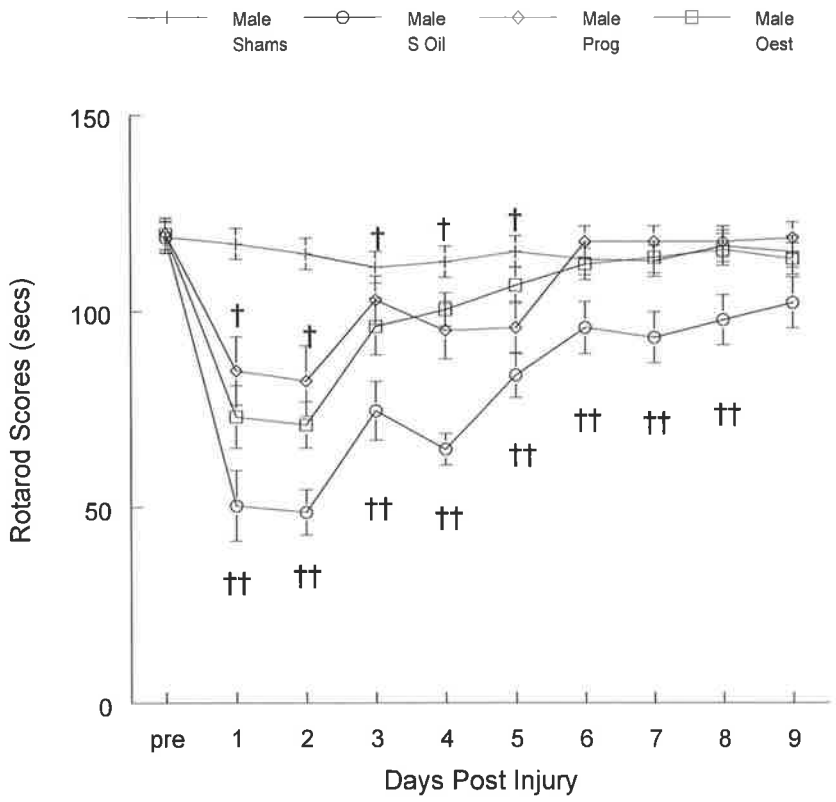


Figure 6.1 Motor (Rotarod) function in male shams and male animals treated with sesame oil, progesterone and oestrogen following TBI. Data are the means ± SEM (n=10 per group). † $p<0.001$, †† $p<0.0005$ compared with respective pre-injury scores.

After injury, there was a significant change in escape latency over time (Pillai's Trace=0.821, $F[9,19]=9.667$, $p<0.01$). Vehicle treated animals demonstrated profound cognitive deficits at 24h after trauma compared with their pre-injury baseline scores, ($t[9]=3.996$, $p<0.001$). This increase in latency to locate the escape tunnel persisted until day six of the assessment period at which point they no longer demonstrated a significant difference from pre-injury values ($t[9]=2.281$, $p>0.05$). In contrast, progesterone treated animals did not have a significant increase in their latency time compared to pre-injury values, (24h: $t[9]=2.231$, $p>0.05$). Similarly, oestrogen treated animals had no significant increase in their escape latency time compared to their pre-injury baseline, (24h: $t[9]=1.954$, $p>0.05$). This difference between the hormone treatment groups and the vehicle treated controls was significant by ANOVA ($F[2,27]=12.316$; $p<0.001$), with a significant difference (post-hoc Tukey's $0.0005<p<0.05$) between the hormone treatment groups and the vehicle treated controls at all points other than days one and five. There was no significant difference between oestrogen and progesterone treated animals.

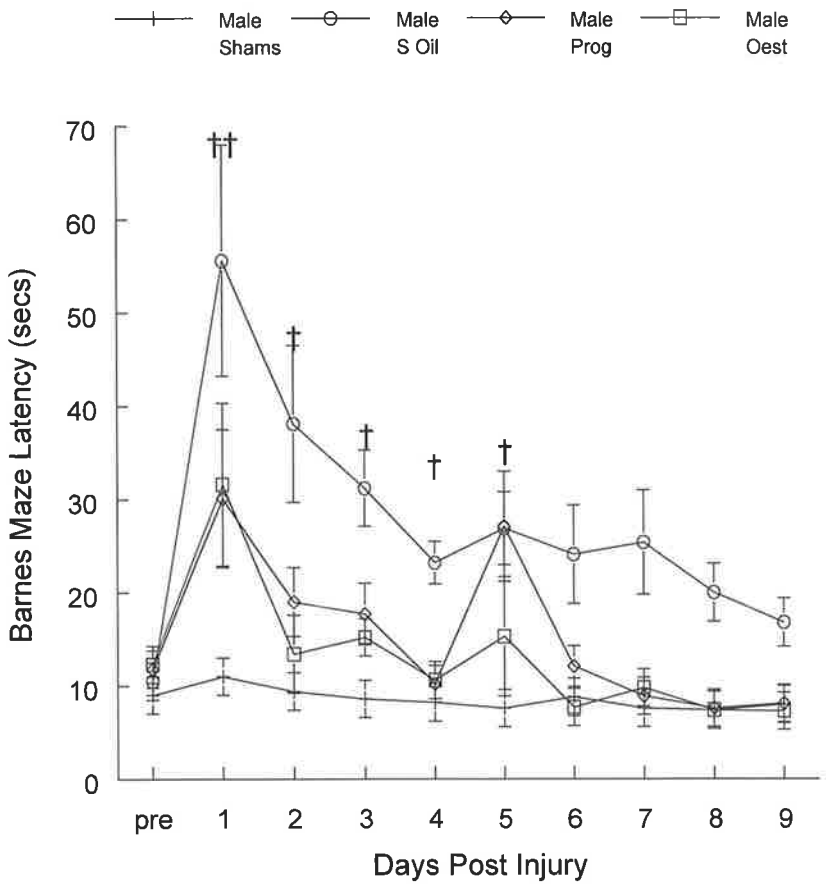


Figure 6.2 Cognitive (Barnes maze) function in male shams and male animals treated with sesame oil, progesterone and oestrogen following TBI. Data are the means ± SEM (n=10 per group). † $p<0.01$, †† $p<0.001$ compared with respective pre-injury scores.

6.3.4 Open Field Outcome

Squares Traversed

Figure 6.3 illustrates the spontaneous exploratory activity for the male treatment groups following TBI. Prior to injury, there was no significant difference amongst the treatment groups during the pre-injury five minute assessment session (shams, 220.00±27.30; sesame oil, 226.20±16.90; progesterone, 232.70±16.30; oestrogen, 194.80±9.00 squares). Shams demonstrated no significant change in the number of squares traversed across the nine day assessment period, confirming that the surgical procedures did not affect spontaneous exploratory activity. Some non-significant habituation (Thompson 1993) was observed.

After injury, there was a significant decline in spontaneous exploratory activity in all of the injury groups (Pillai's Trace=0.949, $F[5,23]=85.061$, $p<0.0005$). All treatment groups showed a significant decrease in the number of squares traversed at 24h compared with their respective pre-injury levels, and no significant recovery was noted over the remainder of the nine day assessment period (see Figure 6.3). There were no significant differences between the progesterone and oestrogen treatment groups, which is similar to that observed in females (Chapter Five).

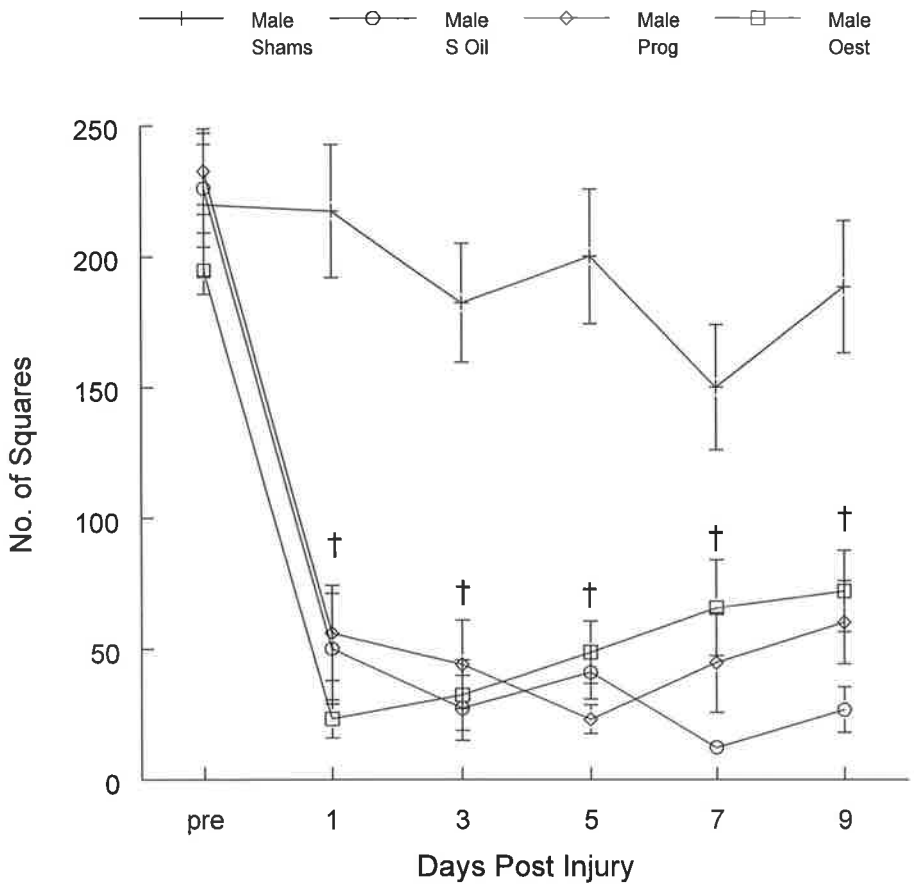


Figure 6.3 Behavioural (Open Field – Squares Traversed) function in male shams and male animals treated with sesame oil, progesterone and oestrogen following TBI. Data are the means ± SEM (n=10 per group). † $p<0.0005$ compared with respective pre-injury scores.

Freezing

As part of the five minute open field test, we also measured the time that an animal remained in a stationary location without exploring their environment as a measure of anxiety (Vallee et al. 1997; Larsson et al. 2002). Prior to injury (Figure. 6.4), none of the animals demonstrated any freezing behaviour in their pre-injury five minute assessment period. Male shams also recorded an insignificant amount of freezing behaviour across the nine day assessment period confirming that the surgical procedures did not affect the freezing pattern of these animals.

After injury, there was a significant increase in open field freezing behaviour compared with pre-injury values, (Pillai's Trace=0.920, $F[5,23]=52.683$, $p<0.0005$). Vehicle treated animals ($t[9]=9.095$, $p<0.0005$), progesterone treated animals ($t[9]=7.236$, $p<0.0005$), and oestrogen treated males ($t[9]=6.536$, $p<0.0005$), each recorded a significant increase in their freezing behaviour at 24h after injury. No animal group recovered to pre-injury levels across the entire nine day assessment phase, although a significant treatment effect was observed ($F[2,27]=4.841$, $p<0.05$). Tukey's post-hoc tests demonstrated that oestrogen treated animals were significantly different from vehicle treated animals ($p>0.01$) on day seven of the assessment period. Progesterone treated animals demonstrated a similar trend when compared with vehicle treated animals, but the difference did not reach statistical significance ($p=0.090$).

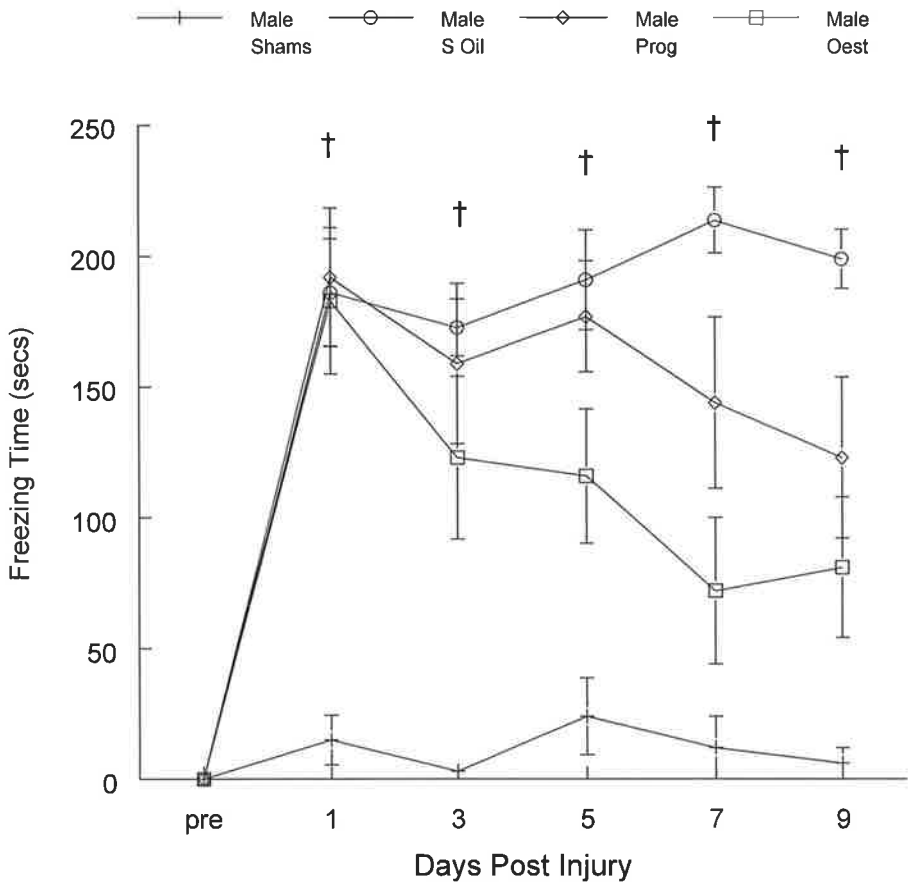


Figure 6.4 Behavioural (Open Field – Freezing Times) function in male shams and male animals treated with sesame oil, progesterone and oestrogen following TBI. Data are the means ± SEM (n=10 per group). † $p<0.0005$ compared with respective pre-injury scores.

6.4 Discussion

The current study has demonstrated that both oestrogen and progesterone improve motor and cognitive outcome in male animals after TBI. Neither hormone was more effective than the other at improving outcome. With respect to open field spontaneous activity and freezing, there was a beneficial effect of oestrogen on freezing, while progesterone caused a non-significant reduction in freezing time. Neither hormone had any effect on spontaneous exploratory activity.

In female animals, the limited effect of the individual hormones on the open field test was not enough to improve performance to that of intact females, and it was postulated that the superior performance of intact females may be due to the simultaneous presence of both gonadal hormones (Chapter Five). This would be a possible therapeutic option in females after TBI. However, in males, a combined effect mediated through hormone receptors is unlikely. An alternative may be that the dose used in the present study is insufficient to facilitate the maximal protective effects of the hormones. Stein and colleagues (Goss et al. 2003) have determined that the optimal dose of progesterone required for functional improvement is between 8 and 16mg/kg administered over at least five consecutive days. In the present study, we have used a dose of 1.7mg/kg administered daily on the basis that this results in female physiological levels in serum. However, since any protective effects in males would be unlikely to involve receptor mediated mechanisms, supraphysiological levels of the hormone may be required to generate maximum benefits.

While there are limited studies in TBI that have examined hormonal effects in male outcome following TBI, most that have been published support a protective role for both oestrogen and progesterone. Emerson and colleagues (1993) demonstrated in male animals that oestrogen improves both biochemical and neurologic outcome after FP induced brain trauma. Subsequently, (Roof et al. 1994) reported that male rats treated with progesterone following cortical contusion injury were less impaired on the Morris water maze task than vehicle treated animals. This protective effect of progesterone on post-traumatic performance in the Morris water maze was subsequently confirmed in studies using both the FP (Shear et al. 2002) and cortical contusion models (Djebaili et al. 2004) of TBI in rats. This is in contrast with earlier studies by the Stein group (Grossman et al. 2000) that demonstrate a lack of effect by progesterone administration on functional outcome in males, and the authors postulate that progesterone's effects may be dependent upon lesion location. A number of other studies in ischaemia have also reported beneficial effects of the neurosteroids in male rats. For example, both acute and chronic administration of 17β-estradiol has been shown to be protective in males following induction of middle cerebral artery occlusion (Toung et al. 1998). Progesterone has also been shown to be protective in this model with the treatment causing a reduction in infarct size and a significant improvement in functional outcome as assessed by the Rotarod test, Zea Longa test and adhesive-backed paper test (Chen et al. 1999).

A number of mechanisms by which the individual hormones confer their neuroprotective properties have been proposed (Roof et al. 2000b). For example, oestrogen has powerful antioxidant properties (Dixon et al. 1991) and the ability to attenuate amyloid β peptide toxicity and glutamate mediated excitotoxicity (Goodman et al. 1996). It has also been suggested that oestrogen reduces apoptosis by up-regulating bcl-2 expression (Cardona-Gomez et al. 2001) and has been reported to act as a neurotrophic factor (Gibbs 1999; Green et al. 2000). Like oestrogen, progesterone has also been shown to protect against glutamate toxicity (Ogata et al. 1993) as well as apoptosis by inhibiting caspase-3 activation (Djebaili et al. 2004). In addition, progesterone is well known to reduce membrane lipid peroxidation (Roof et al. 1997) as well as attenuating oedema (Roof et al. 1992; Wright et al. 2001). Thus, both hormones have a number of different properties whereby they could each contribute to neuroprotection in males after TBI.

In conclusion, we have demonstrated that both oestrogen and progesterone improve functional outcome when administered to male rats after TBI. While the administration of the individual hormones does not increase performance to that of female animals observed in Chapter Five, there was a significant improvement relative to male animals injured and administered equal volume vehicle. One can speculate that there are intrinsic differences in male and female responses after trauma that defines the sex-specific response independent of exogenous hormone administration.

CHAPTER 7

EFFECTS OF PROGESTERONE AND OESTROGEN ON OEDEMA AND BLOOD–BRAIN BARRIER PERMEABILITY FOLLOWING TRAUMATIC BRAIN INJURY

7.1 Introduction

The previous chapters have illustrated the protective effects of the female gonadal hormones on functional outcome in both male and female animals after diffuse TBI. While the mechanisms of action are unknown, a number of reports have suggested that the effects of the hormones on oedema formation after CNS injury may be significant (Roof et al. 2000b; Finklestein et al. 2001). Cerebral oedema is a serious consequence of TBI, resulting in increased ICP and possibly death (Foda et al. 1994). In young victims of trauma, it has been reported that brain oedema is responsible for up to 50% of all deaths (Feickert et al. 1999). Currently, there is no effective treatment in clinical practice, with interventions such as mannitol, corticosteroids, hyperthermia, barbiturates and drainage of CSF having either limited success or being completely ineffective (Foda et al. 1994).

A number of experimental brain trauma studies have demonstrated that the female gonadal hormones, and in particular progesterone, may significantly attenuate post-traumatic oedema formation. For example, normal cycling female rats develop less oedema than males at 24h after TBI (Roof et al. 1992; Roof et al. 1993). Females at the high oestrogen stage of their cycle (proestrus) demonstrated 50% of the oedema developed in males whereas females that were high in progesterone (pseudo-pregnant) showed virtually no evidence of oedema. The attenuation of oedema was apparent following contusion injury in both male and female rats (Roof et al. 1992), even when delayed by up to 24h after injury (Roof et al. 1996). Later studies have shown that at least three days of progesterone administration is required to have a significant effect on oedema development (Galani et al.

2001). No effects of hormonal status on integrity of the BBB have been detected (Duvdevani et al. 1995).

In contrast to the experimental TBI literature, the effects of female gonadal hormones on oedema and brain swelling in human head injury are less clear. A recent report examining this issue has shown that females under 50 years of age have worse oedema and brain swelling than males (Farin et al. 2003) suggesting that the female gonadal hormones have no beneficial effect on oedema formation. Whether this is a reflection of the difference between clinical injury with its highly diffuse nature and the experimental models used to date with their focal lesions is uncertain.

