AN INVESTIGATION OF THE *IN VIVO* ROLE OF VACCINIA VIRUS COMPLEMENT CONTROL PROTEIN IN HEAD INJURY AND ALZHEIMER'S DISEASE.

By

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DECLARATION

I, Nirvana Shanalee Pillay, hereby declare the work on which this thesis is based is my original (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

The complement system is the first line of defence against invading micro-organisms. In diseases such as traumatic brain injury (TBI) and Alzheimer's disease (AD), pathological inflammation is caused by the prolonged or inappropriate activation of the complement system. Prolonged inflammation is a major cause of tissue destruction. Vaccinia virus complement control protein (VCP), a virus-derived complement inhibitor inhibits both the classical and alternate complement pathways. VCP is structurally similar to the family of human complement control proteins containing four short consensus repeats and is functionally similar to the first complement receptor and is able to bind the third and fourth components of the complement system. Complement components C3 and C5 have previously been shown to play a critical role in secondary damage caused by inflammation in TBI. Previous work has also confirmed the ability of beta-amyloid fibrils to activate complement *in vitro*. This study was undertaken to evaluate the therapeutic role of VCP in a mild and severe TBI rat model following standardised fluid percussion brain injury. The efficacy of VCP in a transgenic mouse model that corresponds to an early-onset of familial AD was also investigated.

Training of rats in a Morris water maze (MWM) consisted of a total of 16 trials over a 2 day period before rats were anaesthetised and subjected to mild (1.0-1.1 atmospheres) or a severe (2.7-3.0 atmospheres) lateral fluid percussion injury (FPI) 3.0 mm lateral to the sagittal suture and 4.5mm posterior to bregma. 10µl of VCP (1.7µg/µl) was injected into the cortical injury site immediately after FPI. Two weeks post-FPI the rats were assessed in the MWM for spatial learning and memory. Neurological motor function tests were also carried out after FPI for 14 consecutive days.

In the mild head injury model, saline-treated FPI rats spent a significantly ($p<0.05$) larger amount of time in one of the incorrect quadrants than VCP-treated FPI rats. Neurological evaluations done on the same group of rats using an angle-board revealed that saline-treated FPI rats showed greater inability to stand on an inclined plane, turned more frequently and demonstrated more freezing behaviour than VCP-treated rats. The ambulation

test further revealed that saline-treated rats showed impairment in ambulation and was significantly different ($p<0.05$) than VCP-treated rats from day 9 of testing.

In the severe head injury model, no significant differences between saline-treated and VCP-treated rats were shown in cognitive testing in the MWM. However, sensorimotor tests (in the same group of rats) revealed significant differences between saline-treated and VCP-treated rats in lateral left pulsion ($p=0.001$) and tactile placing ($p=0.002$) on the first five days of testing. In addition, significant differences in right lateral pulsion in the first four days ($p=0.007$) of testing was shown.

In order to test the efficacy of VCP administered via an alternative route instead of direct injections into the lesioned area, a VCP or saline injection administered via the lateral ventricle of the brain after severe TBI in rats was performed. In these rats no significant differences were evident in spatial learning and memory when tested 48 hours later in the MWM. Western blot analysis of the injured cortices showed no evidence of VCP uptake. It is therefore possible that VCP injected in the cerebrospinal fluid via the lateral ventricle may not be entering the brain tissue in sufficient concentration, as is also evident from the lack of cognitive effects in these rats.

The effect of VCP on memory processes in a Mo/Hu APPswe/PS1De9 transgenic mouse model that corresponds to an early-onset of AD was also investigated. Other studies have shown that in these transgenic mice amyloid plaques were present by 6 months.

Training and testing time of APPswe/PS1De9 mice lasted 34 days. Training was done in four phases that included the habituation, pre-training, cued task and spatial acquisition tasks that were carried out on a cheeseboard maze (dry maze condition vs MWM) before intracranial injections of 5µl of VCP (1.7µg/µl) or 5µl saline was administered. The probe phase included the probe task and reverse task. Each of these tasks consisted of a total of 8 trials over a 2-day period. Using the cheeseboard, significant differences in cognitive measures between APPswe/PS1De9 transgenic and wildtype mice were shown in the habituation and cued task phases. The probe phase carried out two weeks later evaluated the effect of VCP on memory. A one-way ANOVA revealed a significant difference between saline-treated and VCP-treated

transgenic mice in one of the 4 trials. However, during the reverse task 2 of the 4 trials showed a significant difference in goal latency between saline-treated and VCP-treated transgenic mice. This data suggests that VCP treatment of 2 or 3 month old APPswe/PS1De9 mice showed improvement in episodic memory and that the use of a dry maze is sensitive in distinguishing cognitive measures between wildtype and APPswe/PS1De9 transgenic mice. Taken together, these results suggest that using VCP in an effectively lower dose compared to previously published results, indicates that VCP may play a neuroprotective role in traumatic brain injured rats and have a positive effect on memory in the APPswe/PS1De9 mouse model of Alzheimer's disease.

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CHAPTER 1
GENERAL INTRODUCTION

To date there is no effective therapeutic agent available to treat the pathological inflammation caused by traumatic brain injury (TBI) and Alzheimer's disease (AD). This study endeavours to evaluate the efficacy of a virus-derived therapeutic agent on the cognitive outcome in TBI and AD animal models.

In this section, epidemiology, risk factors and causes of head injury are addressed. This will offer insight into the devastating morbidity and mortality of the condition. The long term consequences of brain injury are also described in order to provide a glimpse into the challenges faced by affected individuals and their caregivers.

Next a glance into the theories of the pathogenesis of AD will be presented, followed by the hypothesis and aims of this study.

It will also become evident that both TBI and AD are a major health and socioeconomic burden as a result of secondary or delayed damaged in the brain caused by inflammatory effects.

1.1. TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) occurs when there is a sudden physical insult to the head that may result in the disruption of brain functioning. In Johannesburg alone, the estimated incidence of TBI is 316 per 100 000 (Brown and Nell, 1992) and the estimated incidence in the United States is between180 to 250 per 100 000 (Bruns and Hauser, 2003). In the Netherlands, TBI is the fourth leading cause of mortality and is the leading cause of death in children and adults up to 39 years old (van der Sluis et al., 1998). According to the Centre for Disease Control, the leading causes of TBI are falls, motor-vehicle accidents and assaults. According to Levin (2004), TBI in South Africa may largely be due to the high rates of violence and motor vehicle accidents.

With the use of preventative measures such as helmets and seatbelts, the overall mortality rates of TBI victims have decreased. However, the morbidity of TBI survivors have increased and the physical, psychological and social consequences of a non-fatal head injury persist long after the injury (Langfitt and Gennarelli, 1982).

1.1.1. Types of TBI

In humans, head injury can be categorised into focal injury and diffuse injury. Focal injury is characterised by the presence of contusions, lacerations and haematomas and diffuse injury by diffuse swelling, ischemic brain damage and diffuse axonal injury exhibited not only at the site of impact but also in remote regions of the brain. TBI is further graded according to the severity of the injury. A primary injury is when an immediate mechanical damage occurs at the moment of impact, damaging neuronal cells and tissue. A secondary injury has delayed effects because the patient presents clinically only hours or days after the moment of impact or injury. The severity of TBI is classified according to the Glasgow Coma Scale. This is an assessment of eye-opening, motor and verbal responses. It consists of three major categories of mild (score higher than 13), moderate (score between 9 and 12), and severe TBI (score below 9) (Laurer et al., 2000). A common symptom that results from brain injury is post traumatic amnesia (PTA). PTA is characterised by disorientation, impaired attention, memory failure in daily events, illusions, and misidentification of family, friends and medical staff (Brooks, 1984). PTA may vary from an hour to days and its duration is used as a guide to the extensiveness of brain injury. The longer the PTA, the stronger the possibility of extensive damage (Walsh, 1987).

1.1.2. Risk factors and Causes of TBI

In general, males are at a higher risk of sustaining head injuries than females (Guerrero et al., 2000). Records in the emergency department of a hospital in the United States (US) showed that TBI caused by falls were predominant in people aged 0-14 and over 44 years, and were more prevalent in females than males. People aged 15-24 years were more likely to be involved in motor vehicle accidents and were more likely be 'struck by an object'. TBI in 25-44 year old people were more likely to be caused by motor-vehicle related incidents and intentional injuries. Intentional injuries accounted for a larger proportion in males than females.

TBI may be caused from a variety of recreational and occupational activities including: cycling (Rosenkranz and Sheridan, 2003) skiing (Cattermole, 1999), snowboarding (Ferrera et al., 1999), boxing (Martland, 1928;

Mawdsley and Ferguson, 1963), motor vehicle accidents (Hillary et al., 2001) and war (Okie, 2005).

Data of hospitalised individuals involved in bicycle collisions and falls showed that 50 percent did not wear helmets at the time of injury and 75 percent of patients with closed head injury did not use helmets (Rosenkranz and Sheridan, 2003).

As early as 1928 boxing fans, promoters and sport writers identified a peculiar condition that was coined "punch drunk". This condition was recognised in fighters who had particularly received severe and multiple blows to the head. Martland (1928) first reported that almost one half of the boxers developed a mild to severe form of neurological and psychiatric squeals that often necessitated admission to a mental institution (Martland, 1928). By 1963, it was established that repeated head injuries in the ring could manifest in dementia and impairment of motor function (Mawdsley and Ferguson, 1963).

At war, among other hazards soldiers are involved in blasts. Blasts produce a rapid increase in air pressure on the brain resulting in a concussion or contusion. In comparison to the Vietnam War, the overall survival rates of soldiers are greater in the Iraq and Afghanistan war, of the surviving soldiers wounded in combat, brain injury accounted for a larger proportion (22%) than in the Vietnam War (12-14%) (Okie, 2005).

1.1.3 Long-term consequences of TBI

Severely head-injured patients have long-lasting psychological, social and emotional consequences that persist much longer than the physical problems (van der Sluis et al., 1998). Although, mild-injured patients do not get hospitalised, approximately 15% may continue to have similar consequences (Alexander MP, 1995; Guerreo et al., 2000). More than half of patients that had mild head injury complained of memory deficit (Rimel et al., 1981).

A study in the Netherlands reported psychological complaints including cognitive, behavioural and emotional problems in severely head-injured patients. Cognitive problems that include fatigue, poor memory and slowness caused more distress than the behavioural (irritability and impulsiveness) and

emotional (anxiety, depression and sleep disturbances) problems in patients who had sustained severe head injuries (van der Sluis et al., 1998).

Poor memory and slowness are characteristic features of severe head injury, however, symptoms reported after mild brain injury also included memory problems, concentration difficulties, fatigue and headaches (Rimel et al., 1981; Guerrero et al., 2000). A study of patients with brain injury and a duration of unconsciousness ranging from 0 to 210 days revealed that 42% met the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) criteria of a major depressive disorder that included the diagnostic symptoms of psychomotor retardation, sleep disturbances and concentration difficulties (Kreutzer et al., 2001). In patients who sustained a mild head injury and had a Glasgow coma Scale score of 13 to 15 and an unconsciousness of 20 minutes or less, 34% were unemployed as long as 3 months following injury. This high rate of unemployment was attributed to patients having persistent psychological and emotional symptoms (Rimel et al, 1981).

After brain injury, the myriad of symptoms that include physical, cognitive and neurobehavioral impairments of the affected individual also influence family functioning and marital status (Groom et al., 1998; van der Sluis et al., 1998). Two-thirds of marriages ended in divorce within 2 years post brain injury (Anderson-Parente et al., 1990; DiCesare et al., 1990). Livingston and co-workers reported that the proportion of caregivers (30%) that had sufficient emotional problems that needed clinical intervention increased from the first to the fifth year (Livingston et al., 1985).

Stress levels of the caregiver and patient unemployment were strongly related to the presence of neurobehavioral symptoms such as depression, indifference to current condition, socially inappropriate behaviour and pragmatic communication deficits in the patient (Groom et al., 1998).

1.1.4. Morphological and Physiological Changes following TBI in patients and animal models

The underlying neurological and cognitive deficits observed in patients are caused by both the primary and secondary injury following TBI. The primary insult initiates the secondary injury that manifests long after the time of injury and includes the disruption of biochemical and physiological processes in the brain (Faden, 1993; DeKosky et al., 1998). By unravelling the pathophysiology of the TBI potential therapeutic targets could be identified.

Since memory, concentration and problem-solving are mediated partly by forebrain cholinergic and catecholaminergic innervation, researchers identified that the morphological damage or loss to the system occurred in the rat ventro-basal forebrain cholinergic neurons (Schmidt and Grady, 1995). Another report demonstrated that in fatally head-injured patients, cholinergic neurons in the nucleus basalis of Meynert (NBM) were damaged (Murdoch et al., 2002). Similarly, in a closed head injury in a rat model the selective loss of forebrain cholinergic neurons caused significant disturbances in cognitive tasks associated with the neocortical and hippocampal cholinergic function (Schmidt and Grady, 1995). It was also shown that by increasing the severity level of fluid percussion injury in rats, an increasing number of injured neurons were evident in the hippocampus (Hellmich et al., 2005).

Ultra-structure evaluation of rat brains following TBI revealed that the primary injury mechanism is the disruption of the blood-brain barrier (BBB) and hemorrhagic contusions (Dietrich et al., 1994). One hour post-injury rat gene expression for both IL-1 (interleukin-1) and TNF (tumour necrosis factor) were upregulated (Fan et al., 1995; Fan et al., 1996) and the BBB became permeable to blood- borne factors that consequently propagated secondary injury (Tanno et al., 1992; Dietrich et al., 1994; Baldwin et al., 1996). Reactive microglia, astrocytes and macrophages are found in the vicinity of the lesion in rat brains (Bellander et al, 1996; Bramlett et al., 1997; Dunn-Meynell and Levin, 1997).

In transgenic mice whose microglia were fluorescently labelled, microglia become activated, accumulate and fuse around the damaged and dead cells forming a shield at the site of the laser-induced injury (Nimmerjahn et al.,

2005). Activation of microglia cells causes phagocytosis of cell debris and the secretion of pro-inflammatory cytokines such as interleukin 1β (IL-1β) and tumour necrosis factor α (TNFα). IL-1 also promotes the proliferation of microglial cells and activates astrocytes resulting in the elevated production of apolipoprotein E. IL-1 in addition induces the neuronal production of amyloid precursor protein (APP) (Buxbaum et al., 1992; Barger and Harmon, 1997). Increased synthesis of β-APP following FPI in rats was also evident after moderate and severe TBI. It was proposed that β-APP under certain circumstances of abnormal accumulation may give rise to the pathological β-amyloid found in senile plaques in AD (Bramlett et al., 1997). TNFα has been shown to be elevated (Goodman et al., 1990) and present in the cerebrospinal fluid (CSF) and plasma of severely head injured patients (Ross et al., 1994). Interleukin 6 (IL-6) plays a dual role in response to head injury by releasing both pro- and anti-inflammatory mediators that promote tissue repair and also contribute to additional damage by releasing neurotoxic substances (Ott et al., 1994; Schindler et al., 1990).

Besides the focal point of injury, regions more remote from the site of impact showed transient breakdown of the BBB, however, the BBB at the site of impact remained permeable up to seventy- two hours after injury in rats (Tanno et al., 1992). As a result of the disrupted BBB, vasogenic oedema results from the accumulation of oedema fluid in the extra cellular spaces in the brain (Cernak et al., 2004).

In a study using magnetic resonance imaging to examine the roles of cerebral blood volume (CBV) and oedema in head-injured patients, brain swelling due to brain-tissue fluid or oedema was evident in 87% of the patients and the CBV was reduced in proportion to reduction of cerebral blood flow (Marmarou et al., 2000).

Cells that are not mechanically damaged following injury experience metabolic and ionic changes that alter their extra and intracellular environment, which in turn cause undesirable long-term consequences (Hovda et al., 1990; Hovda et al., 1995). These are calcium accumulation (Hovda et al., 1990), increase in extracellular potassium (Katayama et al., 1991) and increase in glucose metabolism (Hovda et al., 1990) resulting in

an ionic flux that induces an increase in glycolysis with consequent lactic acid accumulation.

Proton magnetic resonance spectroscopy was used to quantify the human brain metabolites *in situ* during recovery after TBI at 1.5, 3 and 6 months (Brooks et al., 2000). Various metabolite markers such as, myoinstol (as an index of osmotic imbalance and glial proliferation), choline (as a measure of inflammation) and N-acetylaspartate (NAA) levels (as an indicator of neuronal injury) were quantified in regions remote from the primary injury site. Using these neurometabolite markers together with neuropsychological testing, researchers were able to monitor neuronal injury, inflammation and metabolic dysfunction and predict cognitive functional outcomes (Brooks et al., 2000). Genes encoding molecules for cellular signalling, synaptic plasticity, metabolism, ion channels and transporters in the rat hippocampus were investigated after experimental injury (Li et al., 2004). It appears that differential gene expression relates to the severity of brain injury. The number of genes affected increased as the severity of head-injury increased. However 90% of genes that were up-regulated after a severe TBI and 59% were down-regulated after a moderate TBI. Forty-two percent of the down-regulated genes following a moderate head injury became up-regulated after a severe TBI. Interestingly, the genes that showed differential expression after moderate and severe brain injuries are among the genes that related to the primary responses to injury (Li et al., 2004).

Cell death by apoptosis has been previously documented following experimental rat brain injury (Rink et al., 1995). More specifically, apoptosis by the activation of caspase proteases have been shown to occur after TBI (Namura et al., 1998). Caspases are cysteine proteases that are cleaved into smaller subunits and have enzymatic activity upon activation. Caspase -3 has been strongly implicated in neuronal apoptosis (Namura et al., 1998). Two major biochemical pathways initiate caspase 3 activation. The extrinsic pathway is mediated by caspase 8 and is activated through cell surface death receptors.Caspase-9 mediates the intrinsic pathway that is activated by signals from the mitochondria and develops into the formation of an apoptosomal complex. Knoblach and colleagues compared the activation of caspases 3, 8, and 9 after FPI in rats. Cleaved forms of caspases 3 and 9

were evident at 1, 12 and 48 hours following injury but caspase-8 was undetected.

1.1.5. Potential Therapeutic targets for TBI

Currently there are no neuroprotective agents available to counteract or repair secondary damage caused by TBI. Over the years, however, there have been efforts to elucidate the pathophysiology of TBI in order to establish potential therapeutic targets. Although these targets showed promising results in experimental work, clinical trials proved to be disappointing (Royo et al., 2003).

In response to injury, microglia release reactive oxygen species (ROS) (Théry et al., 1991) and nitric oxide (Boje and Arora, 1992) which cause the release of glutamate that acts on N-methyl-d-aspartate (NMDA) receptors to promote the influx of calcium cations into the cell and ultimate cell death (Choi, 1987; Rothman and Olney, 1987). Both competitive and non-competitive NMDA and non-NMDA receptor antagonists have been tested with success in experimental brain injury only (Bullock and Fujisawa, 1992). The release of glutamate, ROS and cytokines has been shown to be inhibited by cannabinoids (Panikashvili et al., 2001). Magnesium has been shown to be neuroprotective in rats that sustained TBI since it was able to modulate NMDA receptor permeability to calcium and sodium (Bareyre et al., 2000). Other inhibitors, such as non-selective and selective inhibitors of nitric oxide synthase, free radical scavengers and caspase inhibitors have shown success as therapeutic targets in animal models (Royo et al., 2003).

1.2. ALZHEIMER'S DISEASE

According to the DSM-IV, AD is the development of multiple cognitive deficits manifested by both memory impairment and one or more of the following cognitive disturbances: aphasia, apraxia, agnosia and a disturbance in executive functioning. Neuropsychological testing was used to assess the functional status in AD patients in 7 cognitive domains including, immediate and delayed verbal memory, attention, visuospatial abilities, language, executive functioning and praxis (Farias et al., 2003). Each of the domains were assessed by measurement of daily living skills that included the ability to use a telephone, shop, prepare food, complete housework, do laundry ,utilise public transportation, administer medication and handle financial responsibilities. Results showed that individuals with AD not only showed cognitive impairments but functional deficits across a number of domains. AD individuals also performed significantly worse on all functional measures than the non-demented geriatric control group. Interestingly, education rather than age was significantly correlated with functional abilities (Farias et al., 2003). Motor and cognitive regulation was assessed using a Behavioural Dyscontrol Scale in AD patients, mild cognitive impaired patients and elderly groups of individuals (Belanger et al., 2005). Executive functions that require alternating hand sequences, inhibition, learning complex motor sequences, alphanumeric sequencing and awareness of deficit or insight were measured. Results revealed that the AD group performed significantly worse than the mild cognitive impaired and elderly groups. However, the mild cognitive impaired and elderly groups did not significantly differ (Belanger et al., 2005). Depression is more common at the early stages of AD, as they are aware of their cognitive decline and failing abilities (Farias et al., 2003; Lindeboom and Weinstein, 2004).