The current study therefore examines the effects of progesterone and oestrogen on oedema formation and BBB permeability following diffuse TBI in rats. The temporal profile of oedema formation after TBI in both males and females will first be characterised, followed by an examination of female gonadal hormones on this profile in both ovariectomised females and males.

7.2 Methods and Materials

7.2.1 Experimental Design

The experiment was conducted in three parts. The first part involved charting a time course for brain water content in intact female and male animals following diffuse TBI. Intact female (n=30; 5/group; 310–400g) and male (n=30; 5/group; 380–450g) Sprague-Dawley out-bred rats were injured and killed at one of six times points (5h, 24h, 48h, 3d, 4d, 5d)

and compared with surgical sham animals (intact female and male; n=5/group).

In the second part, the effect of the female gonadal hormones on oedema was assessed in ovariectomised female (n=42: 6/group; 350–410g) and adult male (n=54: 6/group; 380–450g) animals. Animals were treated with either sesame oil vehicle, progesterone or oestrogen and assessed for oedema development at 5h, 24h and 72h after injury and compared with male or ovariectomised female shams (n=6/group).

In the third part of the experiment, the BBB permeability at 5h after trauma was assessed in intact females and males, and then the effects of oestrogen and progesterone on barrier permeability determined. Intact females (n=3; 320–380g) and ovariectomised female rats (n=3/group: sesame oil; progesterone; oestrogen; 350–420g) were injured and the permeability of the BBB to EB assessed and compared with intact and ovariectomised shams (n=3/group). Male (n=3/group: sesame oil; progesterone; oestrogen; 380–450g) were similarly assessed and compared with shams (n=3). Rats that died immediately after induced TBI were replaced in the study. All rats were fed and watered *ad libitum* and maintained on a 12h light/dark cycle before being subject to the surgical procedures.

7.2.2 Ovariectomy

Female rats (n=60) were surgically ovariectomised at seven weeks of age as described in detail in Chapter Two. Briefly, anaesthesia was induced using halothane (3% induction followed by 1% maintenance) and a bilateral ovariectomy performed by ligation and dissection of the ovaries. Rectal temperature in all animals was maintained at 37°C with a

thermostatically controlled heating pad. Animals were allowed to recover for approximately nine weeks, after which TBI was induced.

7.2.3 Vaginal Smearing

The stage of oestrus in intact female animals was determined using vaginal smears (Maeda et al. 2000). Briefly, vaginal lavages were collected and analysed at the time of trauma. The epithelium of the vagina was scraped with a disposable inoculating loop and the sample of cells spread onto a clean microscope slide and flushed with 96% ethanol. The samples were then fixed and stained with Papanicolaou stain and analysed using a light microscope (x20). The cell type (leukocytes, nucleated epithelial cells, and/or cornified epithelial cells) was recorded. The stage in the oestrus cycle (metestrus, diestrus, proestrus or oestrus) at the time of the lavage was determined based on the presence or absence of these cell types. At the time of trauma, 66% of the intact females were in diestrus, 17% in oestrus and 17% in metestrus.

7.2.4 Induction of Injury

All animals were injured under halothane anaesthesia using the impact–acceleration model of diffuse brain injury (Foda et al. 1994) as described in detail in Chapter Two. Briefly, the skull was exposed by a midline incision and a stainless steel disc (10mm in diameter and 3mm in depth) was fixed rigidly with polyacrylamide adhesive to the animal's skull centrally between lambda and bregma. The rats were subsequently placed on a 10cm foam bed and subjected to brain injury induced by dropping a 450g brass weight a distance of two metres onto the stainless steel disc. Such an injury has been previously shown to

produce DAI of moderate severity (Foda et al. 1994). During all surgical procedures and in the immediate recovery period, rectal temperature was maintained at 37°C by use of a thermostatically controlled heating pad. Subsequent to injury, all wounds were sutured, anaesthesia was terminated and after stabilising, animals were returned to their cages.

7.2.5 Drug Treatment and Administration

At 30 minutes post-trauma, animals were administered a single subcutaneous injection of either 33.3μg/kg 17-β-estradiol, 166 μg/kg progesterone or equal volume sesame oil vehicle (0.33ml), depending on treatment group. These doses have been previously shown to result in physiological serum levels of the respective hormones (Gibbs 1998; Gibbs 1999).

7.2.6 Oedema Measurement

At the pre-selected time points, animals were re-anaesthetised with halothane and decapitated. All sham animals were decapitated at 30 minutes after surgery. Brains were rapidly removed from the skull, the olfactory bulbs and cerebellum discarded and the cortex and subcortex separated. The cortex and subcortex of each rat was placed separately into pre-weighed and labelled glass vials with quick fit lids to prevent evaporation. After weighing for wet water content, the vials (glass lids removed) were then placed in an oven for 72h at 100°C. Vials and brain segments were then re-weighed to obtain dry weight content. Oedema in each brain sample was calculated using the wet/dry method formula (Elliot et al. 1949):

[(wet wt – dry wt)/wet weight] x 100.

7.2.7 Determination of Blood–Brain Barrier Permeability

EB is a serum albumin tracer and extravasation of EB was used for determination of BBB permeability at 5h after TBI as described in detail in Chapter Two. Briefly, at 4h after injury, 2% EB was injected intravenously at a dose of 2ml/kg. Animals were then re-anaesthetised at 5h with halothane and perfused using saline to remove intravascular EB dye. Animals were then decapitated, the brains removed and homogenised in phosphate buffered saline. Trichloroacetic acid was then added to precipitate protein, and the samples were cooled and centrifuged. The resulting supernatant was measured for absorbance of EB using a spectrophotometer.

7.2.8 Statistical Analysis

Data are shown as means ± SEM. Statistical analysis involved both one-way and two-way between groups ANOVA. Post-hoc comparisons with Tukey's HSD test were used to determine specific differences between groups. A p value of 0.05 was considered significant, however if the assumption of homogeneity of variance was violated a p value of 0.01 was considered significant (Pallant 2001). Significance in mortality was assessed using Fishers exact test.

7.3 Results

7.3.1 Mortality

There were 16 deaths in the current study (n=10 males, n=6 ovariectomised females and no intact females). There was no significant difference amongst the groups. Most deaths occurred in the first 30 minutes after TBI when animals with observed respiratory distress did not respond to manual resuscitation. Animals that died following TBI were replaced.

7.3.2 Pre-injury Brain Water Content

The temporal profile for oedema development in male, female and ovariectomised female animals in the cortex and subcortex is shown in Figures 7.1A and 7.1B, respectively.

There were significant differences in the water content of uninjured hormonally intact female, ovariectomised female and male sham animals in both the cortex ($F[2,12]=5.930$ $p<0.05$) and the subcortex ($F[2,12]=8.740$; $p<0.01$) as determined by ANOVA. Tukey's post-hoc comparisons demonstrated that in the cortex, intact females (78.15±0.11%) had significantly lower water content than the males (78.72±0.08%). The cortical water content of ovariectomised female shams (78.61±0.11%) fell between the male and intact female values and was not significantly different from either. Similarly in the subcortex intact females (75.87±0.35%) and males (77.45±0.20%) were significantly different, with the water content of the ovariectomised females (76.74±0.17%) being between the two values and not significantly different from either.

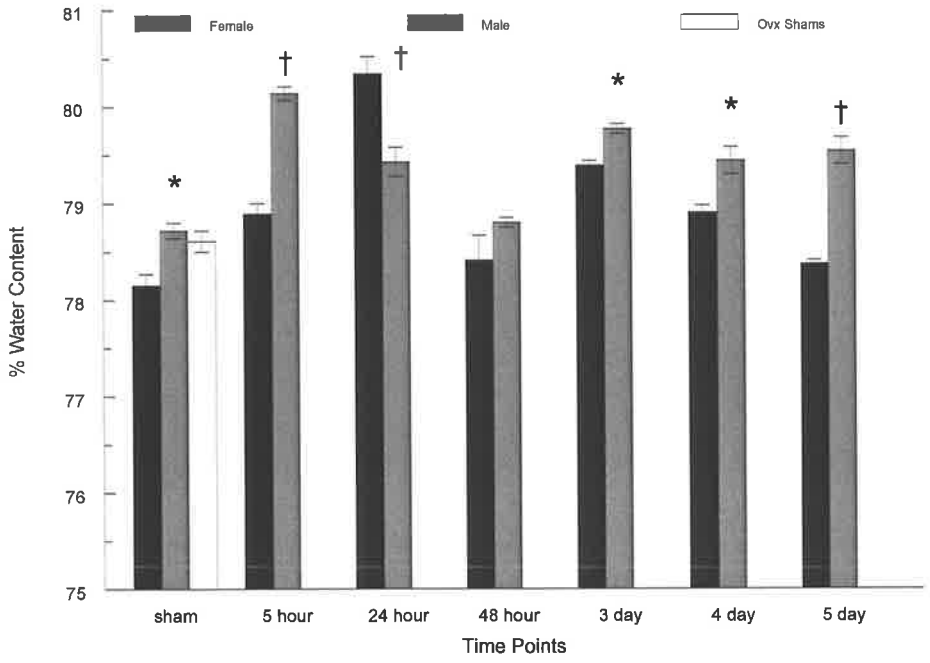


Figure 7.1A Percentage cortical water content in male and female rats recorded over a five day assessment period following diffuse TBI. Data are the means ± SEM (n=5 per time point) (* $p<0.05$ between male and female groups, † $p<0.01$ between male and female groups).

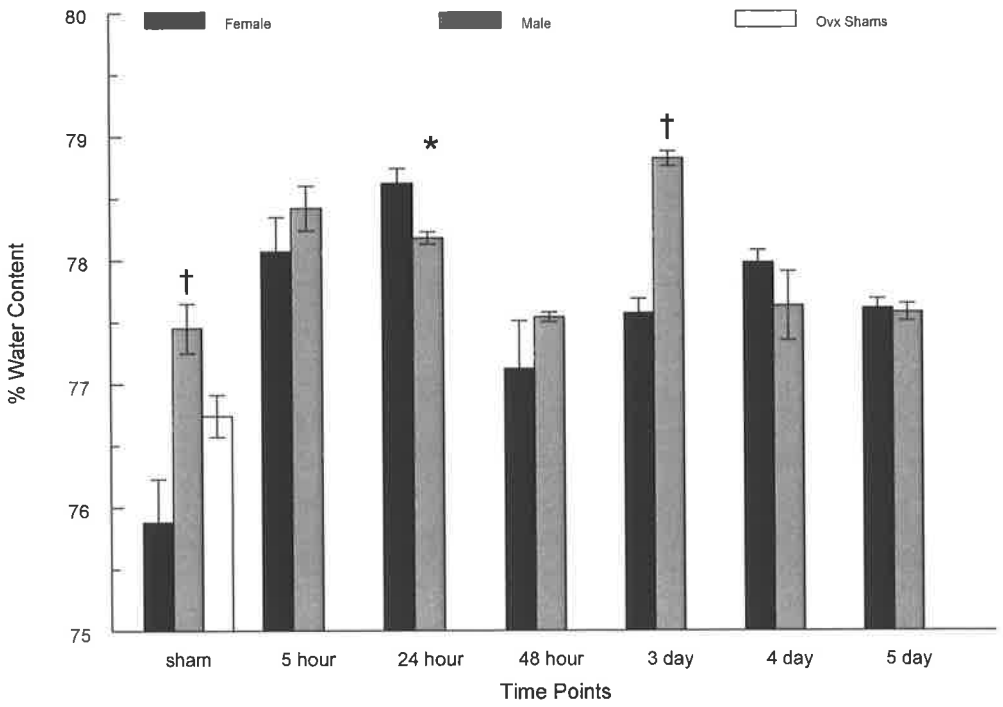


Figure 7.1B Percentage subcortical water content in male and female rats recorded over a 5 day assessment period following diffuse TBI. Data are the means ± SEM (n=5 per time point) (* $p<0.05$ between male and female groups, † $p<0.01$ between male and female groups).

7.3.3 Post-injury Oedema

Intact females had a significant main effect of time in the cortex, (F[6,28]=23.309; $p<0.01$). Tukey's post-hoc comparisons illustrated that brain water content was significantly increased at 5h, 24h, 3d and 4d post-TBI versus intact female shams (Figure 7.1A). The largest increase occurred at the 24h time point. Intact females also exhibited a significant increase in brain water content in the subcortex, (F[6,28]=10.653; $p<0.01$).

Tukey's post-hoc comparisons revealed that subcortical oedema levels significantly increased in females at all time points except 48h when compared with shams (Figure 7.1B).

Male animals also recorded a main effect of time in the cortex (F[6,28]=21.281; p<0.001). Tukey's post-hoc comparisons demonstrated significantly increased oedema at 5h, 24h, 3d, 4d and 5d after TBI versus male shams (Figure 7.1A). Male animals demonstrated the highest level of oedema post-injury at the 5h assessment point. Similarly, there was a significant difference in male subcortical oedema levels (F[6,28]=13.378; p<0.001). Tukey's post-hoc comparisons illustrated that subcortical oedema significantly increased in males at 5h, 24h and 3d after induced TBI (Figure 7.1B).

A significant sex by time interaction was observed (F[6,56]=15.434; p<0.01) in the cortex of both groups (Figure 7.1A) indicating that cortical water content in the sexes had maxima at different time points. Tukey's post-hoc comparisons illustrated that females recorded significantly less oedema after injury than male animals at 5h, 72h, and 5d. In contrast, female animals demonstrated significantly more oedema than male animals at the 24h time point. There were no significant sex differences observed at the 48h point.

In the subcortex, two-way between groups analysis of variance of oedema formation demonstrated a significant sex by time interaction (F[6,56]=6.377; p<0.01) (see Figure 7.1B). Tukey's post-hoc comparisons illustrated that, following injury, females recorded significantly more oedema than male animals at the 24h assessment point, but significantly

less oedema than males at the 72h time point. No significant sex differences were observed at any other time point (5h, 48h, 4d, 5d) in the assessment period.

7.3.4 Effects of Hormones on Oedema

Females

Effects of oestrogen and progesterone on oedema formation following TBI are shown in Figures 7.2A and 7.2B. Measurements were only taken at 5h (the male maximum), 24h (the female maximum) and at 72h, as these were the points that demonstrated a significant difference between the sexes.

Vehicle treated ovariectomised female animals demonstrated a significant increase in cortical water content after injury ($F[3,20]=4.467$; $p<0.05$). Tukey's post-hoc comparisons demonstrated that the significant differences occurred at five hours and 72 hours with no significant oedema present in vehicle treated ovariectomised females at 24 hours (Figure 7.2A). This oedema profile in the ovariectomised female animals was very similar to that observed in male animals (Figure 7.1A). Non-parametric Kruskal Wallace analysis also revealed similar differences in oedema levels in the subcortex of vehicle treated ovariectomised females versus ovariectomised shams ($\chi^2=8.360$; df=3; $p<0.05$).

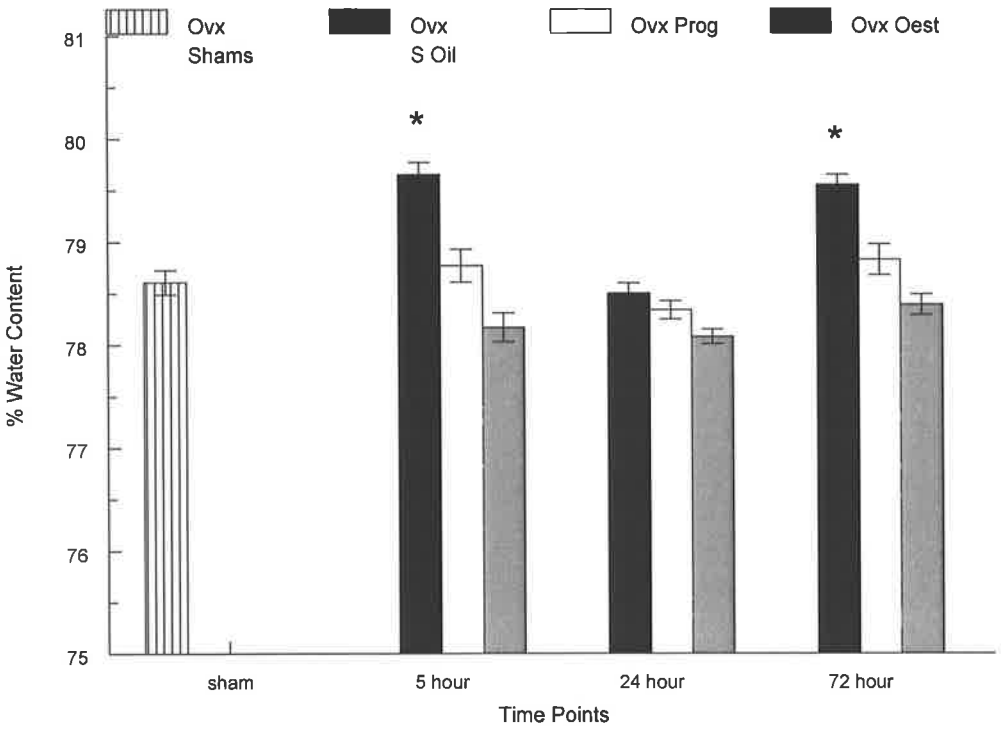


Figure 7.2A Percentage cortical water content in female rats recorded at 5h, 24h and 72h following diffuse TBI. Data are the means ± SEM (n=6 per time point) (* $p<0.05$ compared with ovariectomised shams).

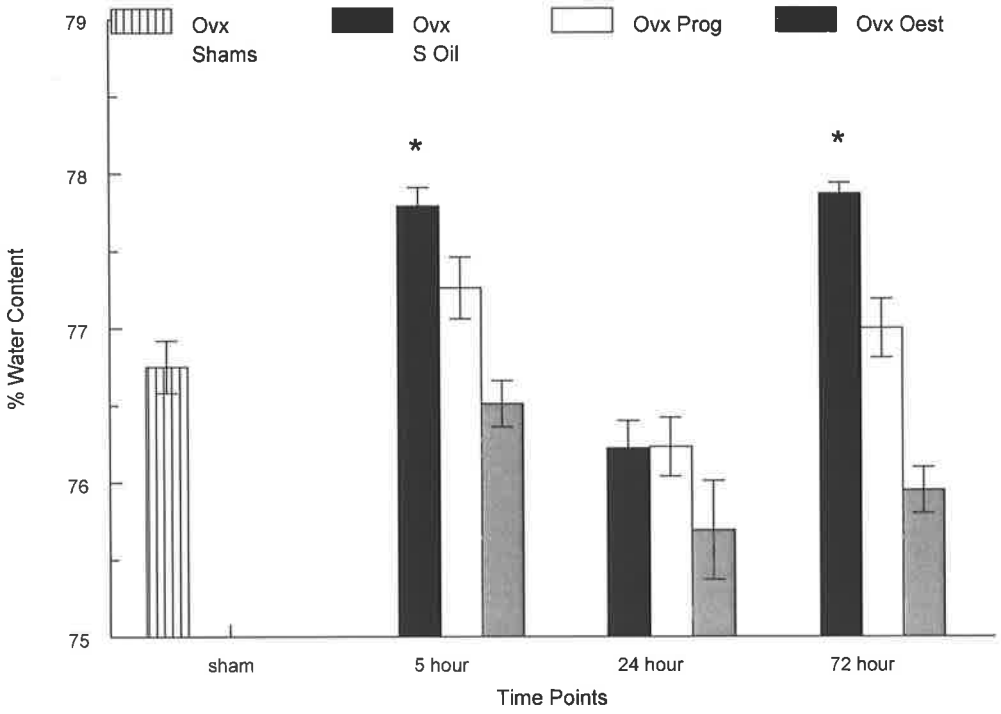


Figure 7.2B Percentage subcortical water content in female rats recorded at 5h, 24h and 72h following diffuse TBI. Data are the means ± SEM (n=6 per time point) (* $p<0.05$ compared with ovariectomised shams).

Two-way analysis of variance revealed a significant main effect for drug treatment in the cortex in animals treated with either progesterone or oestrogen ($F[4,30]=14.442$; $p<0.01$). Tukey's post-hoc comparisons indicated that at 5h after injury, progesterone and oestrogen treated animals had significantly less oedema than the vehicle treated controls. In fact, the water content in hormone treated animals was not significantly different from that observed in the shams. Similarly at 72h, progesterone and oestrogen treated animals had significantly less oedema than the vehicle treated controls. The water content was again not significantly

different from the shams.

A significant main effect for drug treatment was also recorded in the subcortex (F[4,30]=5.106; p<0.01). Tukey's post-hoc comparisons demonstrated that at 72h post-injury vehicle treated animals recorded significantly more oedema than the progesterone and oestrogen treated animals. Progesterone and oestrogen treated animals recorded a similar subcortical water content as that measured in ovariectomised shams.

Males

Effects of oestrogen and progesterone on oedema formation following TBI in males are shown in Figures 7.3A and 7.3B. One-way analysis of variance showed that oedema was significantly increased after injury in the cortex of vehicle treated male animals when compared to the sham animals (F[3,20]=30.024; p<0.01). Tukey's post-hoc analysis revealed that significant differences occurred at 5h, 24h and 72h after injury. This is identical to that observed in Figure 7.1A, suggesting that the vehicle had no effect on oedema formation after injury. Similarly in subcortical tissue, significant oedema was observed in vehicle treated animals compared with shams (F[3,20]=8.681; p<0.01). Tukey's post-hoc comparisons revealed that significant differences occurred at 5h and 72h after injury.

With hormone treatment, a significant main effect for drug was observed in cortical tissue (F[6,50]=20.746; p<0.01). Tukey's post-hoc analysis demonstrated that progesterone and oestrogen treated animals had a significantly lower brain water content than vehicle treated

animals at 5h, 24h and 72h after injury. In fact the brain water content in the hormone treated male animals was not significantly different from sham values. Similarly, a significant main effect for drug treatment was observed in the subcortical tissue of male animals (F[6,50]=11.620; p<0.01). Tukey's post-hoc comparisons demonstrated that progesterone and oestrogen treated animals had a significantly lower water content than vehicle treated animals at 5h and 72h after injury, and were not significantly different from shams.

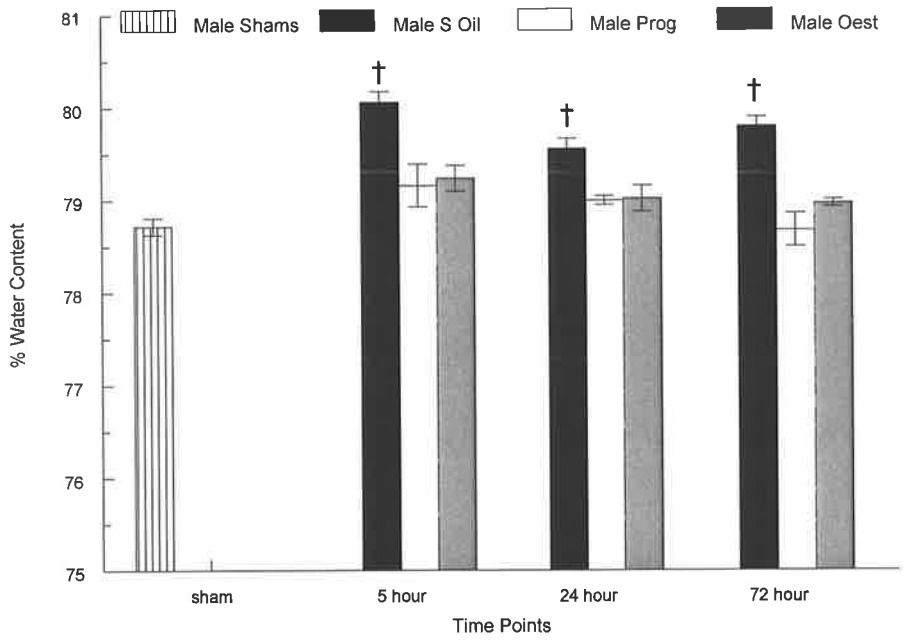


Figure 7.3A Percentage cortical water content in male rats recorded at 5h, 24h and 72h following diffuse TBI. Data are the means ± SEM (n=6 per time point) († p<0.01 compared with male shams).

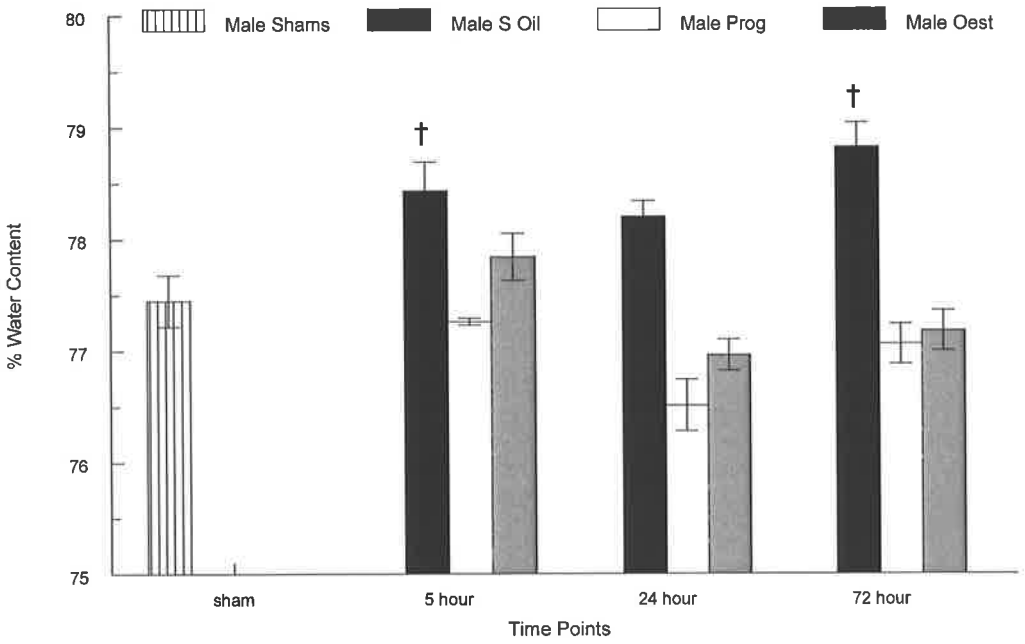


Figure 7.3B Percentage subcortical water content in male rats recorded at 5h, 24h and 72h following diffuse TBI. Data are the means ± SEM (n=6 per time point) († $p<0.01$ compared with male shams).

7.3.5 Effects of Hormones on Blood–Brain Barrier Permeability

Previous studies in impact–acceleration induced brain trauma have shown that increased BBB permeability is apparent immediately after the injury and for up to 6h after trauma (Barzo et al. 1996; Beaumont et al. 2000; O'Connor et al. 2003). Accordingly, effects of hormones on BBB permeability in both males and females after trauma were assessed at 5h post-trauma.

Females

The pattern of EB extravasation in intact and ovariectomised female rats following TBI is shown in Figure 7.4. Results of one-way ANOVA demonstrated a significant group effect ($F[5,12]=4.284$; $p<0.05$). Tukey's post-hoc analysis indicated that vehicle treated ovariectomised females demonstrated a significant increase ($p<0.05$) in BBB permeability to EB relative to ovariectomised shams. Administration of either progesterone or oestrogen significantly inhibited EB extravasation, indicating a reduced BBB opening in response to hormone treatment. This observation was supported by the demonstration that injured intact females had a negligible penetration of EB, with the value being identical to that of non-injured intact shams.

Males

The pattern of EB extravasation in male rats following TBI is shown in Figure 7.5. Analysis using one-way ANOVA revealed a significant group effect ($F[3,8]=45.677$; $p<0.01$). Tukey's post-hoc test showed that male animals had a significant increase in BBB permeability after TBI compared to shams. The increase was identical to that observed in males treated with sesame oil, confirming that the vehicle had no effect on BBB permeability after trauma. Treatment with either progesterone or oestrogen resulted in a significant inhibition of BBB permeability to EB. Indeed, the level of EB extravasation following hormone treatment was identical to that observed in the sham animals.

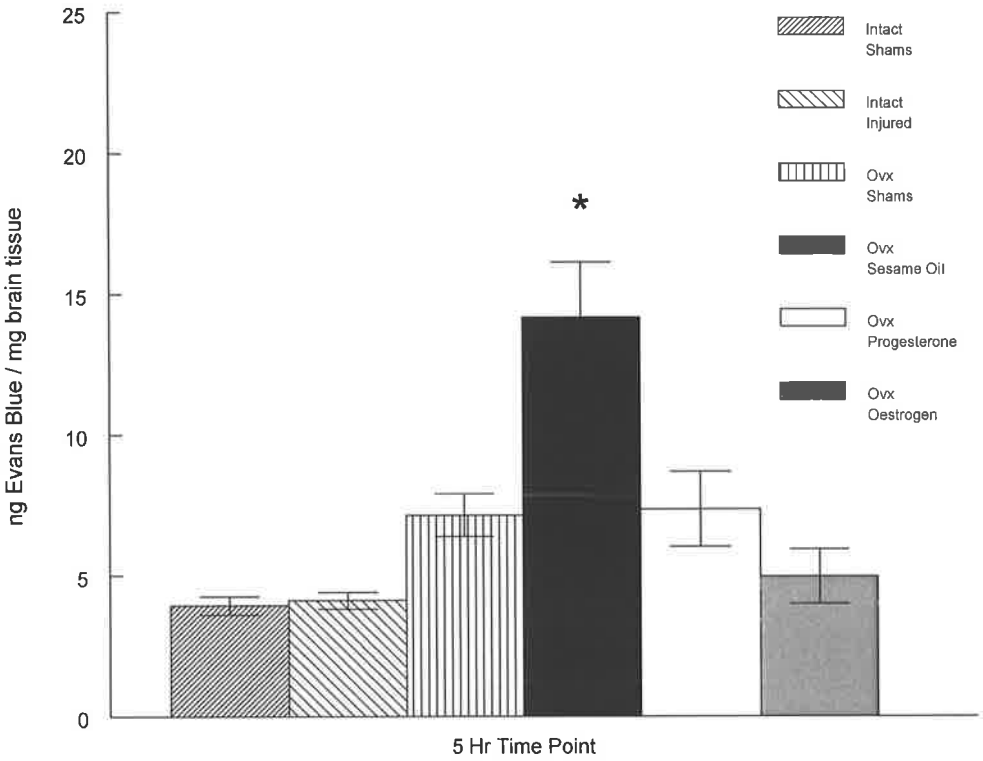


Figure 7.4 EB extravasation levels in intact and ovariectomised females recorded 5 hours after induced TBI. Data are the means ± SEM (n=3 per group) (* $p<0.05$ between intact shams, ovariectomised shams and ovariectomised females treated with sesame oil, progesterone or oestrogen).

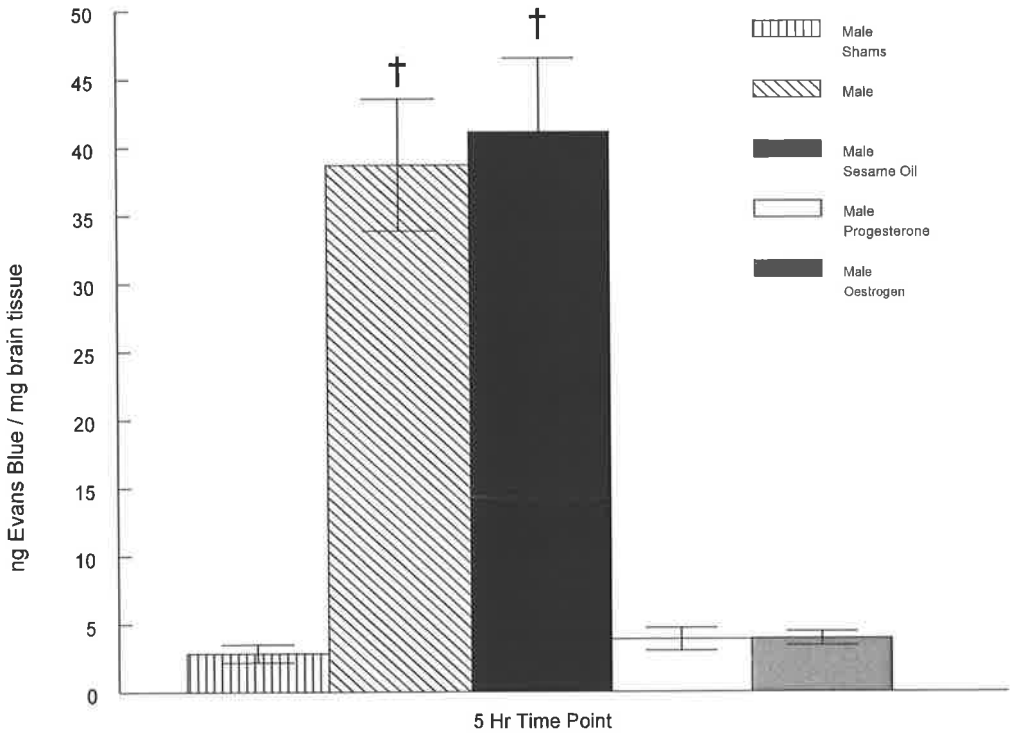


Figure 7.5 EB extravasation levels in male animals recorded five hours after induced TBI. Data are the means ± SEM (n=3 per group) († $p<0.01$ between male shams, and males treated with sesame oil, progesterone or oestrogen).

7.4 Discussion

In the present study, we have demonstrated that females have a different oedema profile after diffuse TBI from males. This variation seems to depend on endogenous hormone levels since injury in ovariectomised females resulted in a male oedema profile. Administration of a single physiological dose of either oestrogen or progesterone profoundly inhibited oedema formation in both female and male animals at all time points up to three days post-trauma. They also inhibited any increased permeability of the BBB to EB dye, suggesting that the hormones have a direct effect on BBB permeability.

A number of studies have now reported effects of female gonadal hormones in CNS injury including ischaemic injury (Chen et al. 1999; Kumon et al. 2000), CCI injury (Roof et al. 1992; Roof et al. 1993; Roof et al. 1996; Galani et al. 2001) and bilateral medial frontal cortex injury (Wright et al. 2001; Shear et al. 2002). These models are generally focal in nature and a number of the traumatic injury models also involve a profound ischaemic component. The degree of oedema in these forms of injury is high, with increases in water content from 6–10% in male animals having been recorded (Roof et al. 1992; Wright et al. 2001). Clinical trauma involves a significant degree of DAI, and rodent models of diffuse TBI have shown small increases in brain water content of less than 3% (O'Connor et al. 2003). The present study has shown that the female gonadal hormones also effect oedema formation in such models of diffuse TBI.

Male animals illustrate a biphasic oedema profile after trauma, with a maximum at 5h, followed by a decline and a subsequent increase in oedema over the following four days. This profile is consistent with previous results in this model of trauma (Barzo et al. 1997b), with the initial oedema coinciding with an opening of the BBB (Barzo et al. 1996) and being considered as a form of vasogenic oedema. The oedema that develops at later time points has been described as a cytotoxic oedema, with reports suggesting that the early vasogenic oedema may be permissive for the development of the subsequent cytotoxic phase (Beaumont et al. 2000).

The profile for oedema formation in females after TBI is considerably different from that noted in males. Female animals displayed a significant increase in oedema at 5h, but this was much less than that observed in the males. Furthermore, unlike the males, the early oedema was not followed by a decline in water content, but an increase by 24h that was significantly greater than that observed in the males. Thereafter, female brain water content levels declined steadily over the ensuing four days, being less than that observed in males at every time point. What is perhaps most intriguing is that ovariectomy conferred a male oedema profile on the ovariectomised animals, with a maximum at 5h and significant oedema at later time points. Clearly, the presence of endogenous levels of hormones not only attenuated the oedema development after trauma, but also altered the temporal profile of oedema formation.

Administration of a single physiological dose of either oestrogen or progesterone resulted in a profound attenuation of oedema in both male and ovariectomised female animals for up to 72h after TBI. This included the early vasogenic phase of oedema as well as the later (72h) cytotoxic oedema that was noted in male animals. In both cases the level of brain water was reduced to that of shams, be it in female or male animals or with oestrogen or progesterone administration. Few studies have examined the effects of exogenous oestrogen administration on oedema development following TBI. However, a number of studies have investigated progesterone and have reported a need for supraphysiological concentrations of progesterone (8–16mg/kg) for up to three days post-trauma to see an effect on oedema (Finklestein et al. 2001). Our present studies have demonstrated that a single physiological dose of 1.7mg/kg is sufficient to produce a significant effect. Previous studies (Gibbs 1998) have shown that this dose of progesterone results in serum concentrations of ~10ng/ml at 5h after injection declining to ~5ng/ml by 24h. Normal serum levels of progesterone range between 7–18ng/ml over the rat oestrus cycle. Serum levels of oestradiol in the rat range between 5–50pg/ml over the oestrus cycle. A single subcutaneous injection of oestradiol at the dosage used in the current study results in a serum concentration that of ~50pg/ml at 5h after injection declining to ~37pg/ml by 24h after injection (Gibbs 1998; Gibbs 1999).

The beneficial effect of the female gonadal hormones on the early oedema was further investigated by examining the effects of the hormones on BBB permeability after trauma. In ovariectomised females and males, there was a significant increase in EB extravasation at 5h after trauma. The degree of extravasation in ovariectomised female animals was less than that observed in the males, despite the degree of early oedema being identical in both groups. Clearly, more processes are involved in the early phase of oedema after TBI than simply increased BBB permeability. Administration of either oestrogen or progesterone significantly attenuated the EB extravasation, in most cases down to sham levels. Our results with progesterone contradict those of Duvdevani and colleagues (1995) who reported that the hormone does not attenuate BBB opening after frontal cortical contusion. One possible explanation for this disparity is that the contusion models cause extensive brain tissue damage and loss at the site of injury, with meningeal tearing and tissue laceration. The BBB is extensively and persistently disrupted under these conditions. In the diffuse TBI model used in the present study, there is no meningeal damage, no tissue laceration, no extensive focal tissue loss and only a transient opening of the BBB.

The mechanisms by which oestrogen and progesterone attenuate BBB permeability and oedema after TBI are currently unknown, however a number of possibilities have been proposed (Roof et al. 1993; Roof et al. 2000b). Lipid peroxidation and free radical generation may increase BBB permeability and oedema formation. Both oestrogen and progesterone have been shown to inhibit membrane lipid peroxidation (Roof et al. 1997; Ayres et al. 1998) and have been described as antioxidants (Betz et al. 1990; Dixon et al.

1991). Oestrogen also has effects on CBF via modulation of nitric oxide formation (Pelligrino et al. 1998), and the ensuing vasodilation is thought to protect against BBB damage. In addition, progesterone has been shown to modulate the astroglial inducer of the BBB (Wolff et al. 1992) and inhibit vessel growth associated with leaky BBB function (Plum et al. 1963). Thus the hormones may attenuate BBB damage and resultant oedema formation by a number of different mechanisms.

In conclusion, we have demonstrated that female gonadal hormones have a profound effect on oedema formation after TBI. Not only does exogenous administration of oestrogen and progesterone attenuate oedema formation and reduce BBB permeability after TBI, in female animals, the endogenous levels of the hormones also alter the temporal profile of oedema formation. This may significantly affect the clinical management of oedema in females following diffuse TBI.

CHAPTER 8

EFFECTS OF OESTROGEN AND PROGESTERONE ON MORPHOLOGICAL OUTCOME FOLLOWING TRAUMATIC BRAIN INJURY

8.1 Introduction

Chapters Five, Six and Seven have shown that oestrogen and progesterone improve functional outcome in both female and male animals after TBI, and that a single physiological dose of either hormone profoundly inhibits oedema formation and any increase in blood brain barrier permeability after TBI. Whether such effects are correlated with attenuation of neuronal cell death is unclear, although a number of recent reports suggest such a neuroprotective role for both hormones.