Tests of language comprehension and language expression were administered to AD patients in order to evaluate how deficits in working memory affect communicative functioning (Bayles, 2003). AD affected individuals performed poorer than unaffected individuals. These differences resulted from difficulties with storing, retrieving and manipulating linguistic

information rather than a loss of linguistic or conceptual knowledge (Bayles, 2003).

A study showed that 24.5% of AD patients admitted to a memory clinic suffer from sleep disturbance (Moran et al., 2005). Neuronal pathways, particularly the cholinergic pathways have been implicated in the initiation and maintenance of sleep. It is thought that damage to these pathways in AD contribute to sleep changes. Furthermore, sleep disturbances were also associated with aggressiveness including verbal outbursts, physical threats and agitation (Moran et al., 2005). According to Hope and co-workers sleep disturbance and night-time behaviour disturbance have been associated with caregiver stress and the reason cited for deciding to admit a patient to institutional care (Hope et al., 1998).

Content analysis of unsolicited letters from caregivers of individuals suffering from AD reveal that their primary stressors emerge from the burdens of providing activities of daily living care; problematic behaviour such as aggressiveness and sexual issues; and difficulties in handling changes in their cognitive status (Anderson et al., 2004). Caregivers also included secondary stressors such as conflicts between family members and the financial burden of AD home-care that further exacerbate their global outcomes of depression, anxiety and a deterioration of physical well-being (Anderson et al., 2004). Spirituality has however, been reported as a coping strategy that reduces caregiver burden in stressful situations particularly in providing care to AD patients (Spurlock, 2005).

1.2.1. The Nature of Memory Processes in AD

In AD, memory processes are affected. Memory is classified into temporal states according to their duration of retention, namely iconic, short-term and long-term as shown in Fig.1.1. Iconic memory is retained even after the visual event has occurred. Short-term memory retains a limited capacity of information only temporarily. In order for short-term memory to be retained it has to be rehearsed (Kandel, 1991). Working memory is a temporary storage of either visual or verbal material that maintains information while it is being processed (Baddely, 1986; Squire, 1987). Baddely (1986) proposed that short-term visual-spatial information is temporarily held in the visuospatial sketchpad and the short-term verbal memories are stored in the phonological loop. A central executive system accesses these short-term and long-term stores and processes the information (Baddely, 1986; Thompson, 2000). Long-term memory is divided into declarative and non - declarative memory. Non-declarative memory is procedural and involves no awareness of task acquisition. Learning is implicit and incidental. Declarative memory is explicit and memory represents the conscious learning or memorisation of material. Declarative memory may be subdivided into semantic and episodic memory. Semantic memory represents organised information such as facts, concepts and vocabulary. Episodic refers to stores of cumulated events on ones life, like an autobiography (Squire, 1987). The initial phase of AD affects episodic memory and then as the pathological process advances, impairments occur in semantic memory, language and visuospatial ability (Lindeboom and Weinstein, 2004). AD patients seem to sustain greater damage to episodic and working memory systems than to non-declarative systems (De Vreese et al., 2001). Furthermore, damage to declarative memory systems occur gradually (Lipinska and Backman, 1997). For most of the progression of the disease, non-declarative memory systems are spared (Squire, 1987).

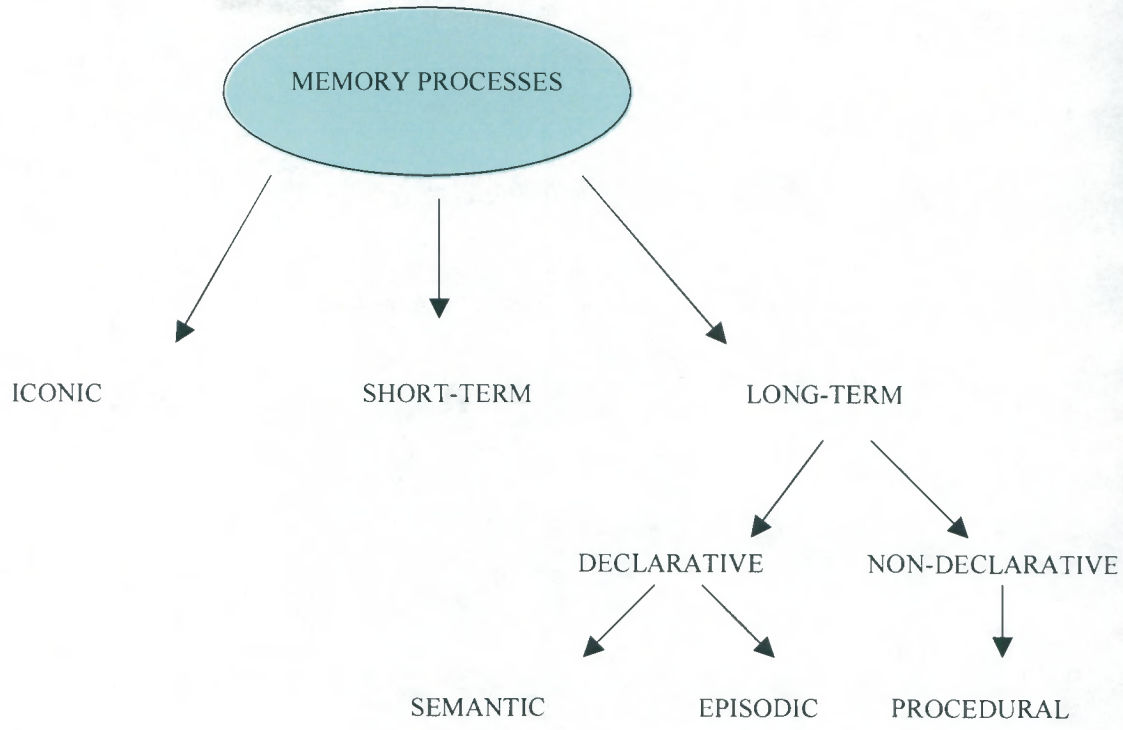


Fig.1.1. Schematic diagram to show the nature of memory processes in humans.

Biopsy samples from Alzheimer's patients have shown reduced acetylcholine synthesis compared to age-matched control samples (Bartus et al., 1992). Human nucleus basalis of Meynert is a major source of cholinergic neurons in the brain and are the major source of extrinsic cholinergic innervation to the cerebral cortex and hippocampus (Whitehouse et al., 1982).

The nucleus basalis magnocellularis (NBM) of the rat contains large cholinergic neurons in the substantia innominata and is similar to the human nucleus basalis of Meynert (Aigner et al., 1991). Acetylcholine (Ach) is synthesised in the terminal endings of cholinergic neurons. The basic chemical reaction of Ach synthesis is catalysed by choline acetyltransferase (ChAT). ChAT activity is significantly reduced in the brains of Alzheimer's patients when compared to age-matched controls and this is indicative of cholinergic neuron loss. Aigner and co-workers (1991) investigated the role

of basal forebrain nuclei in memory by selective neurotoxic lesions in monkeys (Aigner et al., 1991). They demonstrated impairments of recognition memory and delayed sample matching performance. Muir and co-workers (1994) showed that similar lesions in rats caused impairment of memory tasks (Muir et al., 1994). These and other studies in primates (Wilson and Rolls, 1990a and 1990b; Rolls et al., 1979) using visual stimuli associated with rewards or punishments (Rolls et al., 1979) clearly implicate the basal forebrain region in the memory storage process. It therefore appears that if the normal operations of these cells (NBM) are impaired, then normal memory is adversely affected. Abnormal activity or disruption of NBM function could contribute to the memory disorder in AD.

1.2.2. Pathophysiology of Alzheimer's Disease

Alzheimer's disease (AD) is an extremely complex disease. It is characterised by the abnormal extracellular accumulation of the β-amyloid protein (Aβ) in the brain and is the associated with dementia. These amyloid plaques were first described in 1906, by Alois Alzheimer (Alzheimer, 1907). Over the decades, there has been extensive investigation into the initial molecular event that causes the pathogenesis and neurodegeneration in Alzheimer patients. One of the first theories described (the amyloid cascade hypothesis) proposed that the main component of the plaques, accumulated Aβ was the causative agent of the disease and that neurofibrillary tangles (NFT), cell loss, vascular damage and dementia are the direct result of this deposition (Hardy and Selkoe, 2002). Another theory known as the tau or tangle hypothesis argues that aggregated neurofibrillary tangles precedes Aβ accumulation (Mudher and Lovestone, 2002). These tangles are normally expressed in axons and are comprised of microtubules associated with tau protein. In AD they become highly phosphorylated and aggregate into abnormal filaments in the cell body Fig. 1.2. The exact mechanism by which Aβ induces neurodegeneration and cell death has not yet been fully resolved. Recently a close link between Aβ protein and NFT has been established, in which caspase cleavage of tau is initiated by Aβ exposure which further promotes the assembly of tau into pathological filaments in neurons (Gamblin

et al., 2003). Since these two pathological hallmarks of AD were identified there has been a vast amount of research into the process of their formation. The role of complement activation by Aβ and the resultant response seem to be the major factors in sustaining the progressive neurodegeneration in AD (Daly and Kotwal, 1997).