Bramlett and Dietrich (2001) have shown that the presence of endogenous levels of female hormones reduce contusion volumes in females after traumatic brain injury, irrespective of the stage of oestrous cycle the animals were in. Similarly, Wise et al. (2001) showed that low concentrations of oestradiol significantly protected explants of rat cerebral cortex from ischaemic and metabolic damage. Additionally, these authors demonstrated that both young and middle aged rats pre-treated with physiological levels of 17β oestradiol prior to middle cerebral artery occlusion had reduced infarct damage. Dubal et al. (1999), using ovariectomised female rats, found that estradiol decreased the extent of neuronal death following ischaemia by an oestrogen β-receptor-mediated effect on Bcl-2, a proto-oncogene that can block necrotic and apoptotic cell death.

With respect to progesterone, a number of reports have now appeared supporting a role in morphological protection after traumatic injury to the CNS. In rats subjected to incomplete spinal paraplegia injury, progesterone administration resulted in less tissue and white matter damage at the epicentre of the injury when compared with control animals (Thomas

et al. 1999). Similarly in experimental stroke, progesterone administration before or two hours after transient middle cerebral arterial occlusion reduced infarct volume compared with control animals (Jiang et al. 1996). Following traumatic brain injury, progesterone has been reported to reduce necrotic damage (Shear et al. 2002), with the observation of reduced neuronal cell loss being confirmed in various brain regions during subsequent studies (Djebaili et al. 2004; He et al. 2004).

Thus, there exists a significant body of evidence supporting the concept that female gonadal hormones provide morphological protection after TBI, and that this morphological protection may be associated with an improvement in functional outcome. However, no studies have performed comparative studies examining the effects of oestrogen or progesterone on morphological outcome following diffuse traumatic brain injury. Accordingly, the present study examines the effects of both oestrogen and progesterone on dark cell change, as detected by haematoxylin and eosin (H&E) staining, apoptotic cell death as detected by caspase-3 immunoreactivity, and axonal injury as detected by amyloid precursor protein (APP) immunoreactivity, following diffuse traumatic brain injury in male and ovariectomised female rats.

8.2 Methods and Materials

8.2.1 Experimental Design

Effects of the female gonadal hormones on morphology after TBI were assessed in male (n=12; 3/treatment group; 380-450g), intact female (n=3; 310-400g), and ovariectomised female (n= 12; 3/treatment group; 350-410g) animals. An equal number of shams (n=3 per

gender group) were used for comparison. Female rats (n=15) were surgically ovariectomised at seven weeks of age as described in detail in Chapter Two. Briefly, anaesthesia was induced using halothane (3% induction followed by 1% maintenance) and a bilateral ovariectomy performed by ligation and dissection of the ovaries. Rectal temperature in all animals was maintained at 37°C with a thermostatically controlled heating pad. Animals were allowed to recover for approximately nine weeks, at which time traumatic brain injury was induced.

8.2.2 Induction of Injury and Drug Treatment

Sprague-Dawley out-bred rats were fed and watered *ad libitum* and maintained on a 12h light/dark cycle for two weeks before being subjected to TBI. TBI was then induced under halothane anaesthesia using the impact acceleration model of TBI as described in detail in Chapter Two. After injury, rats of each gender group (male and ovariectomised female) were randomly subdivided into four treatment groups (n=3/group) and received oestrogen, progesterone, equal volume vehicle or no treatment. Intact female animals were injured but not treated. Treatment was administered at 30min post trauma, and was made up of a subcutaneous injection of either 33.3μg/kg 17β-estradiol, 1667μg/kg progesterone or equal volume (0.33 ml) sesame oil vehicle. These doses have been previously shown to result in physiological serum levels of the respective hormones (Gibbs 1998; Gibbs 1999). An additional three animals per group (male, female and ovariectomised female) were used as surgical shams: surgically prepared for injury but not injured.

8.2.3 Perfusion Fixation and Paraffin Embedding

Perfusion was performed using 4% paraformaldehyde (tissue fixation) at three days after injury. This time point coincides with maximal caspase-3 immunoreactivity and apoptotic cell death in this model of injury as determined by previous studies in this laboratory (Cernak et al. 2002). Animals were anaesthetised with halothane and placed in the supine position on a wire rack. A bilateral thoracotomy was performed to expose the heart, and a blunt 19 gauge, 37mm needle inserted into the apex of the left ventricle and guided into position within the ascending aorta. Heparin (5000U; David Bull Laboratories, Mulgrave, Victoria, Australia) was injected slowly and the right atrium incised to permit vascular flushing. Paraformaldehyde was then flushed through the animals to complete the perfusion fixation process. Brains were subsequently removed, cut into 2mm coronal sections within a Kopf rodent brain blocker and the resultant sections embedded in paraffin wax.

8.2.4 Histology and Immunohistochemistry

Haemotoxylin and Eosin Staining

Consecutive 5μm sections were sliced from selected wax blocks using a microtome to give slices located between -3mm and -4 mm relative to bregma. The slices were then taken to water through xylene and ethanol and stained in Hemotoxylin for eight minutes. Following blueing in Scott's tap water substitute, the sections were counterstained in Young's Eosin for four minutes. The sections were then differentiated in tap water, dehydrated in ethanol, cleared in zylene and mounted in a synthetic mounting medium. Once dried and excess resin removed, the sections were viewed and digitally captured by light microscopy

(Olympus).

Amyloid Precursor Protein Immunohistochemistry

Consecutive 5µm sections were sliced from selected wax blocks as described above and immunolabelled with APP primary antibody (1:2000; monoclonal antibody 22C11, Boehringer) by overnight incubation at 4°C. After washing the slices in PBS, slices were then incubated with IgG-HRP conjugated secondary antibody (1:400; Sigma-Aldrich) for 1h at room temperature, and the subsequent immunocomplex visualised using diaminobenzidine as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia). Sections were viewed and digitally captured by light microscopy (Olympus).

Caspase-3 Immunohistochemitry

Sections were sliced from selected wax blocks using a microtome as described above and immunolabelled with caspase-3 primary antibody (1:1000; polyclonal antibody, PharMingen) by overnight incubation at 4°C. After washing the slices in PBS, slices were then incubated with IgG-HRP conjugated secondary antibody (1:400; Sigma-Aldrich) for 1h at room temperature, and the subsequent immunocomplex visualised using diaminobenzidine as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia). Sections were viewed and digitally captured by light microscopy (Olympus).

8.3 Results

Prior to injury, H&E sections of male sham animals (Figure 8.1) showed excellent CA3 architecture and an abundance of healthy neurones in both the hippocampus and cortex. A

healthy neurone was classified as having an oval nucleus with speckled chromatin and a well-defined nucleolus surrounded by pale eosinophilic cytoplasm. A dark neurone (dark cell change) was categorized by intense dark purple staining resulting from contraction of the nucleus and cytoplasm with a non-visible nucleolus. Dark cell change in H&E sections was interpreted as representing cell stress, possibly leading to cell death.

After TBI in male animals, extensive dark cell change and vacuolisation was apparent in both the hippocampus and cortex (Figure 8.1), with the hippocampus demonstrating a loss of layer architecture. Similar observations were made in the dentate gyrus and the CA1 hippocampal layer (Figure 8.2). Caspase-3 immunohistochemistry confirmed the presence of cells that were undergoing cell death, most likely through apoptotic means (Figure 8.3). Prior to injury, there were very few caspase-3 positive (dark brown staining) neurones in either the CA3 hippocampus or cortical regions (Figure 8.3), although after injury, significant numbers of caspase-3 immunopositive cells were clearly visible in both regions. At times there were dramatic changes in caspase-3 immunoreactivity after TBI in the dentate gyrus and CA1 region of the hippocampus (Figure 8.4). Densely staining caspase-3 positive neurones were clearly visible in these regions, together with contraction of the nucleus and cytoplasm, and vacuolisation. However, these dramatic changes were not consistently produced in these regions, and the cortex and CA3 were chosen as a more consistent indicator of morphological changes after TBI.

Staining with APP is considered a highly sensitive marker of axonal injury following TBI (Blumbergs et al. 1994), with the corpus callosum consistently showing APP

immunopositive axonal pathology after injury. Prior to injury in male animals, there was no visible staining with APP in the corpus callosum (Figure 8.5), consistent with the absence of any axonal injury as a result of the surgical and fixation procedures. After injury, darkly stained APP immunopositive axons and retraction balls were clearly visible (Figure 8.5), as was a loss of bundle integrity. These features were consistently present in the corpus callosum after TBI and indicate the presence of axonal injury in this white matter tract. Interestingly, there was also a positive neuronal APP response, particularly in the hippocampal CA1 region (Figure 8.6; high power). This profound APP response coincided with the presence of positive caspase-3 immunoreactivity, consistent with the concept that caspase-3 may cleave APP.

In female animals (intact), TBI did not produce a profound dark cell change response (Figure 8.7) as was previously observed in male animals (Figure 8.1). Indeed, there was a conspicuous absence of dark cell change and vacuolisation in both the cortex and the CA3 region of the hippocampus, although some neuronal cell loss was noted as compared to shams. Caspase-3 positive immunostaining was apparent in the female cortex after injury (Figure 8.8), although less so in the hippocampus. The relative absence of caspase-3 immunopositive staining in the hippocampus was confirmed by examination of the dentate gyrus and CA1 layer (Figure 8.9), which also consistently showed far less caspase-3 positive immunostaining than male animals (Figure 8.4). APP positive immunostaining of axonal injury in the corpus callosum was, however, clearly apparent in these injured female animals (Figure 8.10).

To examine whether oestrogen or progesterone was responsible for the morphological differences noted in intact female animals, injury was induced in both male and ovariectomised female animals and followed by a single bolus injection of the hormone or a vehicle administered at 30min after injury. In male animals, oestrogen did not prevent the development of dark cell change in the CA3 and cortex that was apparent in vehicle treated animals (Figure 8.11). In contrast, progesterone clearly attenuated dark cell change. Similarly, caspase-3 immunopositivity after TBI in male animals was not reduced by oestrogen treatment relative to vehicle treated animals (Figure 8.12), but progesterone resulted in a marked attenuation in the numbers of darkly staining caspase-3 positive cells. With respect to axonal damage, there was profound APP immunostaining in the tracts of the corpus callosum in vehicle treated animals (Figure 8.13), and this was reduced by both oestrogen and progesterone administration. Indeed, progesterone completely prevented APP immunostaining after injury, suggesting that the compound has a profound protective effect against axonal injury.

In ovariectomised female animals (Figure 8.14), injury resulted in a greater degree of dark cell change than observed in injured, intact female animals (Figure 8.7). Administration of the vehicle or oestrogen did not alter this response (Figure 8.15), with dark cell change and vacuolization being clearly apparent in the cortex and hippocampus. In fact, there was a tendency to increased injury with oestrogen treatment, particularly in the hippocampus. In contrast, progesterone treated animals showed a profound reduction in dark cell change relative to both the vehicle and oestrogen treated animals (Figure 8.15). Similar results

were observed with caspase-3 staining. Injury resulted in a marked increase in caspase-3 immunopositivity in both the cortex and hippocampus of ovariectomised female animals (Figure 8.16). Administration of vehicle or oestrogen did not reduce the numbers of caspase-3 positive cells, with oestrogen actually exacerbating injury, particularly in the hippocampus (Figure 8.17). In contrast, there was reduction in caspase-3 positivity relative to vehicle and oestrogen treated animals. With respect to axonal injury in ovariectomised female animals, there was an increase in APP immunostaining following injury (Figure 8.18), which was unaffected by vehicle treatment. While there was some reduction in axonal injury with oestrogen treatment (Figure 8.19), progesterone completely abolished APP immunoreactivity in the corpus callosum of these injured animals.

8.4 Discussion

The current study has shown that females have less neuronal injury as assessed by dark cell change, caspase-3 and APP immunoreactivity after TBI than do male animals. When examining the separate effects of oestrogen and progesterone, administration of either hormone to ovariectomised female animals after TBI reduced axonal injury, as reflected by APP immunostaining in the corpus callosum, while progesterone additionally resulted in a profound reduction in dark cell change and caspase-3 staining in both the hippocampus and cortex. Similar effects were seen in male animals, with progesterone being particularly effective at reducing morphological changes after TBI.

A number of studies have now shown that oestrogen is neuroprotective, both at the functional and morphological level (Dubal et al. 1999; Wise et al. 2001; Jover et al. 2002;

Monroe et al. 2002; Rau et al. 2003). While our studies demonstrated that oestrogen has a neuroprotective effect on axonal injury after TBI, we did not observe a significant effect of oestrogen on dark cell change or caspase-3 immunoreactivity in male or ovariectomised females. While the reasons for this disparity are unclear, there are several possibilities. A number of studies reporting neuroprotective effects for oestrogen use long-term treatment either before or after injury (Jover et al. 2002; Monroe et al. 2002; Rau et al. 2003) as opposed to the single bolus administered after injury used in our studies. Accordingly, it may be that long-term oestrogen exposure may be necessary to induce a neuroprotective effect on hippocampal and cortical neurones. Certainly, this is consistent with our observation that normal cycling females have less neuronal injury after TBI than ovariectomised females, an observation that has been previously reported (Bramlett et al. 2001). Alternatively, the dose used in our studies may be insufficient to confer a neuroprotective effect when administered as a single bolus. Previous studies have shown that the neuroprotective effect of oestrogen is mediated through a combination of receptor-dependent and receptor-independent mechanisms (Roof et al. 2000a; Shughrue et al. 2000; Beyer et al. 2003). Since oestrogen receptors are not highly expressed in hippocampal pyramidal neurones (Shughrue et al. 1997), the dose of oestrogen used in the present study may be insufficient to confer any neuroprotective effect, especially since higher doses are required for non-receptor mediated neuroprotection (Behl et al. 1995). This dose limitation would not apply with prolonged exposure to oestrogen since oestrogen itself is known to promote the expression of further oestrogen receptors (Sohrabji et al. 1994), particularly the ER-α receptor implicated in neuroprotection (Dubal et al. 1999). Under these

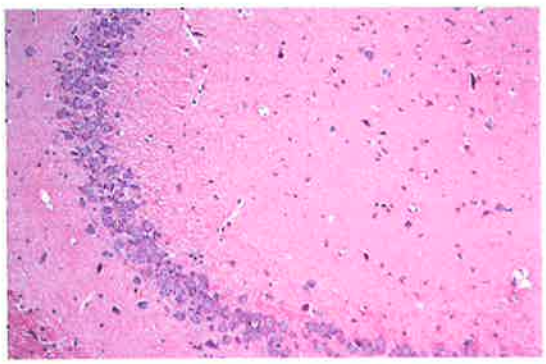
conditions, receptor-mediated effects may be expected to dominate. There is, nonetheless, the danger that increasing dosage may actually exacerbate injury, as has been reported by Picazo and colleagues (2003) in their in vivo studies of kainic acid toxicity in hilar hippocampal neurones. So while a single bolus injection of physiological concentrations of oestrogen is sufficient to ameliorate axonal injury, sustained exposure to physiological levels of oestrogen may be required to minimise cell death in the hippocampus and cortex.

In contrast to the effects of oestrogen, progesterone markedly reduced dark cell change and caspase-3 immunoreactivity in both the hippocampus and cortex after TBI, as well as virtually eliminating axonal injury in the corpus callosum. While no previous studies have reported effects of progesterone on axonal injury, several reports have previously documented the ability of progesterone to attenuate neuronal cell death in various models of brain injury including cortical brain ablation (Asbury et al. 1998) and contusion TBI (Shear et al. 2002; Djebaili et al. 2004). As in our studies with diffuse TBI, the reduction in cell death coincided with a reduction in caspase-3 activity in injured brains (Djebaili et al. 2004). This is consistent with previous observations that physiological levels of progesterone reduce both brain necrosis and apoptosis following excitotoxic insults (Lockhart et al. 2002).

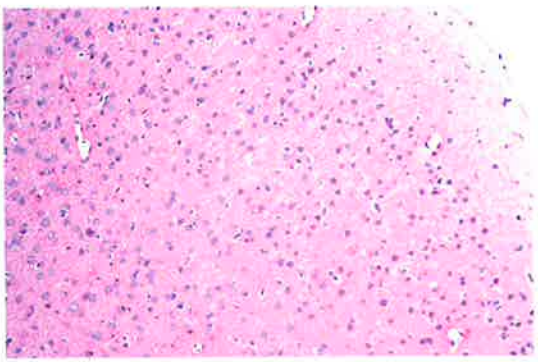
While the mechanisms by which progesterone is neuroprotective has not been fully characterised, a number of possibilities exist. Progesterone has been shown to reduce membrane lipid peroxidation after TBI (Roof et al. 1997) as well attenuating oedema (Roof et al. 1992; Wright et al. 2001). It has also been shown to protect against glutamate toxicity

(Ogata et al. 1993) and its effects on caspase-3 activation and apoptosis have been discussed above (Djebaili et al. 2004). Progesterone also modulates serum magnesium concentration, especially in men where significant increases have been reported with the hormone's administration (Muneyyirci-Delale et al. 1999). Given the central role that magnesium plays in the development of neurological deficits following TBI (Vink et al. 2000), progesterone's affects on magnesium may be critically important. With respect to axonal damage, progesterone has been reported to enhance myelin synthesis in brain (Schumacher et al. 2004), thereby providing neuroprotection and accelerating regenerative processes. Finally, progesterone has recently been shown to reduce the inflammatory response after TBI (Grossman et al. 2004), including the neurogenic inflammatory response (Duval et al. 1998) that has been recently implicated as a major factor in brain oedema formation (Nimmo et al. 2004).

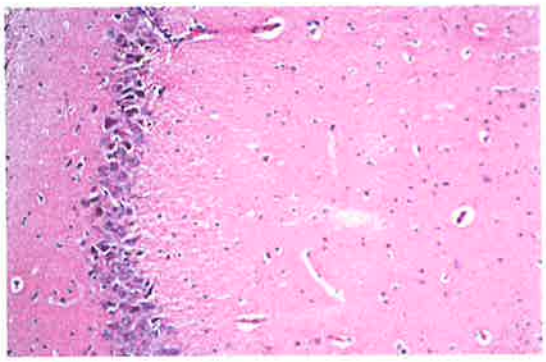
In conclusion, we have demonstrated that both oestrogen and progesterone protect against neuronal cell injury and death following TBI. While both hormones attenuate axonal injury, a single bolus dose of progesterone was particularly effective at reducing hippocampal and cortical injury as opposed to the single bolus physiological dose of oestrogen. This was particularly apparent with apoptotic cell death as identified by caspase-3 immunoreactivity. Based on these effects on neuronal cell morphology and survival, physiological levels of progesterone would be the preferred therapeutic strategy after TBI.



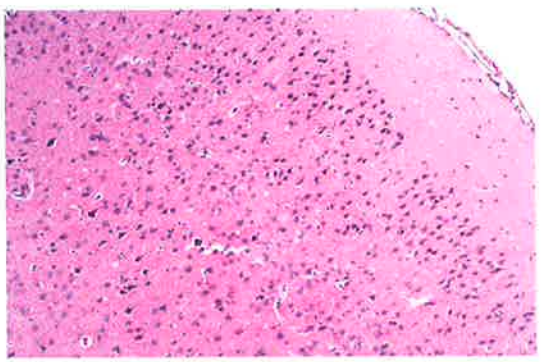
SHAM CA3 HIPPOCAMPUS (x60)



SHAM CORTEX (x60)

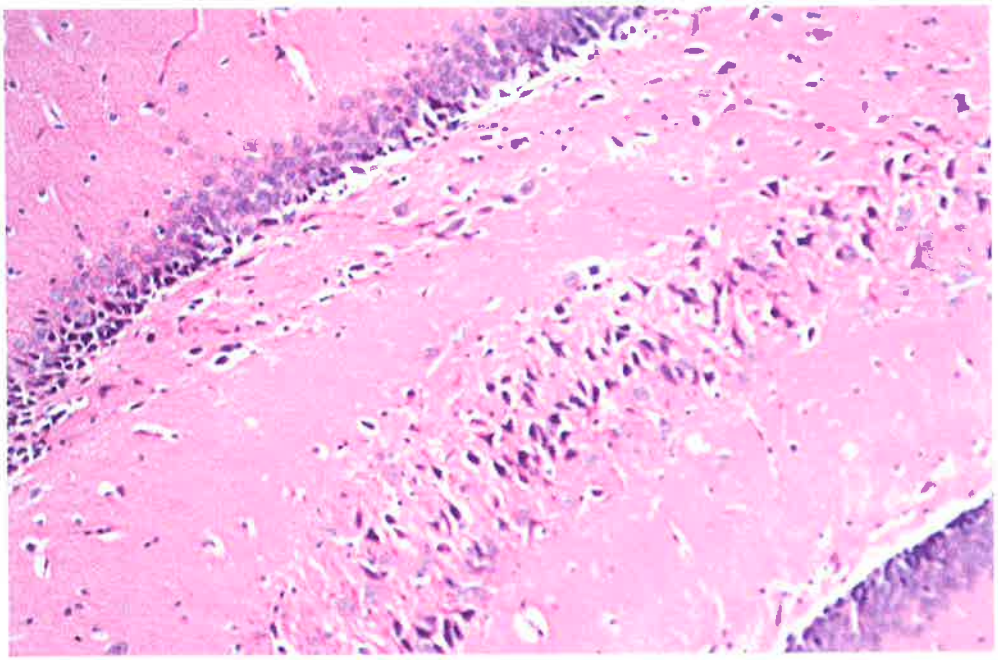


INJURED CA3 HIPPOCAMPUS (x60)

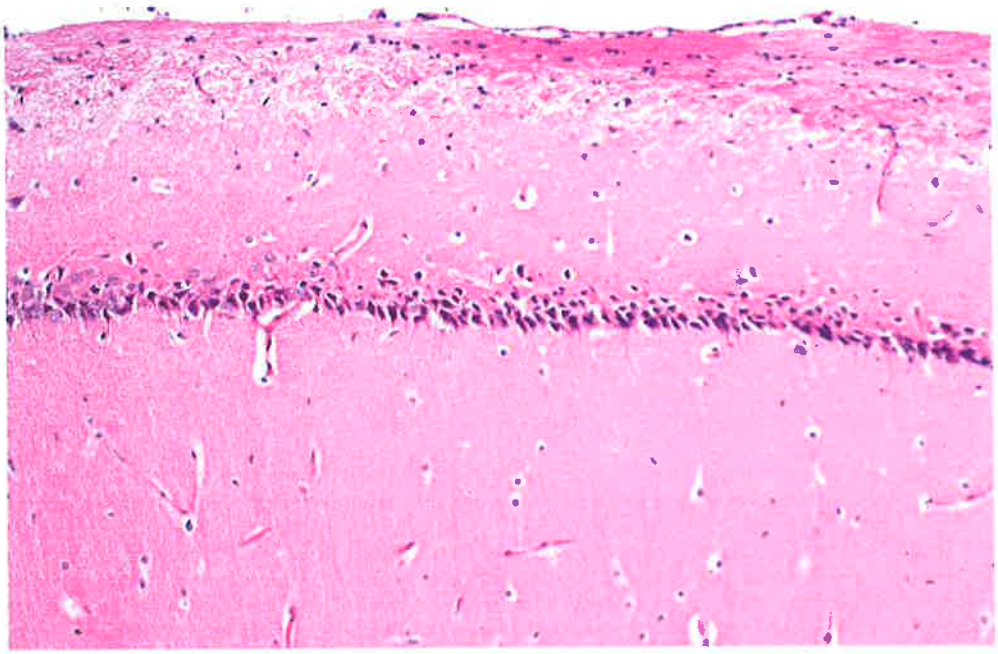


INJURED CORTEX (x60)

Figure 8.1 H&E stained sections from male Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Note the loss of cellular architecture in the CA3 hippocampal layer after injury, and the presence of dark cell change and vacuolization in both the cortex and hippocampus.



INJURED DENTATE GYRUS AND GRANULAR LAYER (x60)



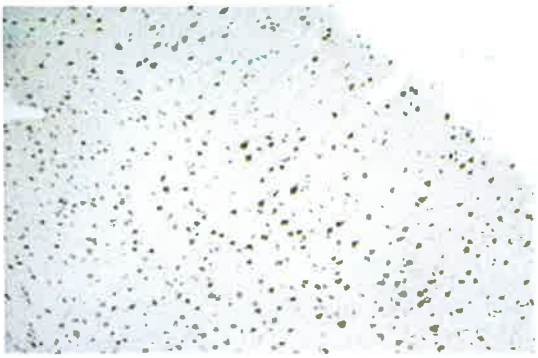
INJURED CA1 (x60)

Figure 8.2 H&E stained sections from male Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Extensive dark cell change and vacuolization is apparent in both the dentate gyrus and the hippocampal layers.

SHAM CA3 HIPPOCAMPUS (x60)

SHAM CORTEX (x60)

INJURED CA3 HIPPOCAMPUS (x60)



INJURED CORTEX (x60)

Figure 8.3 Caspase-3 stained sections from male Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Darkly stained caspase-3 positive cells are clearly visible after injury in the hippocampus and cortex.

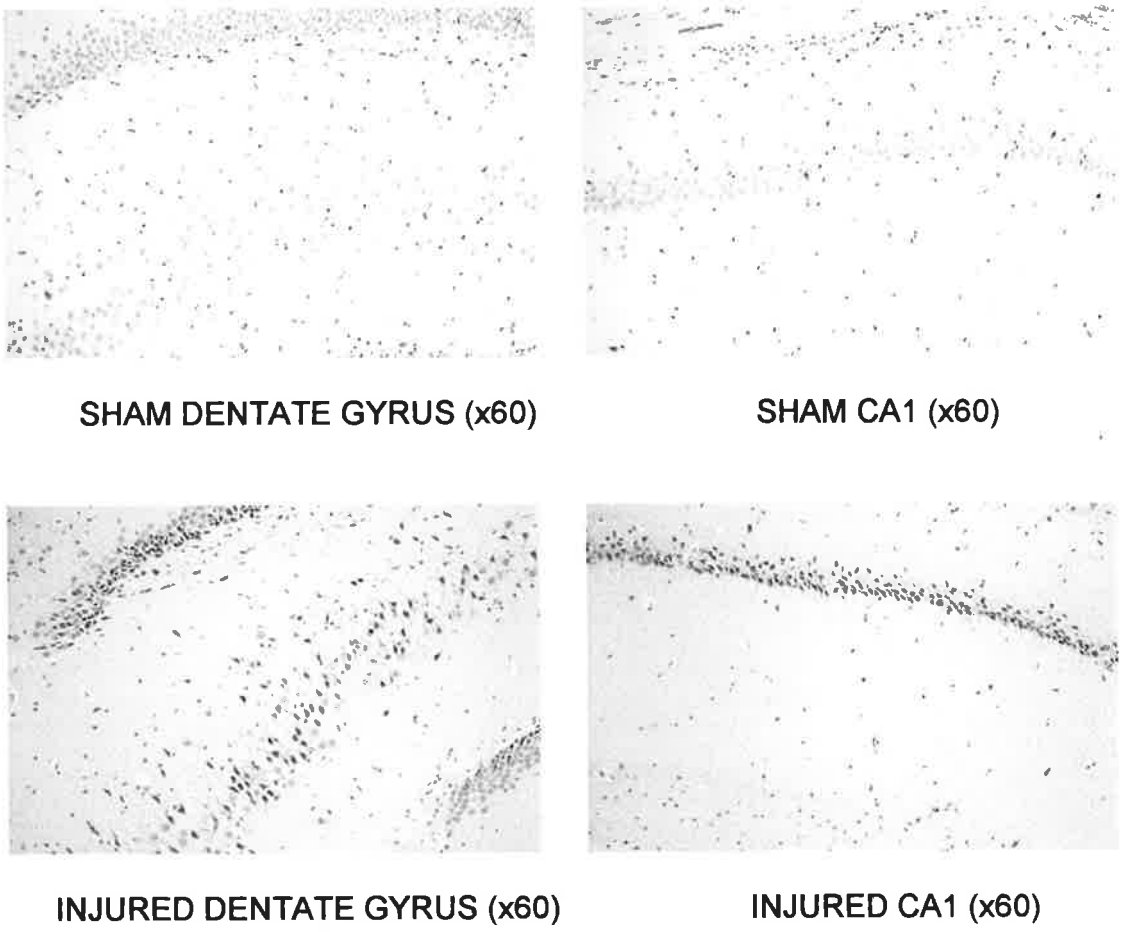
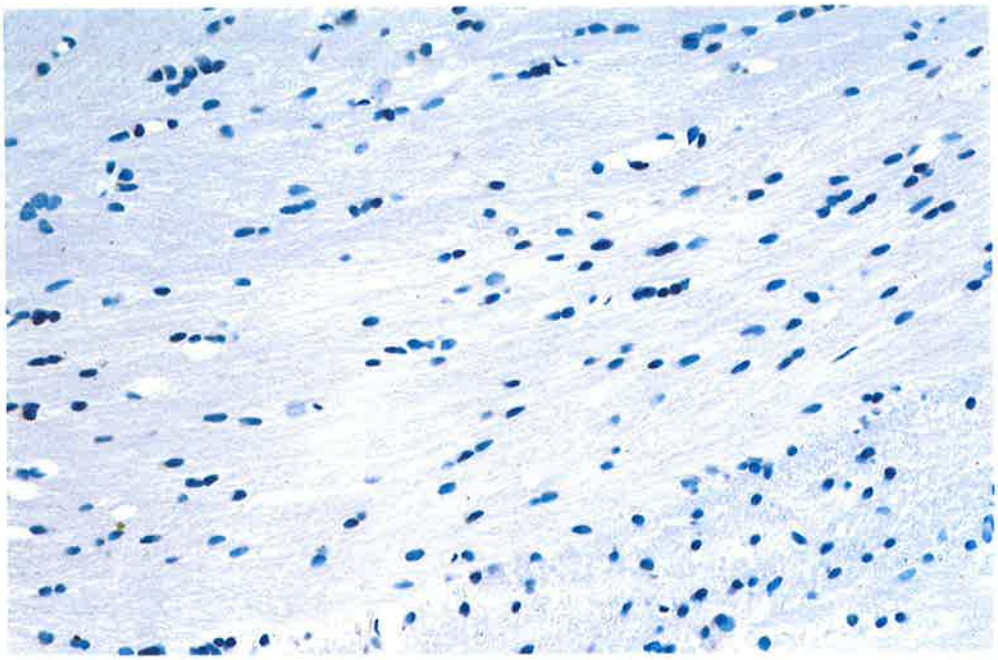
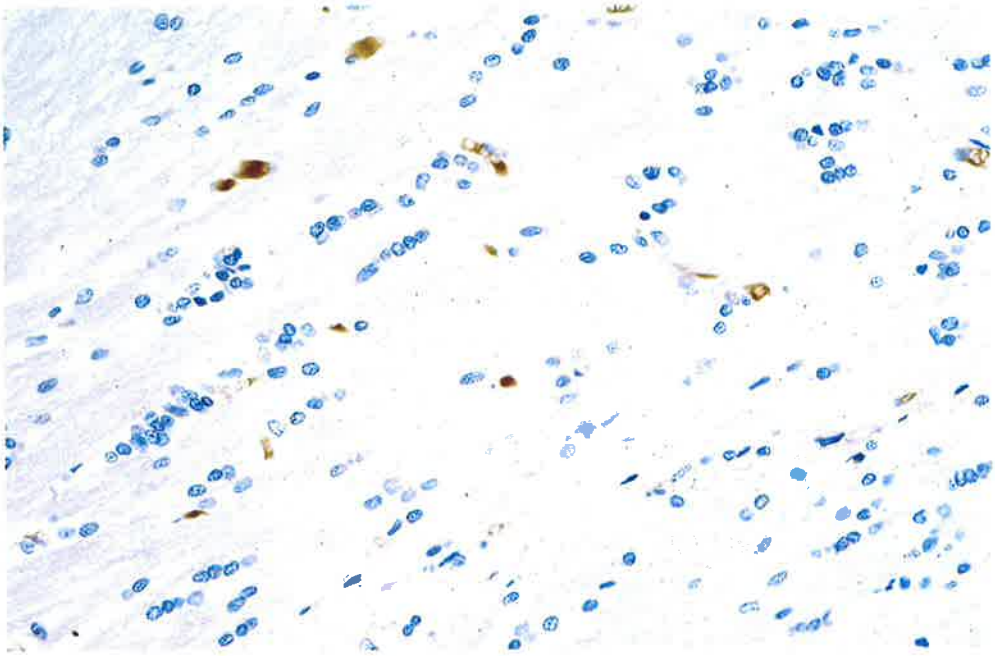


Figure 8.4 Caspase-3 stained sections from male Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Darkly stained caspase-3 positive cells are clearly visible after injury in the CA1, dentate gyrus and granular layers.



SHAM CORPUS CALLOSUM (x90)



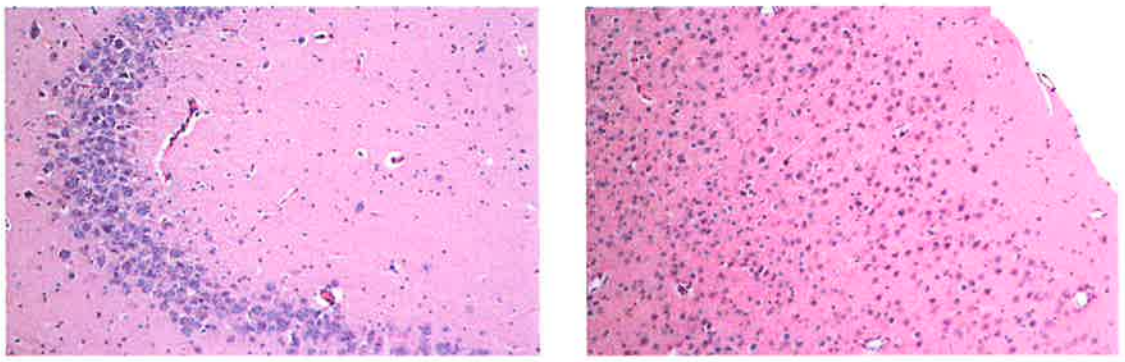
INJURED CORPUS CALLOSUM (x90)

Figure 8.5 APP stained sections from male Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Darkly stained APP positive axons and retraction balls are clearly visible after injury.

INJURED CA1 (CASPASE-3; x90)

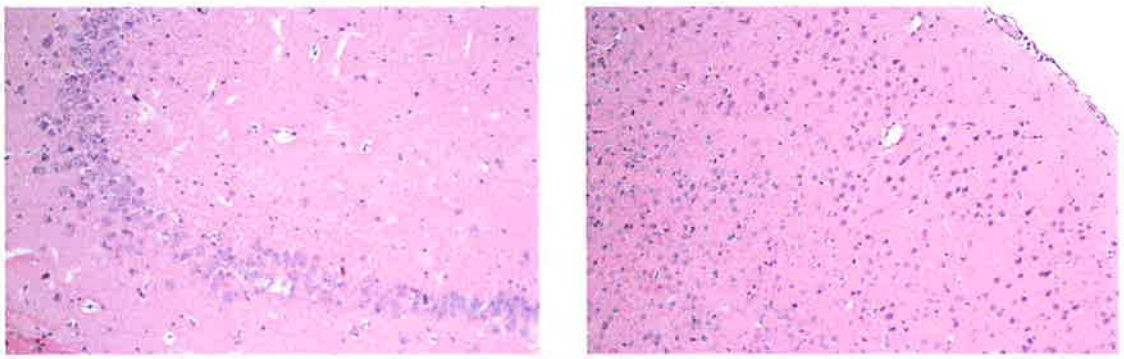
INJURED CA1 (APP; x 90)

Figure 8.6 APP and caspase-3 stained hippocampal CA1 sections from male Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury.



SHAM CA3 HIPPOCAMPUS (x60)

SHAM CORTEX (x60)



INJURED CA3 HIPPOCAMPUS (x60)

INJURED CORTEX (x60)

Figure 8.7 H&E stained sections from intact, female Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. There is a conspicuous absence of significant dark cell change in both the cortex and hippocampus when compared to males.

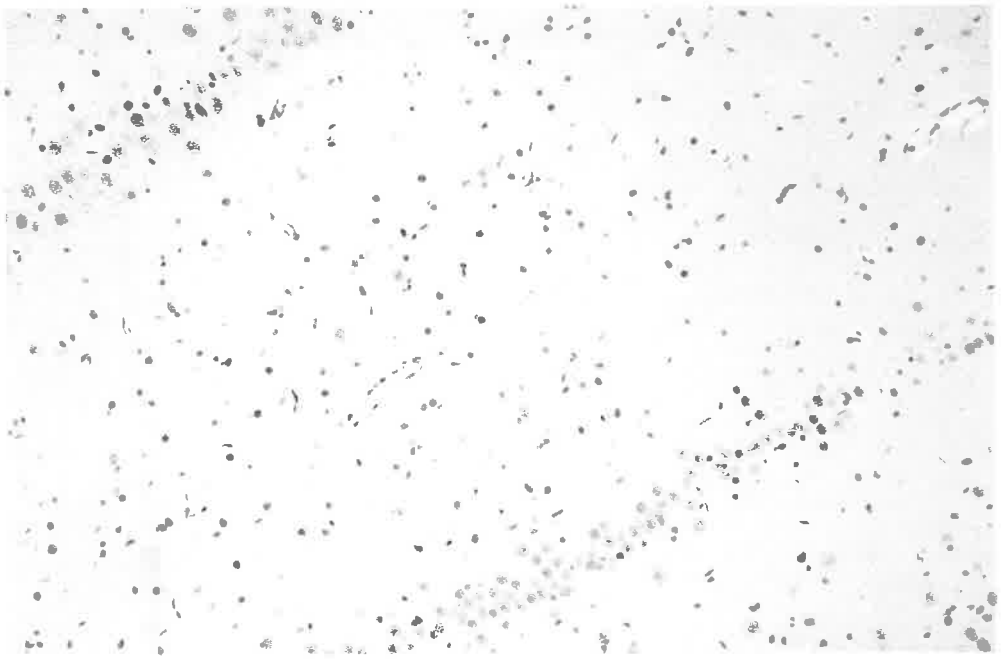
SHAM CA3 HIPPOCAMPUS (x60)

SHAM CORTEX (x60)

INJURED CA3 HIPPOCAMPUS
(x60)

INJURED CORTEX
(x60)

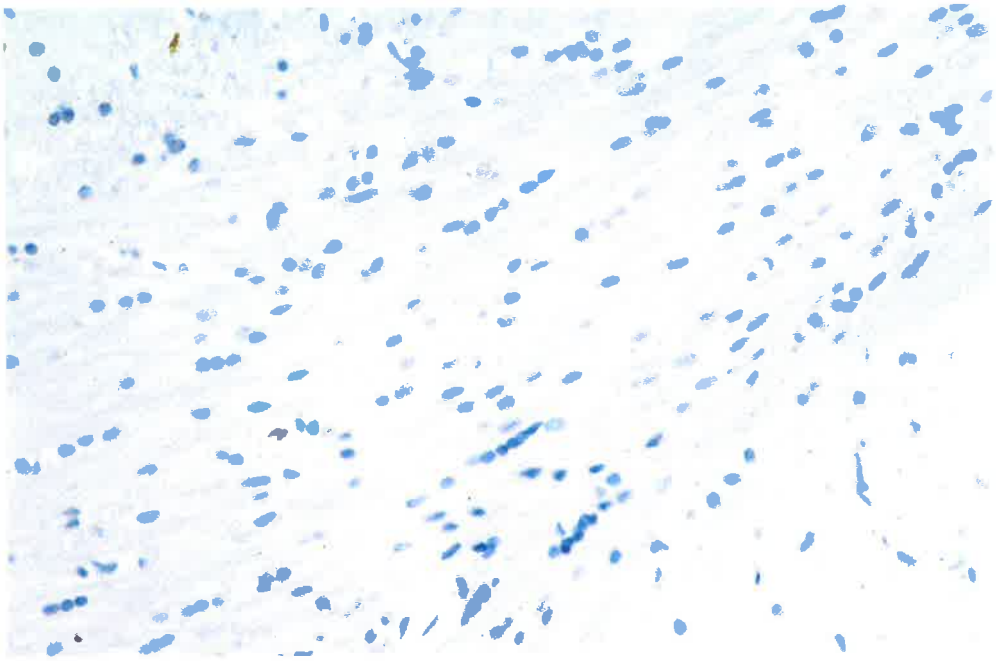
Figure 8.8 Caspase-3 stained sections from intact, female Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Darkly stained caspase-3 positive cells are visible after injury in the cortex, although less so in the hippocampus.