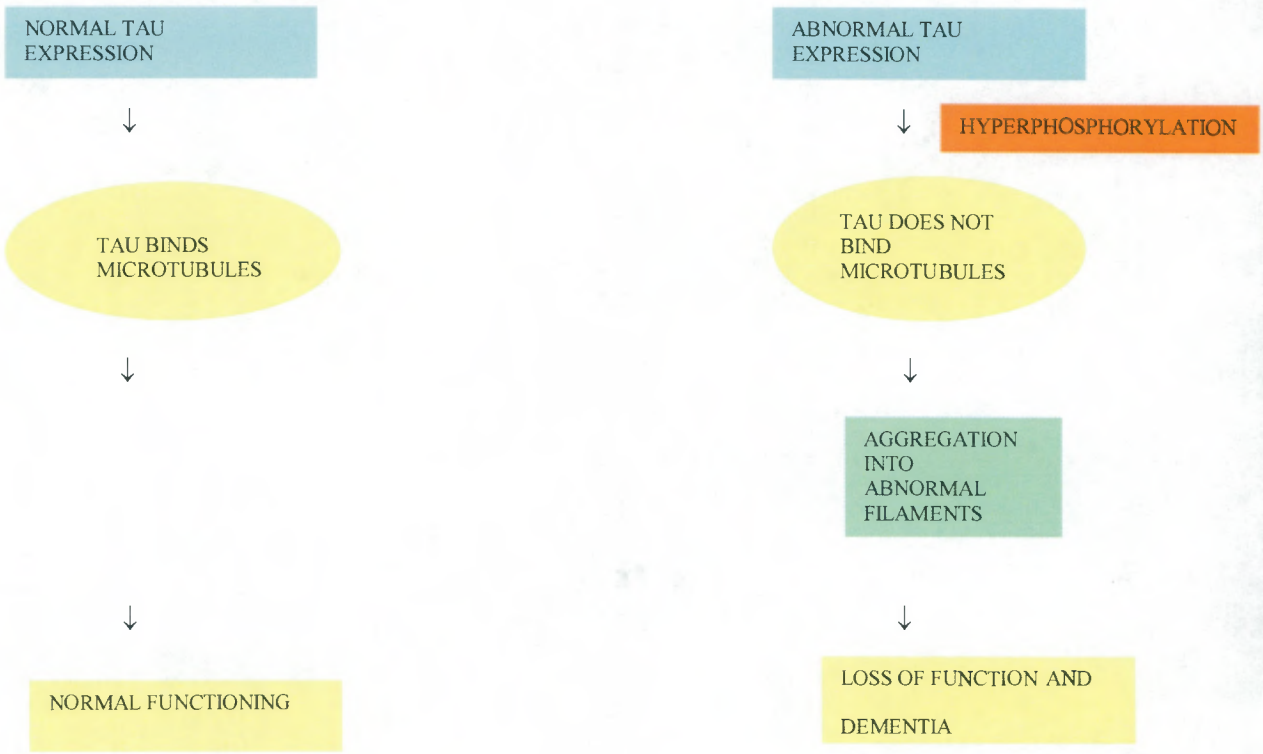


Fig.1.2. The tau and tangle hypothesis. Under normal expression, tau binds microtubules. In AD, this process is disrupted by hyperphosphorylation that causes aggregation of filaments and the ultimate loss of function and dementia.

AD and secretases

Aβ precursor protein or amyloid precursor protein (APP) is a type I transmembrane glycoprotein (Kang et al., 1987). α, β and γ secretases have for long been identified as modulators for processing of an integral membrane protein, amyloid precursor protein (APP) (Passer et al., 2000). Cleavage of the APP by α-secretase produces a large soluble α-N terminal fragment and an 83 amino acid membrane-bound C-terminal fragment, C83. Proteolytic cleavage by β-secretase on APP yields a soluble extracellular β-N terminal fragment and a membrane bound 99 amino acid C-terminal, C99. Further processing of the preferred substrate C99 by γ secretase follows, releasing the neurotoxic Aβ and an APP intracellular fragment domain (γ-CTF or AICD) (Passer et al., 2000; Pinnix et al., 2001). The predicted size of AICD is 57 or 59 residues based on the cleavage site $A\beta_{40}$ and $A\beta_{42}$ but the observed AICD fragment is 50 residues long (Pinnix et al., 2001;Cao and Sudhof, 2001;Kinoshita et al., 2002; Sastre et al., 2001; Yu et al., 2001; Sambamurti et al., 2002). Passer and colleagues showed that AICD fragments occur in the soluble fraction of normal and AD brain (Passer et al., 2000). They also showed that AICD acts as a positive regulator of apoptosis, by causing an overproduction of AICD that may enhance the toxic effects of $A\beta_{42}$ aggregates.

β-secretase, also known as beta site APP cleaving enzyme, (BACE-1) and Asp2 and Memapsin-2 was identified as a membrane-bound aspartic acid protease (Tang et al., 2003; Vassar et al., 1999; Howlett et al., 2000; Sisodia, 2000). Asp2 is expressed predominantly in neurons, but not in glia. It is highly expressed in the hippocampus, cortex and cerebellum (Vassar et al., 1999). Generally, aspartic proteases bind between 6-10 amino acids of their substrates (Howlett et al., 2000). Elucidation of these biochemical events provides opportunity to develop β-secretase inhibitors that are highly specific. Presenilins are integral membrane proteins with eight putative transmembrane domains encoded by genes on chromosomes 14 and 1 (Nishimura et al., 1999). Mutations have now been identified in presenilins where presenilin-1 causes familial AD and these mutations have been shown

to cause an increase in the proportion of $A\beta_{42}$ (Scheuner et al., 1996; Kovacs and Tanzi, 1998).

A complex of four proteins, presenilin-1, nicastrin, anterior pharynx defective (APH-1) and presenilin enhancer (PEN-2) seem to contribute to γ-secretase activity (Mattson, 2003; Goutte et al., 2002; Francis et al., 2002). APH-1 stabilises the presenilin holoprotein in the complex, whereas PEN-2 is required for γ-secretase proteolytic processing (Takasugi et al., 2003). PEN-2 promotes the production of Aβ.

Recently, a new signalling pathway in AD was identified (Neve, 2003). Sumoylation is a post-translational modification that influences protein function. It is biochemically similar to ubiquitination but functionally distinct. Sumoylation is involved in several neurodegenerative diseases but is not yet fully elucidated. However, it appears to regulate protein localisation and stability (Neve, 2003). There are 3 members of the small ubiquitin-like modifier (SUMO) protein family. Preliminary evidence shows that in unaffected brains the distribution of SUMO-3 was found in both the nucleus and soma. In AD brains, SUMO-3 seems to be limited to the neuronal soma only (Neve, 2003). In this model it has been shown that SUMO-3 sumoylation is directly correlated with the regulation of Aβ production. Therefore, abnormal regulation of APP processing may occur in AD. This pathway presents yet another possible target for AD therapy by stimulating SUMO-3 sumoylation via SUMO-3 specific proteases inhibition (Neve, 2003; Li et al., 2003).

Catabolism of APP

The mechanism by which Aβ is broken down is of equal importance in understanding the pathology of AD (Miller et al., 2003; Farris et al., 2003). For several years researchers have focussed on Aβ protein synthesis and its accumulation in the brain. Recently, a number of proteases have been implicated in Aβ protein catabolism shown in Fig.1.3.

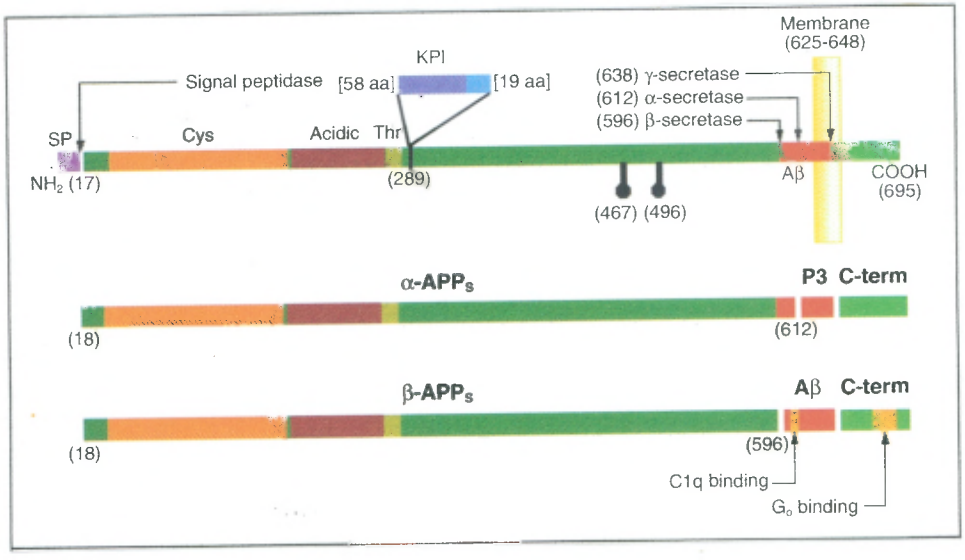


Fig.1.3. A detailed diagram of the major isoform of amyloid precursor protein (APP_{695}) present in neurons. β-secretase cleaves the Aβ domain within APP, liberating a soluble 578 amino acids N-terminal fragment of APP (β-APPs) and a C-terminal bound amino acid fragment, C99. Cleavage of APP by α-secretase also yields a soluble 584 amino acids N-terminal fragment (α-APPs) and C83. C83 cleaved by γ-secretase releases a non-pathogenic P3 protein (as shown in middle diagram). Further processing of the preferred substrate C99 by γ-secretase leads to the release of the toxic Aβ peptide and APP intracellular domain (AICD) (Taken from Daly and Kotwal, 1997).

Neprilysin is an enzyme that is widely distributed in the body. In brain tissue neprilysin is the enzyme responsible for the cleavage of neuropeptides (Kanemitsu et al., 2003; Yasojima et al., 2001). Neprilysin is the rate limiting enzyme that regulates Aβ levels in the brain by altering metabolism. Research shows that low levels of neprilysin may contribute to AD (Yasojima et al., 2001).

Endothelin- converting enzyme (ECE) like neprilysin is a member of the M13 family of zinc-dependent metallopeptidases (Facchinetti et al., 2003). Two types of ECE have been reported, ECE-1 and ECE-2 (Facchinetti et al., 2003; Eckman et al., 2003). ECE-1 and ECE-2 plays a role in restricting Aβ accumulation in the brain (Eckman et al., 2001; Eckman et al., 2003). It has been reported that both ECE-2 and neprilysin genes are linked to chromosome 10.

Originally identified as an insulin-degrading enzyme (IDE), insulysin, is also involved in the degradation of Aβ protein (Eckman et al., 2001; Melchor et al., 2003). IDE has a disease susceptible gene in both AD and type 2 diabetes mellitus (DM2) (Melchor et al., 2003). IDE also regulates the levels of unphosphorylated APP intracellular domain (AICD) but not phosphorylated AICD in the brain. In other words, phosphorylated AICD may offer protection from IDE cleavage. An initial reduction in IDE is capable of placing individuals at risk for AD, DM2 and, in particular hyperinsulinemia. The IDE region of chromosome 10 has been linked to late-onset AD and DM2 (Melchor et al., 2003).

Plasmin has been shown to increase the processing of human APP preferentially at the α-cleavage site (Ledesma et al., 2000). Furthermore, both secreted amyloidogenic and non-amyloidogenic APP fragments are efficiently degraded by plasmin. Reduced levels of plasmin were evident in brain tissue of AD patients, suggesting that plasmin down regulation may cause amyloid plaque deposition.