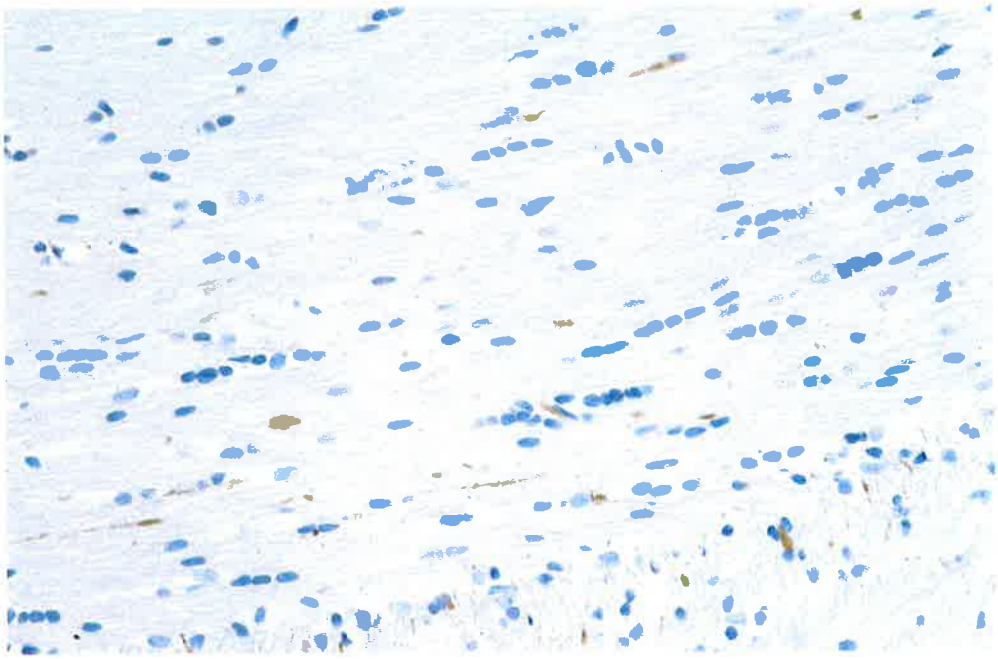
INJURED DENTATE GYRUS (x60)

INJURED CA1 (x60)

Figure 8.9 Caspase-3 stained sections from intact, female Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Very few darkly stained caspase-3 positive cells are visible after injury in either the CA1, dentate gyrus or the granular layers.

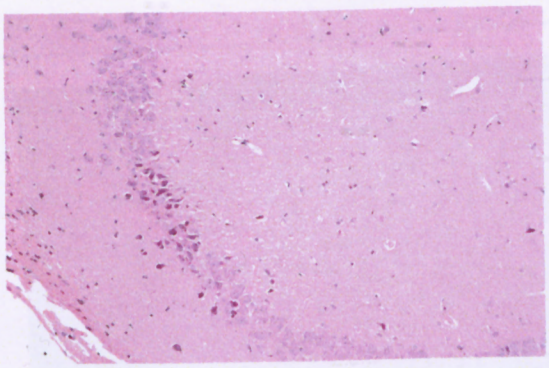


SHAM CORPUS CALLOSUM (x90)

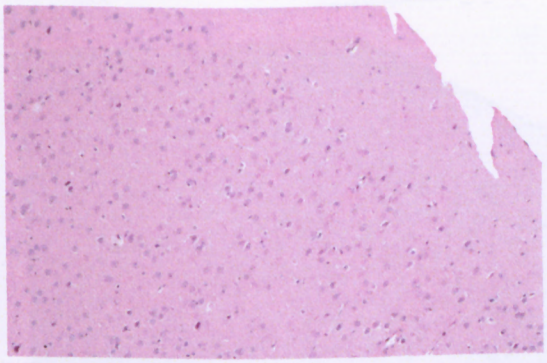


INJURED CORPUS CALLOSUM (x90)

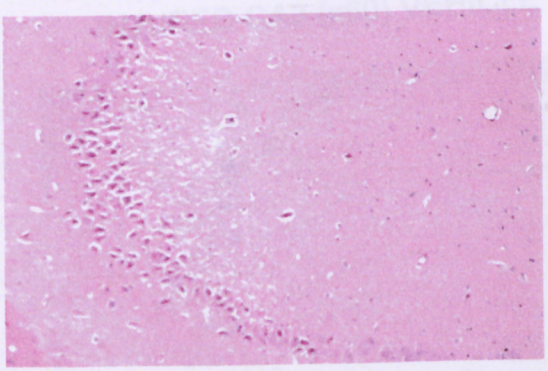
Figure 8.10 APP stained sections from intact, female Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Darkly stained APP positive axons and retraction balls are clearly visible after injury.



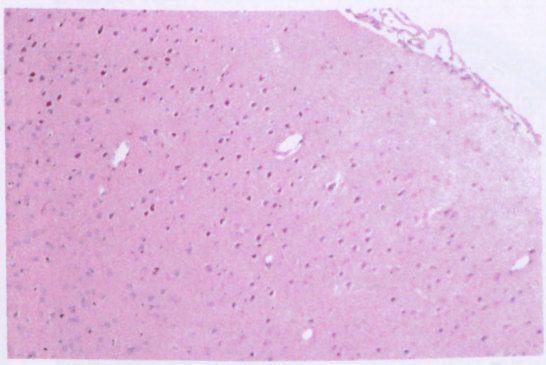
INJURED CA3 (x60) VEHICLE



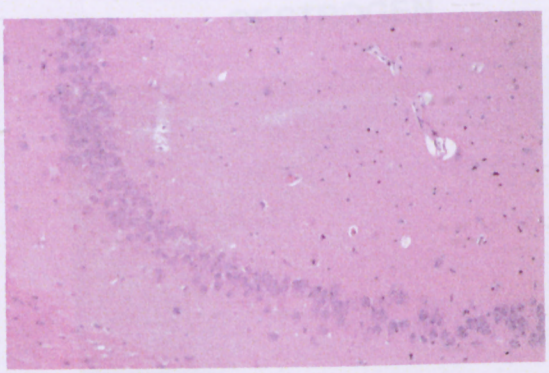
INJURED CORTEX (x60) VEHICLE



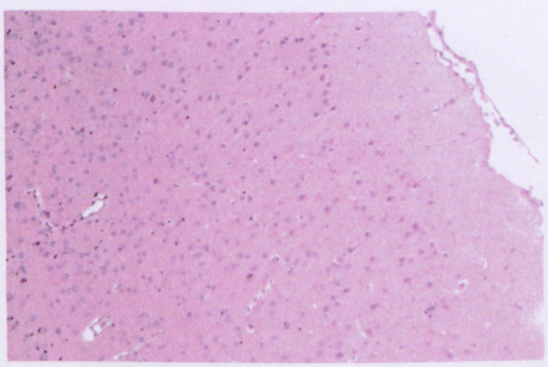
INJURED CA3 (x60)
OESTROGEN



INJURED CORTEX (x60)
OESTROGEN



INJURED CA3 (x60)
PROGESTERONE



INJURED CORTEX (x60)
PROGESTERONE

Figure 8.11 H&E stained sections from male Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Rats were administered subcutaneous oestrogen, progesterone or vehicle (sesame oil) at 30 mins after injury. Dark cell change, vacuolization and neuronal cell loss was reduced with progesterone treatment.

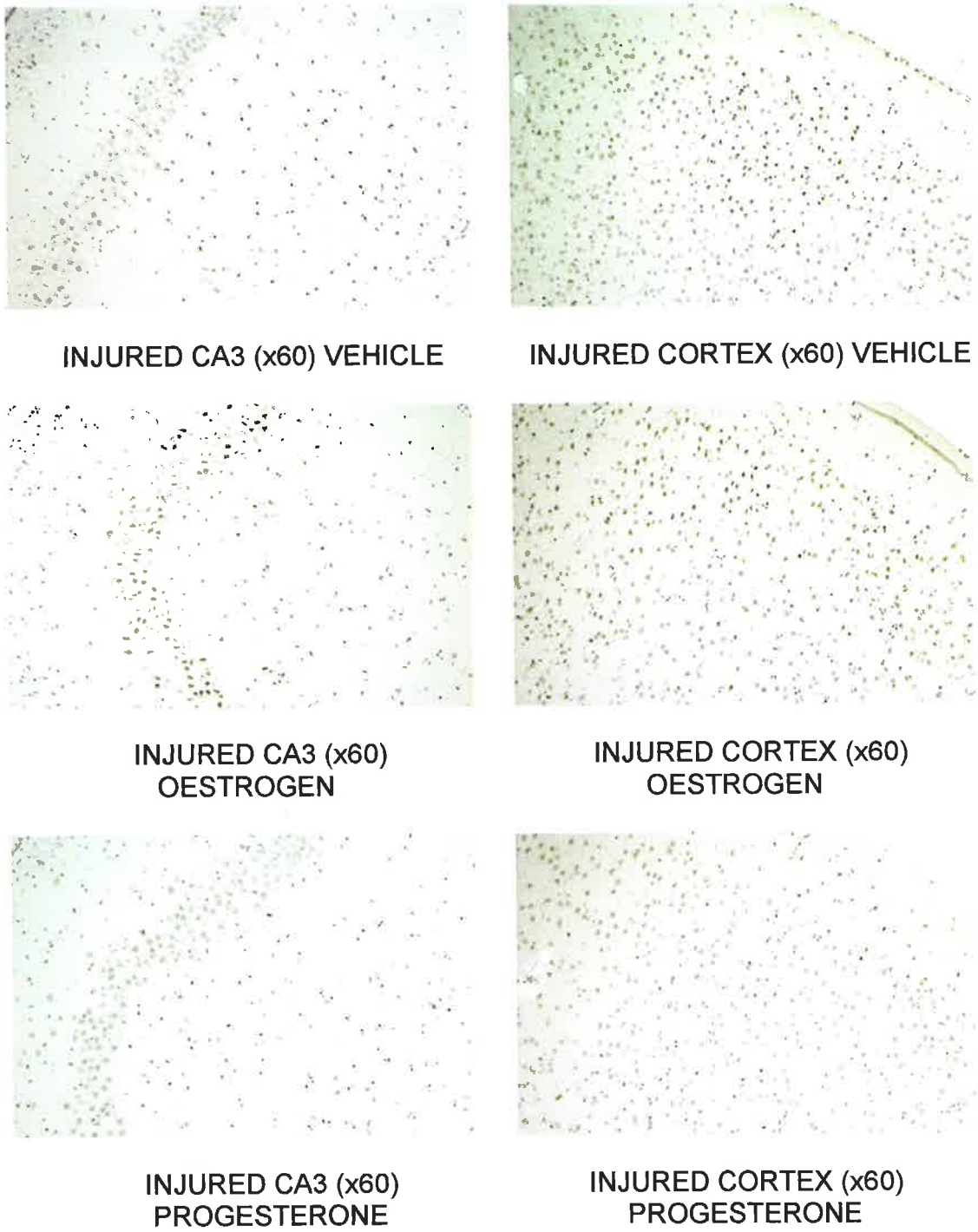
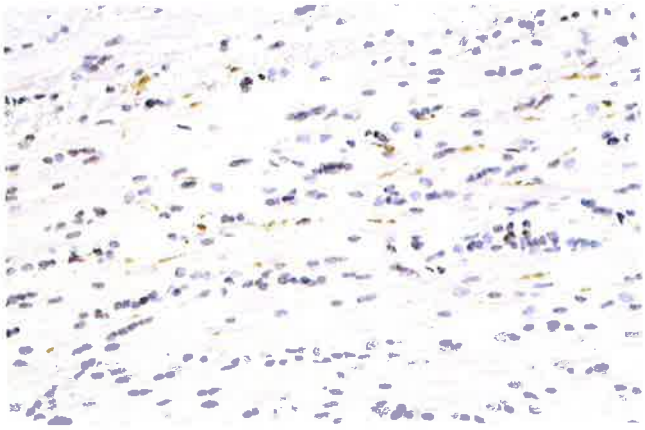
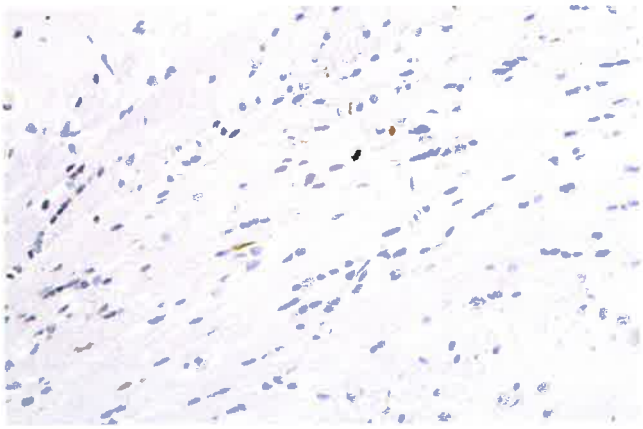


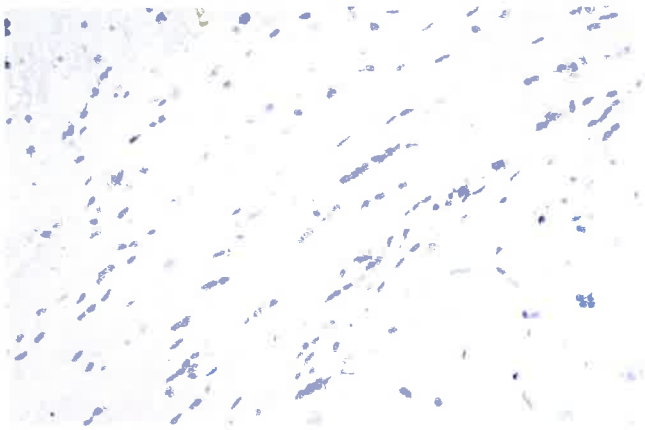
Figure 8.12 Caspase-3 stained sections from male Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Rats were administered subcutaneous oestrogen, progesterone or vehicle (sesame oil) at 30 mins after injury. The number of caspase-3 positive cells was reduced with progesterone treatment.



INJURED CORPUS CALLOSUM (x90): VEHICLE



INJURED CORPUS CALLOSUM (x90): OESTROGEN



INJURED CORPUS CALLOSUM (x90): PROGESTERONE

Figure 8.13 APP stained sections from male Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Rats were administered subcutaneous oestrogen, progesterone or vehicle (sesame oil) at 30 mins after injury. The degree of APP positive axonal staining was profoundly reduced with progesterone treatment.

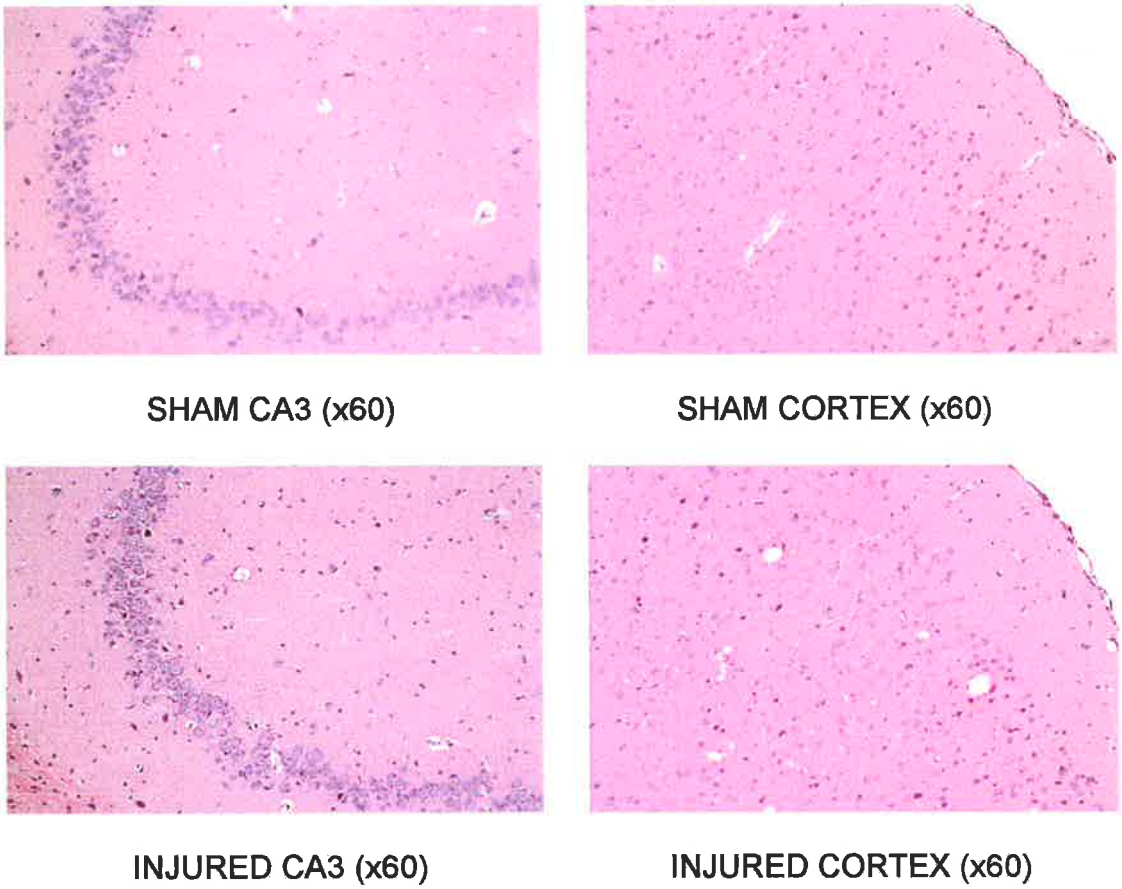


Figure 8.14 H&E stained sections from ovariectomised, female Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Injury resulted in dark cell change and vacuolization in both the hippocampus and cortex.

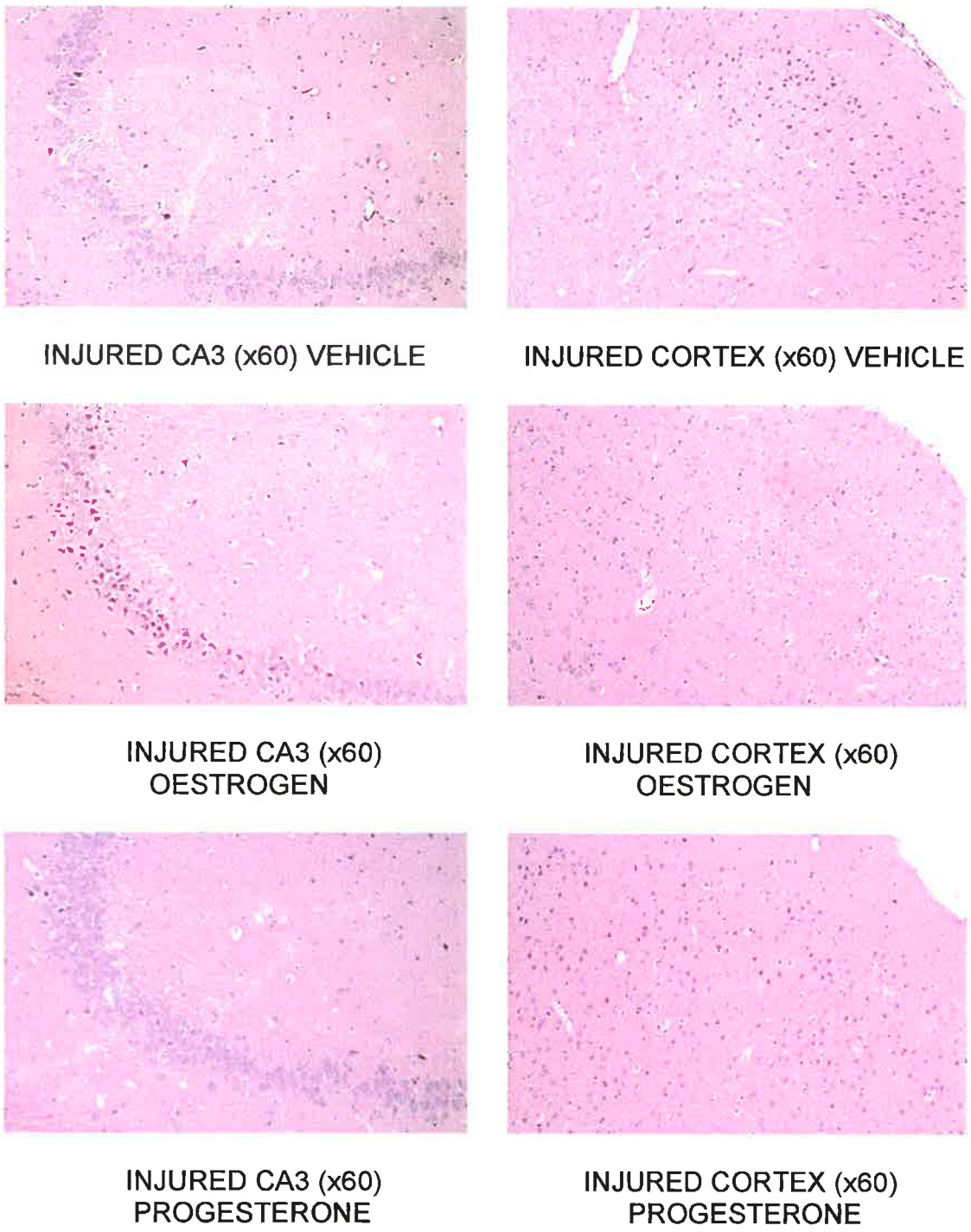


Figure 8.15 H&E stained sections from ovariectomised, female Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Rats were administered subcutaneous oestrogen, progesterone or vehicle (sesame oil) at 30 mins after injury. Dark cell change, vacuolization and neuronal cell loss was markedly reduced with progesterone treatment.