The toxic accumulation of Aβ caused by the failure of these enzymes (ECE, IDE, neprilysin and plasmin) in the clearance of Aβ in neurons may stimulatethe complement system, thereby causing inflammation (Miller et al., 2003; Farris et al., 2003).

AD and Metal Toxicity

Besides the focus on the catabolism of amyloid plaques by enzymes, researchers have also investigated the toxicity in AD caused by metals such as copper, zinc, iron and aluminium (Exley, 1999 ; Barnham et al., 2003 ; Waggoner et al., 1999 ; White et al., 1999; Burdette and Lippard, 2003 ; Sparks and Schreurs, 2003 ; Lee et al., 2002 ; Bush and Tanzi, 2002 ; Bush, 2003 ; Cuajungco and Faget, 2003). Copper and zinc (and iron, to a lesser extent) interact with APP and Aβ peptide in normal physiology (Exley, 1999).

It is hypothesised that aluminium (Al) induces the excessive activation of glutamate receptors resulting in high calcium influx into the cell. This leads to a cascade of events that will affect synaptic plasticity such as long-term potentiation and long-term depression eventually culminating in rapid neuronal loss (Exley, 1999). It is clear that brain Al homeostasis could impact upon brain function, however, the possible role of Al in the initiation and progression of AD is uncertain. Al is more likely to have a secondary rather than a primary effect on the development of AD.

The zinc model of Aβ pathology, proposes that genetic or environmental factors may trigger aberrant Aβ metabolism thereby liberating zinc ions and disrupting cerebral zinc and copper homeostasis (Bush, 2003; Cuajungco and Faget, 2003). At low pH environments, copper ions tend to displace zinc resulting in copper-induced damage and formation of toxic fibrillar Aβ aggregates. This results in oxidative damage causing necrosis. Oxidative damage occurs when the reactive oxygen production exceeds cellular anti-oxidant defences (Smith et al., 2000).

Biosynthetic Mechanisms in AD

The disruption in biosynthetic mechanisms has also been implicated in the pathophysiology of AD.

This model proposes that hypofunctioning of retinoid (vitamin A), is a central factor in the development of Aβ toxicity and late onset AD (Goodman and Pardee, 2003). Retinoid cannot be synthesised by the human body and must therefore be available from the diet. There have been suggestions that the expression of AD-related genes depend on the amount of retinoic acid in the brain, which is dependent upon the retinoid metabolic cascade. The mechanism of transport of retinoic acid from the intestine to the brain seems to be modified in AD. Metabolic evidence of the brains of patients with AD shows a rise in retinaldehyde dehydrogenase (RALDH), the enzyme synthesiser of retinoic acid. When starved neuronal cell lines are replenished with retinoic acid, RALDH increases making this consistent with a feedback type of control mechanism (Goodman and Pardee, 2003).Retinoic acid also regulates IDE which is involved in the degradation of Aβ. Apolipoprotein E (ApoE) is a transport protein for retinyl esters in chylomicrons. It is suggested that increased expression may be the result of feedback mechanisms dependent on the reduced amounts of retinal in aging individuals. This lipoprotein transport system affects APP processing and Aβ plaque formation AD (Goodman and Pardee, 2003).

In addition high levels of cholesterol may contribute to the pathogenesis of AD (Jarvik et al., 1995; Kuo et al., 1998). ApoE is the major protein component of very low density lipoprotein (VLDL) and the principal cholesterol carrier protein in the brain. It is thus clearly established that apoE is involved in lipid transport and metabolism in the plasma and the brain (Mahley, 1988; Puglielli et al., 2003). There are three allelic forms of the apoE gene: ε2, ε3 and ε4. Only apoE ε4 increases the risk of AD in a dose-dependant manner but is neither necessary nor sufficient to cause AD (Puglielli et al., 2003; Bertrand et al., 1995; Fagan et al., 2002). Unlike apoE ε2 and ε3, apoE ε4 has the ability to bind Aβ and cross the blood brain barrier (Martel et al., 1997; Fagan et al., 2002). ApoE concentrations in the brain are demonstrated to be lower in AD patients carrying an apoE ε4 allele than

unaffected control patients and AD individuals with apoE ε3 genotype (Bertrand et al., 1995 ; Refolo et al., 2000). These lower levels of apoE may disrupt the lipid homeostasis, of which the cholinergic system is dependant upon for synaptic plasticity (Martel et al., 1997; Fagan et al., 2002). In addition, a direct relationship between the amount of plasma cholesterol and Aβ was found suggesting that hypercholesterolemia is associated with reduced Aβ accumulation. Only in the presence of apoE does a high cholesterol diet show to affect and accelerate APP-processing, therefore resulting in increased Aβ (Howland et al., 1998; Refolo et al., 2000).

Molecular genetics of AD

The aforementioned sections describe the role of numerous critical molecules in the increased production or accumulation of Aβ. Table 1.1. summarises the gene defects and the chromosomes associated with their effects on Aβ. It is noteworthy that the enzymes involved in Aβ clearance are linked to chromosome 10.

Table 1.1. Summary of gene defects on chromosomes linked to AD and their effect on Aβ.

Chromosome	Gene defect	Protein	Molecular effect on Aβ	
1	Mutation	Presenilin 2	Only increases Aβ42	Scheuner et al.,1996;Kovacs and Tanzi,1998
10	10q23	Endothelin converting enzyme 2 Neprilysin Insulysin	Increased accumulation (expected but not shown)	Eckman et al,2001 Iwata et al., 2000 Iwata et al.,2001 Miller et al.,2003
14	Mutation	Presenilin 1	Only increases Aβ42	Scheuner et al.,1996;Kovacs and Tanzi,1998
19	Polymorphism	ApoE4	Increased production	Strittmatter,and Roses, 1996; Selkoe, 1997; Roses,1997.
21	Mutation	APP	Increased Aβ or Aβ42 production	Scheuner et al.,1996;

AD and Angiogenesis

Angiogenesis occurs in conditions of hypoxia. It triggers vessel growth in order to sustain oxygen supply to tissues (Carmeliet, 2003). In AD, pathological angiogenesis is thought to occur in response to cerebral perfusion (oligaemia) and vascular injury (inflammation). It is proposed that the activation of the brain endothelium in AD leads to deposition of the Aβ plaque and secretion of a neurotoxic peptide that destroys neurons (Vagnucci and Li, 2003). Two mechanisms of angiogenesis are postulated to occur in the diseased brain. The first suggests that reactive oxygen species in the Aβ plaque causes damage in endothelial cells of the brain. A thrombogenic region develops in the vessel wall, leading to excessive production of thrombin. Thrombin activates the vascular endothelial cells to secrete APP via a receptor-mediated, protein kinase C-dependent pathway (Vagnucci and Li, 2003). A second mechanism that stimulates angiogenesis is the release of inflammatory mediators such as tumour necrosis factor α (TNF-α), interleukin 6, interleukin 1β and monocyte chemoattractant protein-1 (Grammas and Ovase, 2001). These inflammatory mediators may be induced by neuronal death, by β-amyloid binding the C1q component of the complement cascade and by peroxidative and free radical injury of microvessels (Vagnucci and Li, 2003). It is not certain whether inflammation is activated as result of tissue destruction or if inflammation causes neurodegeneration in AD. The endothelium-mediated neurodegeneration (END) hypothesis proposes that pathological insults such as injury, toxins and inflammatory factors damage the brain endothelial cells, triggering the release of nitric oxide, reactive oxygen species and cytokines resulting in neuronal cell death (Grammas, 2000).

1.2.3. Therapeutic targets for AD

AD has a complex etiology that has slowly been unravelled over the years. The molecular mechanisms of AD pathogenesis have given researchers clues to possible therapeutic targets for AD.

Cholinesterase inhibitors are drugs that are currently used in symptomatic treatment of patients with mild or moderate AD (Cummings, 2004). These drugs act by prolonging cholinergic neurotransmission by slowing the biochemical breakdown of Ach. A non-competitive NMDA antagonist, memantine, has recently been developed for use in moderate-to-severe AD. Memantine acts by reducing glutamatergic excitotoxicity and by so doing attenuates the effects that may lead to neuronal cell death and cognitive dysfunction (Reisberg et al., 2003).

Active and passive immunisation using monoclonal antibodies of Aβ in AD mouse models have proven to be efficacious in mouse models of AD (Schenk et al., 1999; Janus et al., 2000; Morgan et al., 2000)). The vaccination had profound effects in lowering Aβ in mice (Dodart et al., 2002). However, trials of vaccination with an Aβ 1- 42 synthetic peptide vaccine and adjuvant administered to mild-to-moderate AD patients showed CNS inflammation resembling postvaccinal meningoencephalitis (Monsonego and Weiner, 2003). This lead to the termination of the Phase II trial. Subsequent evaluation of a subset of patients who developed high titres of plaque-binding Aβ antibodies showed a slowing of cognitive and behavioural decline (Hock et al., 2003).

Other strategies such as the use of metal chelators, anti-inflammatory agents, lipid-lowering drugs, anti-apoptosis compounds are all currently been investigated as therapeutic targets (Silvestrelli et al., 2006).

Since several factors contribute to AD pathogenesis, it is important to develop a combination therapy that acts on more than one target site for AD treatment.

1.3. THE ROLE OF COMPLEMENT IN TBI AND AD

The complement system is the first line of defence against invading micro-organisms. It is composed of serum proteins and membrane-bound receptors that through the anaphylatoxin receptors (C3aR and C5aR) play a role in modulating neuronal function (Barnum, 2002). The complement fragments C3a, C4a and C5a mediate inflammation by stimulating smooth muscle contraction; histamine release from mast cells, vasodilation and increased vascular permeability (Wetsel, 1995). C3a promotes cytokine synthesis by adherent monocytes at local inflammatory sites (Takabayashi et al., 1996). C5a stimulates the secretion of TNF-alpha from human mononuclear cells *in vitro* and induces gene expression and protein synthesis for human IL-1 (Okusawa et al., 1987; Okusawa et al., 1988). After experimental brain injury in the rat gene expression for both IL-1 and TNF were upregulated as early as 1 hour post-injury (Fan et al., 1995; Fan et al., 1996). The blood brain barrier was disrupted and reactive microglial, astrocytes and macrophages were found in the vicinity of the lesion (Bellander et al, 1996). Complement factors C3d and C9-IR were also present at the lesion site thus indicating the activation of the complement cascade. The activated complement system results in the formation of the membrane attack complex (C5b9) that targets neurons for cell lysis. Keeling et al. have shown neutrophil infiltration and an accumulation of C3 in cortical and hippocampal brain sections after a moderate FPI in rats (Keeling et al., 2000).

In head-injured patients, raised levels of C3 and factor B complement components were found in the ventricular cerebrospinal fluid (Kossmann et al., 1997). Furthermore, complement components C1q, C3b and C3d were found in the vicinity of activated microglial, activated macrophages and astrocytes in the injured human brain (Bellander, 2001).

Inappropriate or prolonged activation of the complement system may lead to fatal effects due to severe inflammatory tissue destruction (Kirschfink, 1997). It has further been shown that in the antibody-independent, classical activation of the complement cascade, only the fibrillar form of β-amyloid binds C1q (the subunit of the first component of complement) by ionic interactions (Bergamaschini et al., 1999; Tenner, 2001). More precisely

contact occurs between residues 14-26 of the collagen-like portion of C1q and the β-pleated structure of Aβ (Jiang et al., 1994). More recent evidence shows that the globular head domains of C1q can also bind fibrillar Aβ, especially at higher fibrillar Aβ concentrations (Tacnet-Delorme, 2001). The alternate pathway is also activated when Aβ becomes covalently ester-linked to C3b (a subunit of the third component of complement) (Bradt et al., 1998). By establishing precise interactions with Aβ and complement component proteins, strategies for blocking the activation and progression to the formation of the proinflammatory C5b-9, membrane attack complex (MAC) may have therapeutic benefits. In support of this idea a protective role of anti-inflammatory medication for AD is suggested in a study of 90-year-old monozygotic female twins (Jarvenpaa et al., 2003). PET and MRI scans revealed that hippocampal, temperoparietal and frontal cortical atrophy was moderate in the AD affected twin, whereas the hippocampus was intact and cortical atrophy was mild in the unaffected twin. The twins' life histories were extraordinarily similar, except that the unaffected twin used non-steroidal anti-inflammatory drugs (NSAIDs) since the 70's for her rheumatoid arthritis. Previous work has confirmed the ability of Aβ fibrils to activate complement *in vitro*. This activation can be prevented by the addition of a vaccinia virus complement control protein (VCP) (Daly and Kotwal, 1998). These data also demonstrate that Aβ is capable of causing a much greater influx of immune cells in a mouse model with an intact complement system than in mice lacking the fifth component of complement (C5) (Daly and Kotwal, 1998). Altogether this supports investigating the role of VCP in blocking the detrimental effects caused by inflammation after head injury or AD.

1.4. AIMS OF THE STUDY

The main hypothesis is that VCP is able to improve the sensorimotor function and cognitive outcome of spatial learning and memory following (1) mild or severe head injury in rats and (2) in a transgenic AD mouse model. The proposed mechanism for the hypothesis of VCP working to affect AD progression is that it will block Aβ mediated complement activation. The following aims were developed to address this hypothesis and the proposed mechanism.

1. Production and purification of functional recombinant VCP in a yeast expression system for use as a therapeutic agent that confers both complement –binding and heparin-binding activity.

2. Optimisation of injury severity and survival rates in rats following head injury using a locally produced fluid percussion device.

3. Determine VCP affects on sensorimotor and cognitive function following mild and severe brain injury using a Morris Water maze.

4. Investigate the effect of an intra-ventricular route of VCP administration on cognitive outcome in a severe head injury model.

5. Determine the effect of VCP administration in a familial transgenic AD mouse model.

CHAPTER 2
VCP PRODUCTION AND PURIFICATION

2.1. VACCINIA VIRUS COMPLEMENT CONTROL PROTEIN

The vaccinia virus has over an evolutionary period of time emerged with mechanisms to evade the consequences of immune surveillance. The virus has supposedly acquired the DNA encoding the host protein and manipulated the DNA to retain only the most essential domains in order to evade the consequences of viral evasion (Kotwal, 1996). The virus' secretory protein was identified as the first microbial protein to have complement-inhibitory activity (Kotwal and Moss, 1988).

VCP is structurally similar to human complement 4b binding protein (C4b-BP) and is functionally similar to complement receptor 1(CR1), decay accelerating factor (DAF), factor H (fH) and membrane cofactor protein (MCP) as depicted in Fig.2.1 (McKenzie et al, 1992). VCP consists of 4 short consensus repeats (SCRs). In order to examine the properties of VCP, a recombinant truncated VCP (VCPt) was previously engineered and consisted of only the last 3 SCRs (Smith et al., 2000). VCPt lacks the complement inhibitory activity, but retains only the heparin binding activity of VCP.

VCP inhibits the classical pathway of complement activation by binding C3 and C4 and acting as a cofactor for factor 1 cleavage of C3b and C4b (Sahu et al., 1998). The alternative pathway of complement activation is inhibited through the same mechanism, resulting in the cleavage of C3b into iC3b, therefore preventing the formation of the alternative pathway C3 convertase (Sahu et al., 1998). Additionally, VCP has the ability to bind heparin and heparan sulphate proteoglycans further contributing to its' anti-inflammatory function.

For instance, VCP binds to heparin granules in mast cells, and in so doing may possibly allow tissue persistence over a longer period of time (Smith et al., 2000; Jha et al., 2003). Studies have shown that binding to heparin-like molecules on the surface of endothelial cells can reduce chemotactic migration of leukocytes (Hicks et al., 2002). VCP is able to inhibit antibody binding to major histocompatibility complex (MHC) class I molecules on human endothelial cells (Isaacs et al., 1992). It has also been shown to block interactions of pig aortic endothelial cells, neutrophils and natural killer cells preventing cell death (Al-Mohanna et al., 2001). VCP remains fully active

after adverse exposure to high temperatures, freeze-thawing events, and lyophilisation. It has a shelf-life of 30 days at room temperature (the longest time point tested) (Smith et al., 2002). These findings demonstrate advantages for shipping and storage conditions. Furthermore, VCP has been shown to tolerate extreme acidic conditions and is possibly very important for oral administration, considering the acidic gastric environment (Smith et al., 2002). These studies suggest that VCP retains functionality after adverse conditions and is therefore robust and stable. Clearly then, evidence as cited above for the multi-functionality and stability of this immunomodulatory protein, makes the use of VCP promising in the treatment of complement-mediated diseases such as xenograft transplant rejections (Anderson et al, 2002; 2003), head injury (Keeling et al., 2000), Alzheimer's disease (Daly and Kotwal, 1998), peritonitis (Scott et al., 2003) and spinal cord injury (Reynolds et al., 2003; 2004).

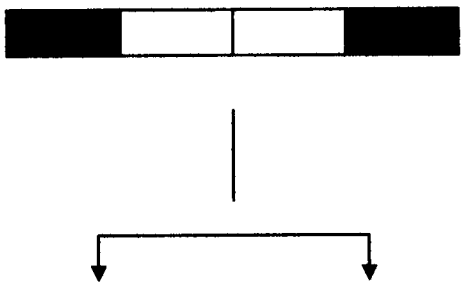


Fig.2.1. Schematic representation of vaccinia virus complement control protein (VCP). Each block represents a short consensus repeat (SCR) or a Sushi-domain. Blue blocks show heparin binding sites (SCR 1 and 4). VCP is structurally similar to human complement 4b binding protein (C4b-BP) and is functionally similar to complement receptor 1(CR1), decay accelerating factor (DAF), factor H (fH) and membrane cofactor protein (MCP).

2.2. MATERIALS AND METHODS

2.2.1. OPTIMISATION OF VCP EXPRESSION IN *Pichia pastoris*

Pichia pastoris is a methylotrophic yeast that was developed as a host system for DNA transformations (Cregg et al., 1985).

VCP has been previously cloned into *Pichia pastoris* cells, using the secretory expression vector pPIC 9 (Smith et al., 2000). In brief, the vector was introduced at the EcoR and Not I sites and the VCP gene was cloned in frame with the initiation codon of the alpha signal sequence. (refer to appendix A for pPiC9 vector map). The *Pichia* cell line utilises a metabolic pathway that employs methanol as a carbon source (Ellis et al., 1985). It uses the promoter from the methanol-induced alcohol oxidase (AOXI) that controls the gene that codes for the expression of the enzyme alcohol oxidase. This enzyme catalyses the oxidation of methanol to formaldehyde. Expression of AOXI gene is tightly regulated and induced by methanol. The clones were maintained on minimal methanol histidine (1.34% yeast-nitrogen base (YNB), $4x10^{-5}$ biotin, 0.4% histidine and 0.5% methanol) plates in a 30°C incubator. Two different growth media were tested for optimal expression (see appendix B for preparation of buffers, media and solutions).

A single colony of *Pichia pastoris* containing the VCP gene insert was first incubated in 100ml of buffered-minimal glycerol media (BMGY; 100mM potassium phosphate pH 6.0, 1.34% YNB, $1x10^{-5}$ % biotin, 1% glycerol) or yeast peptone dextrose (YPD:1% yeast extract, 2% peptone,2% dextrose) for 48 hours at 30°C and 220 rev/min shaking. After vigorous shaking, this starter culture with high cell density (determined by inspection) was then inoculated in one litre of BMGY or YPD and was allowed to grow for a further 48 hours. The cells were harvested by centrifugation in a Sorvall RC58 Plus centrifuge (Kendro, Connecticut U.S.A) for 30 minutes at 10 000 rev/min. The induction phase was then initiated when cells were resuspended in 250ml of a buffered minimal methanol media (BMMY:100mM potassium phosphate, pH 6.0, 1.34% YNB, $4X10^{-5}$% biotin) containing 4% methanol, and was then incubated in similar conditions for 48 hours. The VCP-supernatant was harvested by centrifugation for 20 min at 10 000 rev/min. In order to analyse

expression levels and to determine the optimal time post-induction to harvest, the supernatants were collected at the following time points 24hr, 48hr, 72hr and 96hr.

Test Sample Preparation

Test and control samples were prepared in individual tubes by adding to each tube 16.5µl sample and 7.5µl of loading buffer mix (26µl β-mercaptoethanol and 52µl loading sample buffer (Invitrogen, USA)). 5µl of low range protein molecular weight marker (Bio-Rad, USA) was used in every gel. By using a marker of known molecular weight, ranging from 14.4kDa to 97.0kDa, it was possible to estimate the molecular weight of VCP. All samples were incubated in a water-bath for 5 minutes at 70°C before sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Sodium Doecyl Sulphate Polyacrylamide Gel Electrophoresis

VCP was first detected by Coomassie Brilliant Blue stain binding non-specifically to proteins on a sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE), before further downstream processing commenced.