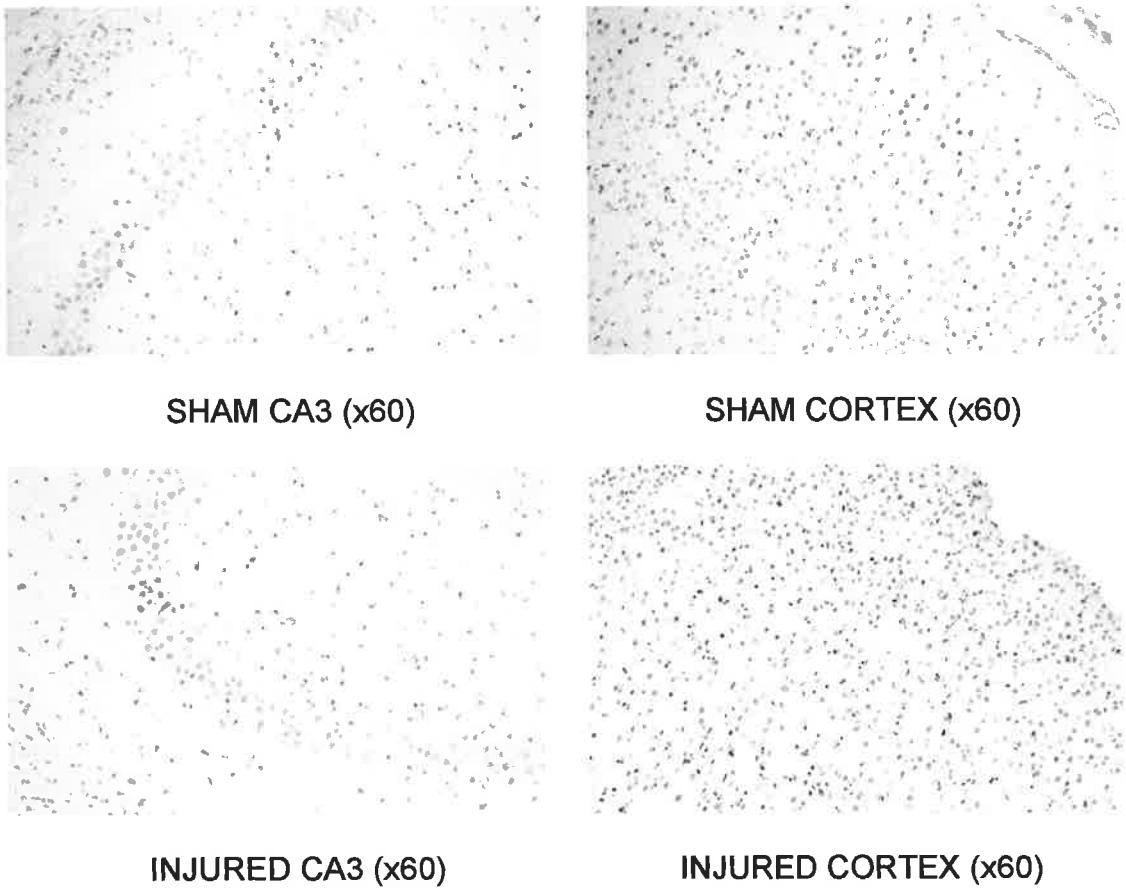


Figure 8.16 Caspase-3 stained sections from ovariectomised, female Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Injury resulted in increased caspase-3 immunoreactivity in both the hippocampus and cortex.

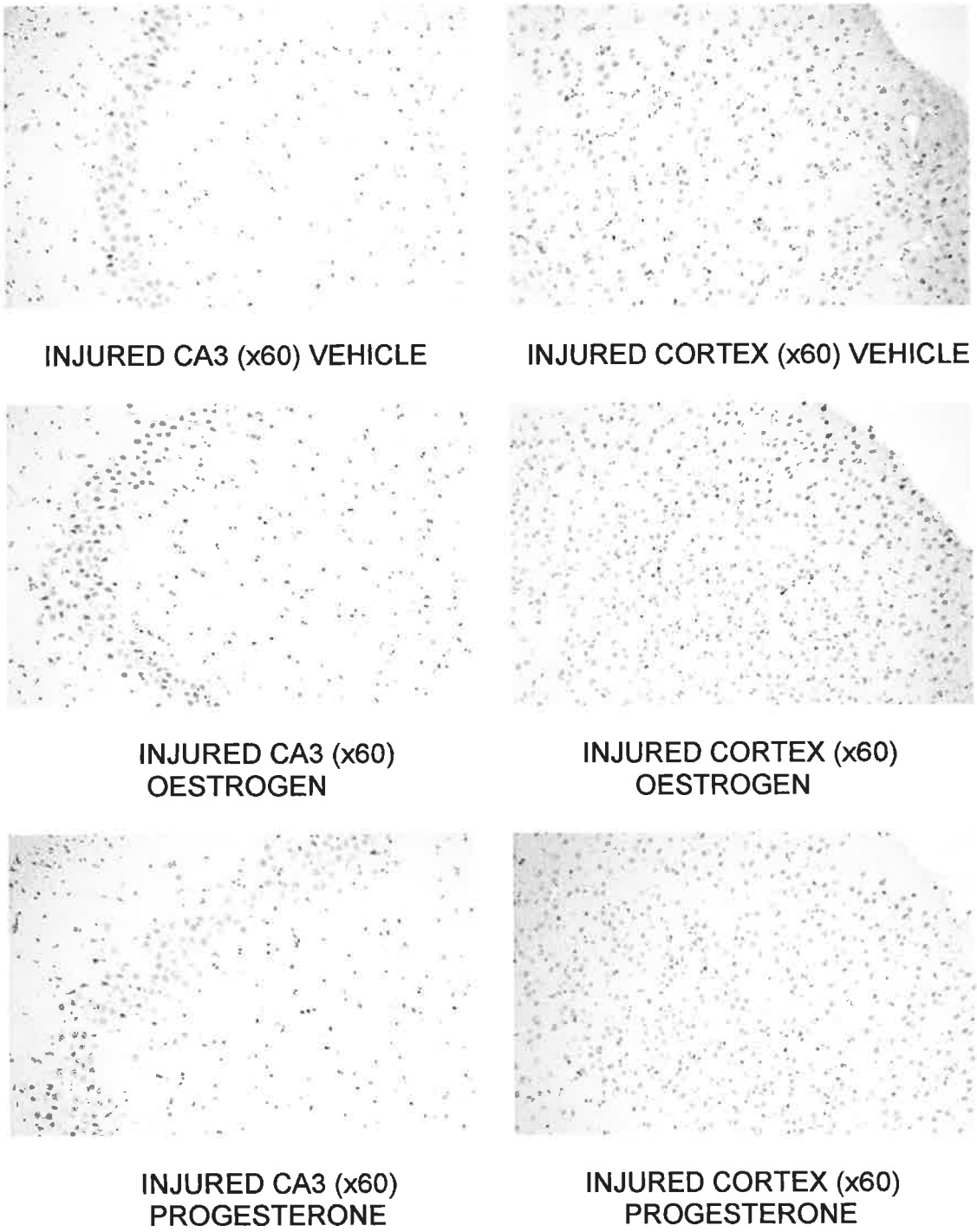
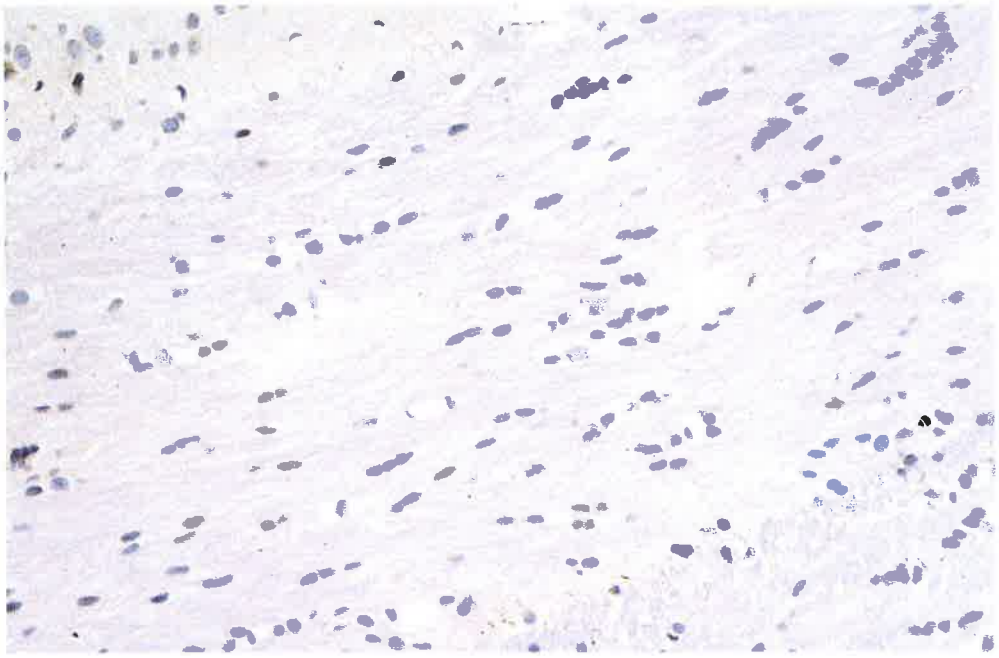
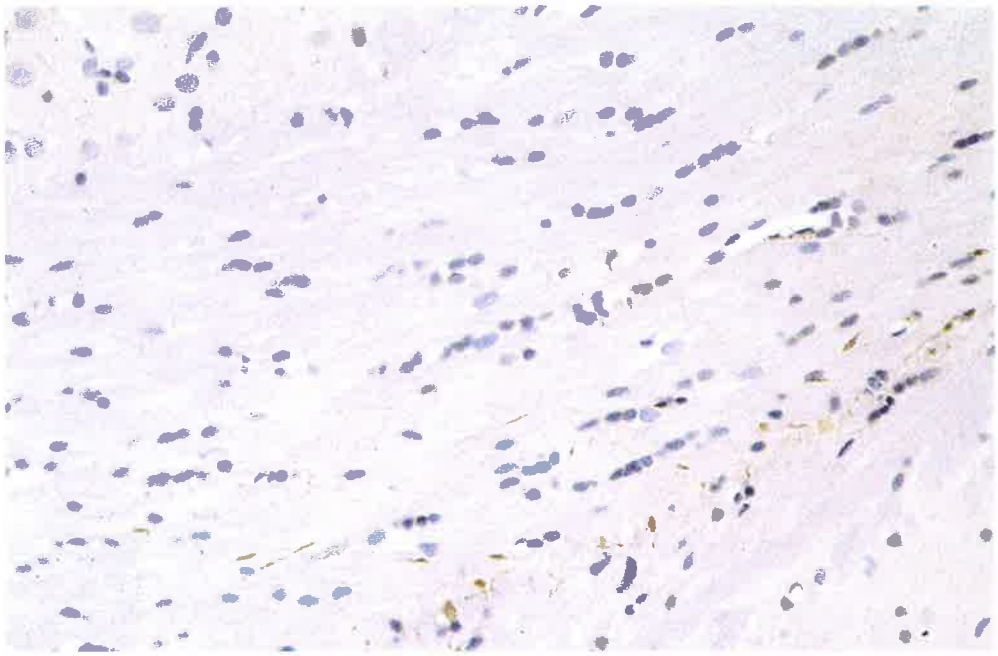


Figure 8.17 Caspase-3 stained sections from ovarectomised, female Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Rats were administered subcutaneous oestrogen, progesterone or vehicle (sesame oil) at 30 mins after injury. The number of caspase-3 positive cells was reduced with progesterone treatment.

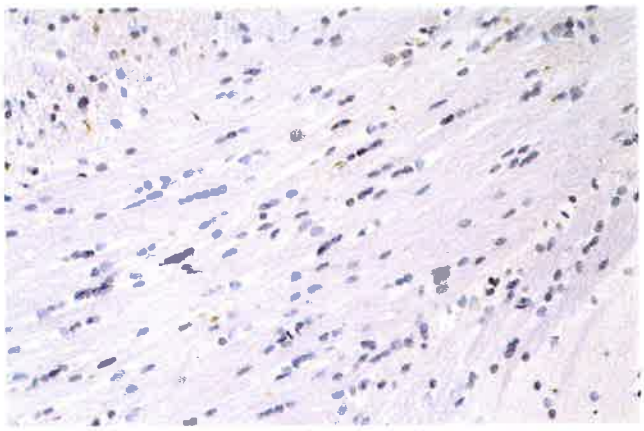


SHAM CORPUS CALLOSUM (x90)

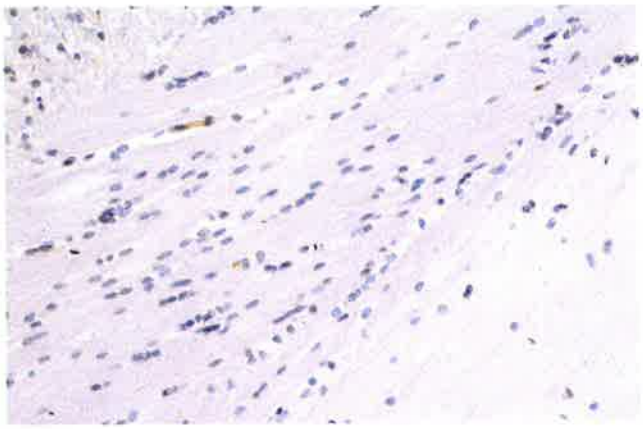


INJURED CORPUS CALLOSUM (x90)

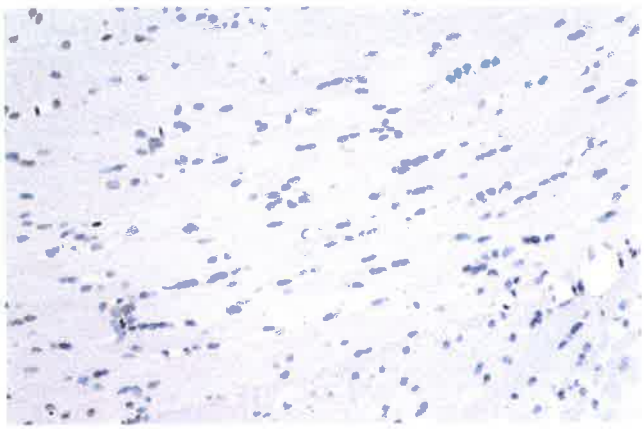
Figure 8.18 APP stained sections from ovariectomised, female Sprague-Dawley rats 3 days after severe diffuse traumatic brain injury. Injury resulted in axonal injury as observed by the increased APP positive staining.



INJURED CORPUS CALLOSUM (x90): VEHICLE



INJURED CORPUS CALLOSUM (x90): OESTROGEN



INJURED CORPUS CALLOSUM (x90): PROGESTERONE

Figure 8.19 APP stained sections from ovariectomised, female Sprague-Dawley rats 3 days after severe diffuse TBI. Rats were administered oestrogen, progesterone or vehicle (sesame oil) at 30 mins after injury. The degree of APP positive axonal staining was profoundly reduced with progesterone treatment.

CHAPTER 9

GENERAL DISCUSSION

Chapter 9

This thesis examines the effects of exogenously administered oestrogen and progesterone on motor, cognitive and behavioural outcomes in rats following experimental diffuse traumatic brain injury, as well as their effects on oedema formation, blood brain barrier permeability and morphological outcome. These studies were performed in male and female animals, as well as ovariectomised female animals to remove the confounding effects of the cycling female hormones. Initial studies required the characterization of the most appropriate paradigms for assessment of motor, cognitive and behavioural outcomes, as well as determination of the most appropriate anaesthesia to be used throughout the study.

Prior to characterizing the effects of the female gonadal hormones on outcome, we had to establish (a) the most appropriate training and assessment parameters for motor, cognitive and behavioural outcomes following diffuse TBI; (b) any baseline differences between female and male rodents' motor, cognitive and behavioural abilities; and (c) the optimal anaesthesia to be used in the study to ensure that these factors individually, or in combination, did not confound the results of the research. Accordingly, Chapter Three demonstrated that the TBI injury model used in the study produces persistent functional deficits that are similar to results previously demonstrated in this laboratory (Heath et al. 1995; Heath et al. 1999). Additionally we show that the functional outcome measures chosen to assess motor, cognitive and stress/anxiety after diffuse TBI are effective in detecting differences in the animals' pre- and post-injury performances. The rotarod test has been previously described as the most sensitive test for the detection of motor deficits

following fluid percussion induced rodent brain injury (Hamm 1994; Hamm 2001) and has been successfully used by our laboratory in a number of diffuse TBI studies to assess the effects of pharmacological agents on motor deficits (Heath et al. 1998; Heath et al. 1999). In addition to requiring the animals to increase the speed of their co-ordinated walk with time, the diameter of the rods introduces a grip component to the test which has previously been used as a motor test in its own right (Hall et al. 1988). Thus, the rotarod test in the present study is a combination of two independent motor tests, and unlike many tests that have been designed to detect left side versus right side functional differences, can be applied to diffuse TBI models that do not produce lateral injury. The decision to use the Barnes circular maze to assess cognitive function was based on work done previously by Fox and colleagues (1998) in TBI in the mouse. The Barnes maze was originally designed for use in rats (Barnes 1979) and the protocol used for the present studies was adapted from those original investigations with modifications for brain trauma (Fox et al. 1998). The technique is a simple and inexpensive method that is less affected than other maze tests by the presence of gross motor deficits. Moreover, when pre-training is required, the training period is relatively short (7–10 days). Unlike Fox and colleagues (1998), however, we found that a more consistent response was generated with the addition of an aversive sound (a loud, high-pitch auditory tone) as opposed to sole use of a bright light stimulus. Finally, the open field test is a simple test of stress/anxiety previously shown to be effective in the detection of post-traumatic deficits in both fluid percussion induced (McIntosh et al. 1989) and diffuse TBI (Vink et al. 2003).