A 5%-12% polyacrylamide gel was cast. This was chosen because VCP with size 35kDa will separate at this acrylamide concentration (Sambrook and Russell, 2001). SDS is an anionic detergent that denatures proteins without breaking peptide bonds and coats the protein with a negative charge. The stacking gel contains Tris-Cl ions and the resolving gel contains Tris-glycine ions. The ionisation of glycine occurs at a high pH in the resolving gel. An applied electric field causes the polypeptides to accumulate and stack at the boundary of the stacking and resolving gel. The glycine ions migrate through the stacked polypeptides and travel through the resolving gel immediately behind the chloride ions. In this way, the denatured proteins are resolved according to their size and shape. The stacking gel consisted of 2.1ml distilled water; 50µl of 30% acrylamide mix (29.2g acrylamide, 0.8g N,N' methylene bisacrylamide); 380 µl 1.0M Tris pH 6.8; 30µl of 10% sodium dodecyl sulphate (SDS) and 10% of freshly prepared ammonium persulphate (APS). The resolving gel consisted of 1.6ml distilled water; 2ml 30%

acrylamide mix; 1.3ml 1.5M Tris pH 8.8; 500µl 10% SDS and 10% fresh APS was prepared in a glass beaker. Free radicals are generated by APS.
The glass plates and spacers were cleaned with 70% ethanol before being assembled in the Bio-Rad gel apparatus. TEMED, a catalyst, was added shortly before being poured into the assembled gel apparatus in order to accelerate polymerisation of acrylamide and BIS-acrylamide. The gel comb was inserted to make wells and once the gel solidified the comb was removed and samples were loaded in each lane. 1X NuPage SDS running buffer (Invitrogen, USA) was used for electrophoresis at 120V.
Electrophoresis lasted one and a half hours, after which the polyacrylamide gel was carefully removed from the plates. Coomassie Brilliant Blue R-250 dye was used to stain the proteins on the gel for 30 minutes on the Orbital shaker SO3 (Stuart Scientific, UK). The fixing of the protein is approximately proportional to the amount of protein on the gel (Sambrook, 2001). The excess dye was then allowed to diffuse from the gel by destaining with a high methanol mixture (454ml methanol, 75ml glacial acetic acid, made up to 1litre of distilled water). The gel was dried at 70°C for one hour on a Slab Gel Dryer (ThermoSavant, UK).

2.2.2. VCP PURIFICATION

After visualising VCP on the SDS-PAGE gels, all VCP-supernatants were pooled and then passed through a 10K Pellicon 2 cassette, a device that uses tangential flow for ultra-filtration (Millipore, Ireland). The filtration system was flushed with distilled water before the pooled supernatant was passed through the system via the feed tube. The filtrate was collected in an Erlenmeyer flask. The membrane was regenerated by rinsing with distilled water before recirculating 0.1M NaOH in the system for about 20 mins. The Pellicon 2 cassette was then stored in 0.05M NaOH at room temperature. As needed, 5ml of ultra-filtrated VCP was purified by injection, through a 0.22 µm filter unit, into a 5ml HiTrap heparin column (Amersham Pharmacia, Uppsala Sweden). The protein was eluted with NaCl ranging from 100mM NaCl to 1M NaCl using a AKTA prime purification system (Amersham Pharmacia, Uppsala Sweden) The protein was then concentrated using a Centriprep centrifugal filter device YM10 (Millipore, Ireland).

2.2.3. HAEMOLYSIS ASSAY

This assay was performed to evaluate the functional activity of VCP in haemolysis inhibition, as previously described (Kotwal et al., 1990) with a few modifications. This assay measures the ability of VCP to lyse 50% of a standardised suspension of sheep red blood cells coated with anti-RBC antibody. When this complex is exposed to complement in the human serum, it will activate the components resulting in lysis of the RBC and the release of haemoglobin. The degree of lysis is proportional to the concentration of total complement in the serum.The supernatant buffer was removed from 3ml of sensitised sheep RBC (Diamedix, USA) and was used as reaction buffer. All preparations were performed on ice. Serum was diluted in a 1:30 proportion with reaction buffer. Each reaction consisted of 75µl of RBC, 5µl reaction buffer, 15µl serum and 5µl sample to a final volume of 100µl. The positive control, excluded VCP and the negative control excluded VCP and serum. The control samples and 4 test samples were incubated for 1 hour at 37°C. After centrifugation at 13 000 rev/min for 1 minute, 150µl of supernatant was transferred to a 96-well plate and was read at wavelength, 405nm on an Anthos 2010 microplate reader (Salzburg, Austria).

2.2.4. PROTEIN ESTIMATION ASSAY

The Bio-Rad protein assay is a simple colorimetric assay for measuring total protein concentration. This assay is based on the Bradford dye-binding procedure (Bradford, 1976). The protein concentration was determined using a Bio-Rad protein estimation assay kit (Bio-rad, California U.S.A) and absorbances were read at 595nm on an Anthos 2010 microplate reader (Salzburg, Austria).

2.3. RESULTS

2.3.1. SDS-PAGE ANALYSIS

After a period of optimisation of selecting robust clones, a comparison between growth media, YPD and BMGY were analysed by Coomassie blue staining of VCP on a SDS-PAGE gel. By visual inspection, there were seemed to be no significant differences in expression levels when BMGY or YPD was used during the growth phase Fig.2.2.

The time point study was done to ensure that protein was expressed only when the induction phase commenced and to monitor protein expression levels. The time point study analysis of expression culture every 24 hours revealed that after 72 hours (Lanes 7 and 8) protein expression was clearly visible on a SDS-PAGE gel Fig 2.3.

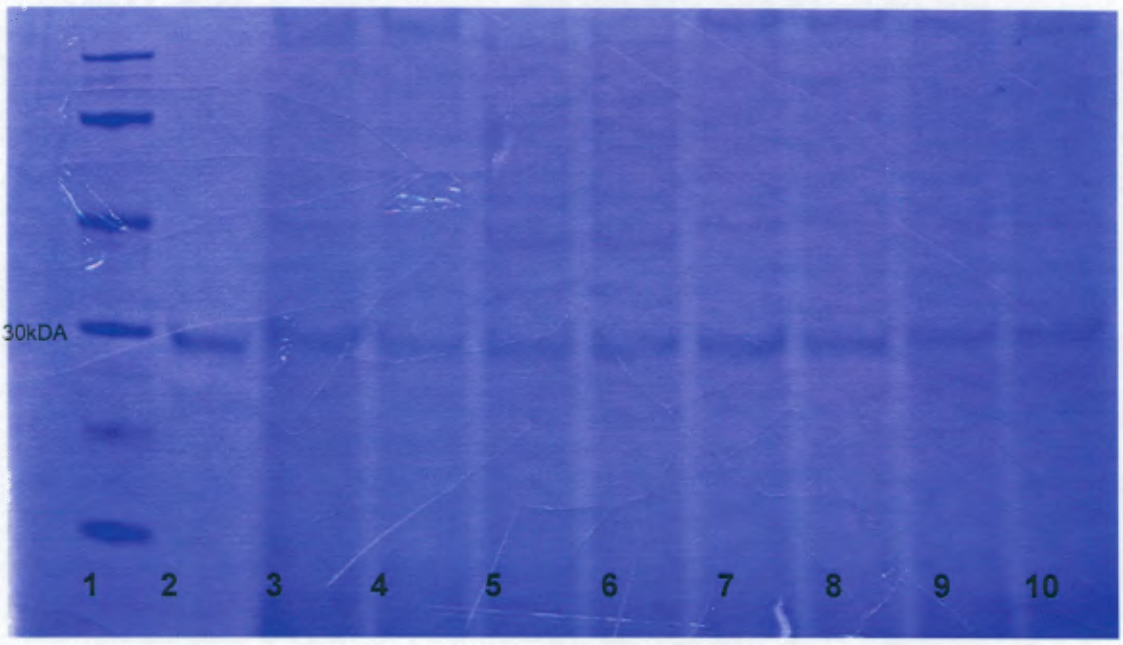


Fig 2.2 Expression of clones grown in two different media after following induction with 1% methanol. Lanes 1: Protein molecular marker ranging from 14.4 – 97 kDa. 2: VCP standard. 3: Clone 1 expressed after 72 hr in YPD. 4: Clone 1 expressed after 48 hr in YPD. 5: Clone 1 expressed after 72 hr in BMGY. 6: Clone 1 expressed after 48 hr in BMGY. 7: Clone 2 expressed after 72 hr in YPD. 8: Clone 2 expressed after 48 hr in YPD.9: Clone 2 expressed after 48 hr in BMGY.10: Clone 1 expressed 24 hr in YPD.

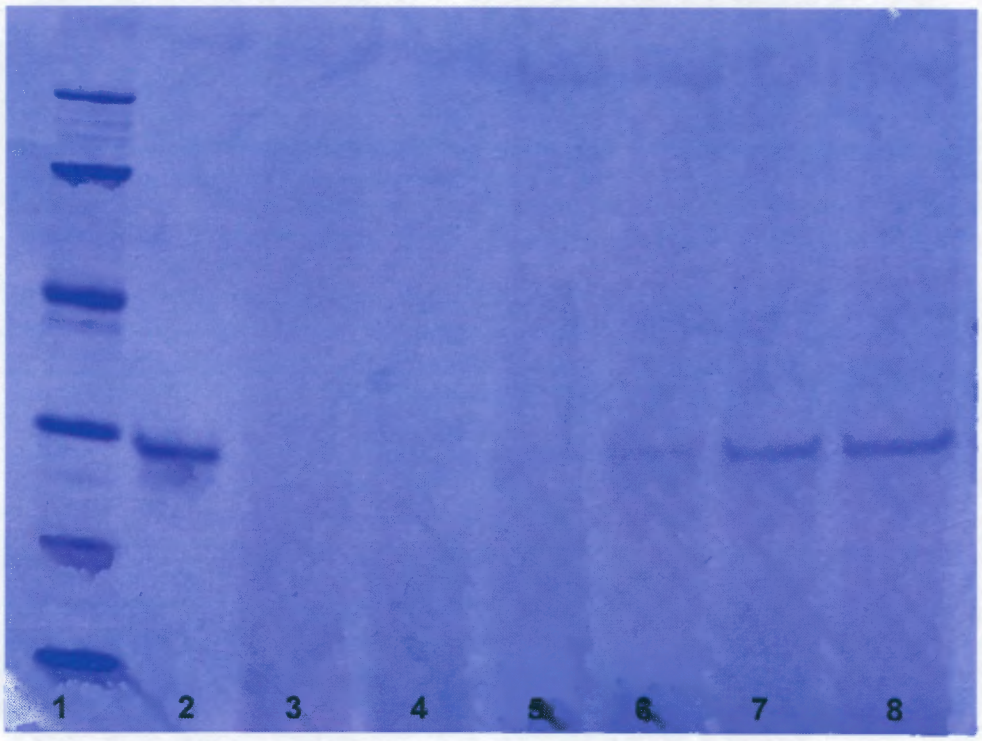


Fig 2.3 SDS-PAGE indicating time-point study of expression culture every 24 hours.
Lanes 1: Protein marker ranging from 14.4 -97kDa. 2: VCP standard. 3: Pre-induction sample.4: 0hr in BMMY media. : 24hrs BMMY .6: 48hrs in BMMY .7: 72hrs in BMMY .8: 96 hrs in BMMY.