We also show that while daily testing of motor and cognitive function following TBI promotes rapid functional recovery with repeated exposure to the task, the rate of functional improvement was mostly independent of pre-injury training, with a significant effect only shown with assessment of motor function. Pre-injury training did not affect the degree of motor deficit immediately after trauma, but it did promote a faster rate of recovery after trauma, suggesting that early and frequent exposure to familiar motor tasks may facilitate motor recovery. This was not the case in the cognitive assessment task where pre-injury training had no effect on rate of cognitive recovery in injured animals. In contrast to motor performance, it would appear that familiarity with the cognitive task does not improve rate of recovery after TBI. It did, however, reduce measurement variance between groups immediately after injury, suggesting that repeated trials on consecutive days may be necessary to achieve statistical significance in untrained animals on cognitive tasks. We chose to use trained animals in our studies to achieve more rapid results in the motor tests and less variance in the cognitive tests.

Finally in this Chapter, we demonstrate that the open field test is a useful tool for differentiating the stress response of injured animals following TBI. In comparison with baseline spontaneous exploratory activity, injured animals showed significantly reduced activity over the entire four-week assessment period. This result was not affected by exposure to the task on either a daily or weekly basis. Consistent with the decrease in activity in the open field, freezing time significantly increased. Such an increase in freezing time has been previously attributed to stress (Vallee et al. 1997; Larsson et al. 2002), and

was not observed in sham animals who continued to explore their environment over the entire four week assessment period.

In Chapter Four, we show that functional recovery following experimental TBI is influenced by the sex of the animals, choice of anaesthesia and measurement task used in the assessment. Specifically, we demonstrate that: (a) irrespective of the anaesthetic used, females rats perform better than male rats before and after injury on motor and behavioural assessment using the rotarod test and open field tests; (b) when post injury motor scores are normalised as a percentage of pre injury function, there is little difference in performance between the sexes after TBI; (c) pentobarbital exacerbates female mortality after TBI and is an inappropriate anaesthesia choice for research involving gender differences in rodents; (d) isoflurane appears to be neuroprotective in female rats following experimental TBI and may confound comparative gender studies; and (e) there are no significant effects or interactions between halothane, task and gender following TBI.

The observation that female animals do better at the rotarod and open field tests both before and after injury compared to their male counterparts is an important one for comparative gender studies. Such gender differences in sensorimotor and cognitive tasks have been previously reported, and attempts have been made to identify the hormone responsible. Specifically, female mice perform better than males in sensorimotor tasks (McDermott et al. 1994), with oestradiol implicated as the responsible hormone. Similarly, oestrogen has also been shown to improve maze performance in mice (Heikkinen et al. 2002). Others have shown that following traumatic brain injury, progesterone improves

functional outcome after injury (Shear et al. 2002; Djebaili et al. 2004). In contrast, oestrogen has been shown to improve blood flow and improve survival after TBI in female rats under isoflurane anaesthesia (Roof et al. 2000b). Thus, both hormones have been implicated in neuroprotection, although no comprehensive study comparing the two hormones has been conducted. Interestingly, in the study under isoflurane anaesthesia (Roof et al. 2000b), the mortality rate in male and female animals under isoflurane anesthesia was identical to what we observed in our experiments, confirming that the methods employed in those studies were comparable to ours. In our studies, however, we demonstrate that isoflurane is protective in female animals and that this may confound any interpretation. Further confounding any comparative studies is our finding that the pre-injury performance in females is significantly better than in males, making it necessary to normalise the data to permit gender comparisons. Having established the pre-requisites for the study, we undertook a comparative study of oestrogen versus progesterone on functional outcome in female animals using halothane as the anaesthetic.

The effects of exogenously administered oestrogen and progesterone on motor, cognitive and behavioural outcome following induced diffuse TBI in female rats is reported in Chapter Five. The study was conducted in two parts. First, we clarified the pre- and post-injury status of ovariectomised females compared with intact females and male animals. With respect to the rotarod motor test, ovariectomised animals demonstrated scores that were identical to males when using the raw data. However, raw data may not be indicative of the relative deficit in each group, and the data was therefore normalised against each

group's pre-injury performance. When transformed in this manner, ovariectomised females recorded significantly greater deficits after injury than males. Similarly, in cognitive tests, ovariectomised females recorded significantly greater deficits in the Barnes maze after injury than did their male counterparts, while in the open field test, ovariectomised females were again identical to males. Clearly, the removal of the gonadal hormones from intact females significantly affects their functional outcome after trauma, reducing it to equal or worse than an injured male. This finding supports the concept that the female gonadal hormones may profoundly influence functional performance after TBI.

In the second part of the study, we examined the effects of physiological doses of either oestrogen or progesterone on posttraumatic functional outcome in ovariectomised female animals. Previous studies in TBI have successfully used doses of progesterone that range between 4mg/kg and 16mg/kg (Stein 2001). This dose will result in peak serum concentrations ranging from 24ng/ml to 96ng/ml (Gibbs 1998; Gibbs 1999), compared to peak levels of 7–18ng/ml in a normal cycling rat. Our aim was to reproduce a condition as close as possible to a normal cycling female rat, since intact cycling females performed significantly better than male rats in functional tests after TBI. Accordingly, we administered progesterone at a subcutaneous dose of 1.667mg/kg which gives a peak serum concentration of ~10ng/ml at 5h after administration (Gibbs 1998; Gibbs 1999). Similarly, serum levels of oestradiol in the rat range between 5–50pg/ml, and a subcutaneous injection of oestradiol at 33.3µg/kg used in the current experiments results in a serum concentration that peaks at ~50pg/ml at 5h after injection (Gibbs 1998; Gibbs 1999). Thus

the concentration of the hormones used in the present study ensure that non-receptor mediated effects associated with supraphysiological levels of the hormones were avoided.

Even with these low doses of the gonadal hormones, we found a significant improvement in motor and cognitive outcome in ovariectomised animals after TBI, with neither hormone being more effective than the other in improving outcome. We also observed the improvement in outcome even though the hormones were administered after the traumatic event, suggesting that hormone exposure prior to injury is not required to facilitate functional improvement. We conclude that it is the presence of the hormone after injury as the tissue attempts to recover that plays a role in motor and cognitive recovery after TBI. No effects of the individual gonadal hormones were observed on spontaneous exploratory activity, even though intact females performed better in this test than did ovariectomised females or males. This suggests that both hormones may need to be present to confer a beneficial effect on this parameter.

Having established that both oestrogen and progesterone may account for the superior functional performance of female animals following TBI, Chapter Six addressed whether exogenous administration of these hormones might produce motor, cognitive and behavioural improvement in male rats following TBI. While there are limited studies in TBI that have examined hormonal effects in male outcome following TBI, most that have been published support a protective role for both oestrogen and progesterone. Emerson and colleagues (1993) demonstrated in male animals that oestrogen improved both biochemical and neurologic outcome after fluid percussion induced brain trauma. Subsequently, Roof et

al (1994) reported that male rats treated with progesterone following cortical contusion injury were less impaired on the Morris water maze task than vehicle treated animals. This protective effect of progesterone on posttraumatic performance in the Morris water maze was subsequently confirmed in studies using both the fluid percussion (Shear et al. 2002) and cortical contusion models (Djebaili et al. 2004) of TBI in rats. Other studies have failed to demonstrate an effect of progesterone administration on functional outcome in males (Grossman et al. 2000), although the authors postulate that this lack of effect may have been influenced by the lesion location. To the best of our knowledge, no single study has compared the effects of both hormones on functional outcome in a diffuse model of TBI. Again we have used identical experimental conditions as described for the female study, including the model of injury, anaesthesia, drug dosages and functional assessments.

Our results demonstrate that both the female sex hormones improve motor and cognitive outcome following TBI in male animals, but as for the female ovariectomised animals, neither was superior to the other in terms of functional recovery. There was a reduction in freezing time in male animals treated with oestrogen after TBI, but this did not translate to a significant effect on overall performance in the open field test. It is important to note that while male animals' functional recovery significantly improved when compared with male vehicle controls, they did not improve to the level of the female animals as seen in Chapter Five. One can speculate that there are intrinsic differences in male and female responses after trauma that are independent of exogenous hormone concentration. The mechanisms associated with this difference still remain to be identified.

One of the mechanisms by which progesterone and oestrogen are thought to improve outcome in females after TBI is by attenuating blood–brain barrier (BBB) permeability and reducing oedema formation (Galani et al. 2001; Stein 2001). Chapter Seven characterises oedema formation in female and male rats after TBI and then examines the effects of oestrogen and progesterone on these parameters. We demonstrate that females have a different oedema profile after diffuse TBI from males. Females have an oedema maximum at 24hr after TBI whereas males have an earlier maximum at 5h post-trauma. After achieving maximal water accumulation at 24h after trauma, the oedema in female animals then steadily declines over time. In contrast, male animals developed a second phase of oedema after the initial 5h peak, commencing at 48hrs after trauma and persisting over the next three days. Such a biphasic vasogenic followed by a cytotoxic profile has been previously reported in male animals after diffuse TBI (Barzo et al. 1997b; Barzo et al. 1997a; Marmarou 2003). This biphasic profile was not apparent in female animals. Obviously, this would impact on which time point was most suitable for comparing male and female oedema after diffuse TBI.

What is perhaps most intriguing is that ovariectomy conferred a male oedema profile on the ovariectomised animals, with a maximum at 5h and significant oedema at later time points. Clearly, the presence of endogenous levels of hormones not only attenuated the oedema development after trauma, but also altered the temporal profile of oedema formation.

Moreover, administration of a single physiological dose of either oestrogen or progesterone profoundly inhibited oedema formation in both female and male animals at all time points

up to three days post trauma. Few studies have examined the effects of exogenous oestrogen administration on oedema development following TBI. However, a number of studies have investigated progesterone and have reported a need for supraphysiological concentrations of progesterone (8–16mg/kg) for up to three days post trauma to see an effect on oedema (Stein 2001). Our present studies have demonstrated that a single physiological dose of 1.7mg/kg is sufficient to produce a significant effect.

We also observed a significant effect of the hormones on BBB permeability after TBI. In ovariectomised females and males, there was a significant increase in Evans Blue extravasation at 5h after trauma. The degree of extravasation in ovariectomised female animals was less than that observed in the males, despite the degree of early oedema being identical in both groups. Administration of either oestrogen or progesterone significantly attenuated the Evans Blue extravasation, in most cases down to sham levels. Our results with progesterone contradict those of Duvdevani and colleagues (1995) who reported that the hormone does not attenuate BBB opening after frontal cortical contusion. One possible explanation for this disparity is that the contusion models cause extensive brain tissue damage and loss at the site of injury, with meningeal tearing and tissue laceration. The BBB is extensively and persistently disrupted under these conditions. In the diffuse TBI model used in the present study, there is no meningeal damage, no tissue laceration, no extensive focal tissue loss and only a transient opening of the BBB.

Characterisation of the morphological effects of oestrogen and progesterone was undertaken in Chapter Eight. Previous studies have shown that normal cycling females

have less neuronal morphological damage than either ovariectomised females or male animals (Bramlett et al. 2001). Our results in Chapter Eight are consistent with these studies. However, we extend these earlier studies by describing the effects of both oestrogen and progesterone treatment on female ovariectomised and male animals after TBI, using H&E, caspase-3 and amyloid precursor protein as our morphological markers. While our studies demonstrated that oestrogen has a neuroprotective effect on axonal injury after TBI, we did not observe a significant effect of oestrogen on dark cell change or caspase-3 immunoreactivity in male or ovariectomised females. While the reasons for this are unclear, it could be that the oestrogen concentration used in the current study is too low. This, however, contradicts the beneficial effects reported in the previous chapters investigating functional outcome and oedema. Alternatively, it may be that long-term oestrogen exposure may be necessary to induce a neuroprotective effect on hippocampal and cortical neurones. Indeed, a number of previous studies have used long-term oestrogen treatment either before or after injury to achieve a beneficial effect (Jover et al. 2002; Monroe et al. 2002; Rau et al. 2003) as opposed to the single bolus administered after injury used in our morphological studies.

In contrast to the effects of oestrogen, progesterone markedly reduced dark cell change and caspase-3 immunoreactivity in both the hippocampus and cortex after TBI, as well as virtually eliminating axonal injury in the corpus callosum. While no previous studies have reported effects of progesterone on axonal injury, several reports have previously documented the ability of progesterone to attenuate neuronal cell death in various models

of brain injury including cortical brain ablation (Asbury et al. 1998) and contusion TBI (Shear et al. 2002; Djebaili et al. 2004). As in our studies with diffuse TBI, the reduction in cell death coincided with a reduction in caspase-3 activity in injured brains (Djebaili et al. 2004).

Mechanisms of neuroprotection

While the mechanisms associated with the neuroprotective actions of the hormones are unknown, a number of possibilities have been proposed (Roof et al. 2000b). In considering oestrogen, the hormone has powerful antioxidant properties (Hall et al. 1991) as well as the ability to attenuate excitotoxicity and amyloid β peptide toxicity (Goodman et al. 1996). It also reduces apoptosis by upregulating bcl-2 expression (Garcia-Segura et al. 2001) and has been reported to act as a neurotrophic factor (Gibbs 1999; Green et al. 2000). Oestrogen has effects on cerebral blood flow via modulation of nitric oxide formation (Pelligrino et al. 1998), and the ensuing vasodilation is thought to protect against BBB damage. Finally, recent reports suggest that oestrogen is a powerful ant-inflammatory agent in the CNS (Baker et al. 2004; Wen et al. 2004), including inhibition of neurogenic inflammation (Bjorling et al. 2001), which has been recently implicated as a major factor in BBB disruption and brain oedema formation following TBI (Nimmo et al. 2004).

Like oestrogen, progesterone has also been described as an antioxidant (Betz et al. 1990; Hall et al. 1991) and has been shown to inhibit membrane lipid peroxidation (Roof et al. 1997; Ayres et al. 1998). It has also been shown to protect against glutamate toxicity (Ogata et al. 1993) as well as apoptosis by inhibiting caspase-3 activation (Djebaili et al. 2004). With respect to axonal damage, progesterone has been reported to enhance myelin synthesis in brain (Schumacher et al. 2004), thereby providing neuroprotection and accelerating regenerative processes. In addition, progesterone has been shown to modulate the astroglial inducer of the BBB (Wolff et al. 1992) and inhibit vessel growth associated with leaky BBB function (Plum et al. 1963). Finally, progesterone has also recently been shown to reduce the inflammatory response after TBI (Grossman et al. 2004), including the neurogenic inflammatory response (Duval et al. 1998).

Effects of oestrogen and progesterone on neurogenic inflammation have not been previously considered in the neurotrauma literature, in part because little is known about the role that neurogenic inflammation may play in oedema formation following TBI. However, recent evidence suggests that neurogenic inflammation plays a critical role in BBB breakdown and subsequent oedema formation (Nimmo et al. 2004). Neurogenic inflammation is a neurally elicited reaction that has typical characteristics of an inflammatory reaction including vasodilation, protein extravasation and tissue swelling (Figure 9.1).

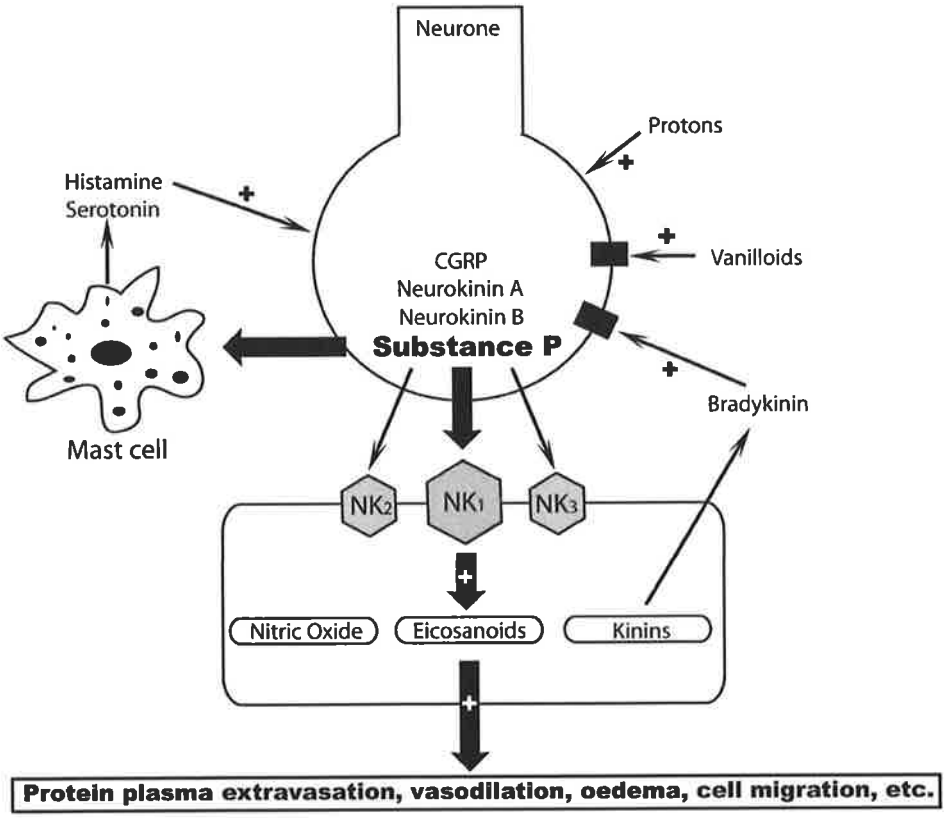


Figure 9.1 The mechanisms associated with neurogenic inflammation. Substance P and other neuropeptides are released and facilitate an inflammatory response by effects on both the target cell (receptor mediated) and mast cells. Some released factors then feedback to further stimulate release of the neuropeptides. NK = neurokinin.

Studies in peripheral tissue have demonstrated that neurogenic inflammation is the result of the stimulation of C-fibres, which causes the release of neuropeptides (Campos et al. 2000). Although a number of neuropeptides have been implicated in this process, it is generally accepted that substance P is primarily associated with an increase in microvascular

permeability leading to oedema formation (Campos et al. 2000). Virtually all blood vessels of the body are surrounded by sensory nerve fibres that contain these neuropeptides. Cerebral arteries, in particular, appear to receive a dense supply of these neurones, and it is therefore consistent that these neurones have a role as mediators of the inflammatory process.

Several studies have now shown that both oestrogen and progesterone can modulate this pathway at several levels. Neurokinin concentrations differ in brains from female and male animals and can be altered by ovariectomy (Duval et al. 1996). Later studies confirmed that the neurokinin concentrations are modulated by both oestrogen and progesterone levels (Duval et al. 1998). Progesterone has also been shown to inhibit substance P induced plasma extravasation (Limmroth et al. 1996). Both hormones are inhibitors of classic inflammation (Baker et al. 2004; Grossman et al. 2004; Wen et al. 2004), while effects of oestrogen on bradykinin (Green et al. 1999) and nitric oxide (Pelligrino et al. 1998) have also been reported. Finally, at the level of initial stimulation, oestrogen has been shown to be important for vanilloid receptor function (Schroder et al. 2003), which along with mechanical stimulation, may be one of the initial steps in the inflammatory process.

While a purported role for the gonadal hormones in neurogenic inflammation is speculation at this stage, the fact that both hormones have profound effects on oedema formation and BBB permeability following diffuse TBI suggests that these aspects are worthy of future studies.

Conclusion

Intact female rats have a better outcome than male rats after diffuse TBI using a standardised model of anaesthesia and outcome measures. This improved outcome relative to males can be observed at the functional level (motor, cognitive and behavioural), the physiological level (oedema and BBB permeability) and the morphological level (dark cell change, axonal injury and caspase-3 immunoreactivity). Ovariectomy deleteriously affects outcome on all of these levels, such that an ovariectomised animal adopts a male response after injury. This can be reversed by administration of physiological concentrations of either oestrogen or progesterone after injury to these ovariectomised animals. Administration of oestrogen or progesterone to a male animal was also beneficial to outcome. At the morphological level, progesterone was identified as being more effective than oestrogen at reducing neuronal cell death, at least when administered after TBI. We conclude that physiological levels of both oestrogen and progesterone improve functional, physiological and morphological outcome after TBI and that this improvement may be associated with the ability of the female hormones to suppress BBB opening and oedema formation after trauma.

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