2.3.2. HAEMOLYSIS ASSAY

The haemolysis assay was performed to determine the functional integrity of VCP. As expected there was no lysis in the negative control and absolute lysis in the positive control. The percentage of inhibition of the lysis of sheep RBC, an indication of VCP activity, was calculated for each of the 4 test samples using measurement readings at absorbance 405nm.This was calculated by dividing the difference of the sample reading from the negative reading by the difference of the negative reading from the positive reading. The percentage of inhibition for samples 1-4 ranged from 66% to 97%.

Table 2.1 Shows the evaluation results from the Anthos 2010 plate reader. The measurement filter was at 405nm and the units of measurement in optical density (OD).

Negative Control	Positive Control	Sample 1 VCP	Sample 2 VCP	Sample 3 VCPt	Sample 4 VCPt
0.028	0.308	0.247	0.214	0.242	0.300

2.3.3. PROTEIN ESTIMATION ASSAY

The protein estimation assay was done to establish the protein concentration in each of the samples following purification Fig. 2.4. The VCP sample that was chosen for use in this study was estimated to have a concentration of 1770µg/ml as indicated in Fig.2.5.

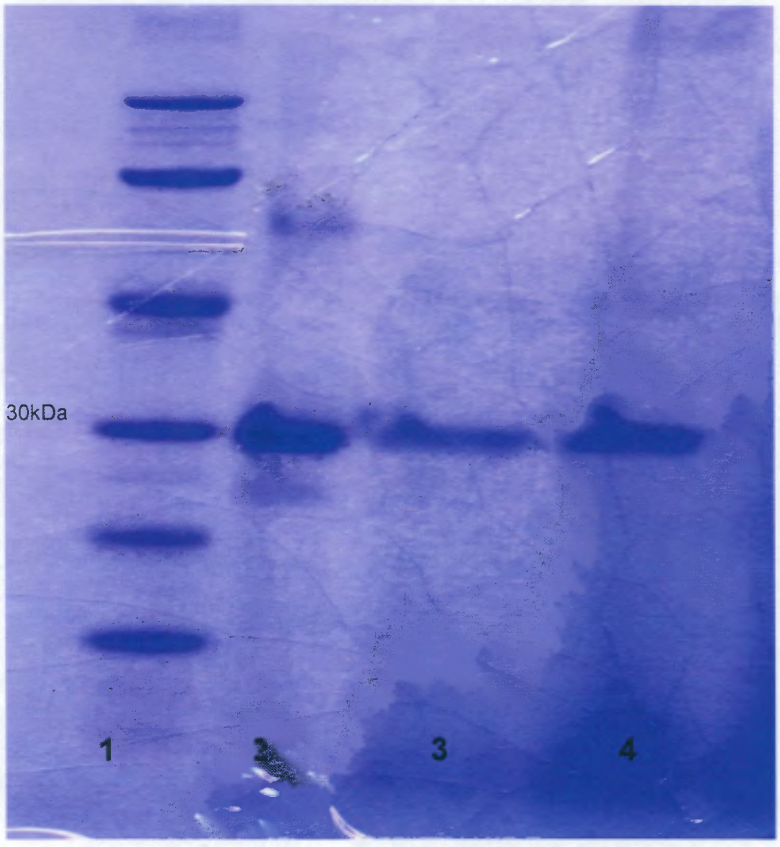


Fig 2.4. SDS- PAGE gel indicating control VCP and VCP expression in *Pichia pastoris*. Lanes 1: Protein marker ranging from 14.4kDa to 97kDa. 2. VCP control. 3. VCP expression. 4. VCP expression following purification.

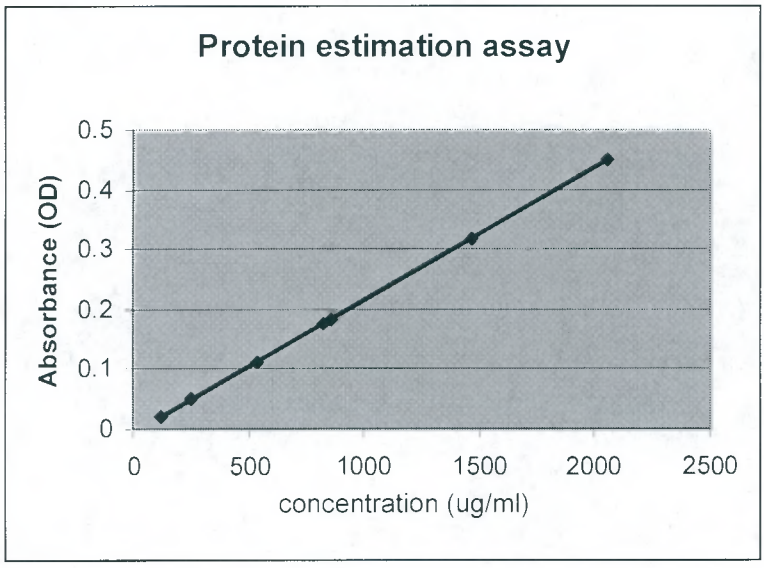


Fig 2.5. Evaluation results of Protein estimation assay using measurement filter 595nm and units of concentration µg/ml. The calculated linear correlation was 99.4%.

2.4. DISCUSSION

Functional VCP was successfully produced and purified in order to administer therapeutically in a mild and severe head-injury rat model and in a transgenic Alzheimer's disease mouse model. Although both full-length and VCPt was produced (only haemolysis results shown), we have decided to use full- length VCP as it retains both complement inhibitory and heparin-binding activities. Furthermore, a previous study by Hicks and colleagues (2002) has demonstrated that there were no significant differences between VCP and VCPt treatment following moderate TBI in rats. Interestingly, VCPt was still able to attenuate impairments in spatial memory, although lacking the first SCR. This suggests the ability to bind heparin-like molecules in the brain is potentially the principal mechanism for functional improvements.

Since VCP has properties that promote therapeutic potential due to its similarities to human complement homologues, it has been widely applied to many complement-mediated diseases. In cardiac xenotransplantation, treatment with VCP has been shown to block xenorejection (Anderson, 2002; 2003). An *in vitro* study demonstrated that VCP is able to block the activation

of the complement cascade by Aβ, thereby preventing the tissue damage caused by neuro-inflammation (Daly and Kotwal, 1998). Following experimental peritonitis in mice, VCP administration revealed significantly better bacterial clearance than saline-treated mice (Scott et al., 2003). The effects of VCP were also evaluated when injected into the spinal cord tissue following injury. Results reveal that VCP inhibits macrophage infiltration and reduces spinal cord deterioration (Reynolds et al., 2004).

CHAPTER 3
OPTIMISATION OF INJURY SEVERITY USING A LOCALLY PRODUCED FLUID PERCUSSION DEVICE

3.1. ANIMAL HEAD INJURY MODELS

In order to test the therapeutic effects of VCP in CNS injury, we required an animal injury model that closely resembles human TBI. Two types of animal injury models are currently used to mimic human TBI, one that causes focal damage to the brain (contusions or lacerations) and diffuse injury; the other from shearing of tissue as a result of the inertial force present at the moment of impacting on the brain tissue.

Models that mimic focal injury to the brain, such as contusions or lacerations include devices that use gravitational force, rotational acceleration forces, pressurised air and also trephination coupled to the impact of fluid on the brain. The weight drop closed head injury, rigid indentation injury and fluid percussion injury models all mimic focal damage of the brain but are also associated with concussive events.

The weight drop closed head injury device is an easy and fast to use model that uses gravitational force of a free falling weight to induce injury (Shapira et al., 1988). The height and the mass of the weight allows for variation in severity of the injury. The energy delivered to the skull is directly proportional to the severity of the lesion. The major drawback is that there is no control of the number of skull fractures produced, especially, when severe injury is subjected because the vertex of the rat skull is extremely thin, making it more susceptible to fracture easily (Tang et al., 1997a) The main advantage is that when mild injury is induced, brain-damage is diffuse and no local lesion or contusion in the brain is observed (Tang et al., 1997a). The use of weight drop injury device in rodents to mimic concussive-like brain injury in humans has shown injury characteristics similar to those found in humans. These include neurobehavioral depression, an acute brain oedema and chronic learning and memory deficits with no motor disability (Tang et al., 1997b).

Controlled cortical impact injury (CCI) employs a pneumatic impactor that involves the use of pressurised air as the source of mechanical energy for the generation of head injury (Lighthall, 1988; Smith, 1995). This technique provides greater control of time, velocity and depth of brain deformation than

the free-falling weight (Laurer, 2000). The CCI technique is the preferred model to mimic focal-type damage. Cortical contusions with intraparenchymal petechial haemorrhages are accompanied by epidural and or subdural haematoma formation. This is achieved by increasing the velocity of air and hence degree of brain distortion (Lighthall, 1988).

The fluid percussion brain injury (FPI) model is currently the most popular method for producing TBI. Injuries induced by the lateral (parasagittal) fluid-percussion in the rat closely resemble the features of head injury in humans and this method is therefore best suited for studying the pathophysiology of TBI. The fluid percussion device (FPD) produces a pulse (21-23 ms) of increased intracranial pressure with displacement and deformation of neural tissue. This model has been shown to produce mild to severe injury that is quantifiable and reproducible (see Table 3.1) (McIntosh et al., 1989). Furthermore, when moderate injury in rats was induced, both focal cortical contusion and diffuse subcortical neuronal injury was demonstrated, making the injury extent comparable to human head injury. In these regions the loss of neurons are evident in the ipsilateral cortex and hippocampus 12 hours after injury. This time-period provides opportunities for therapeutic intervention, in which the progression of pathological effects may be blocked or regenerative processes may be promoted (Hicks et al., 1996). Since this was the first time to use a locally produced FPD, it was necessary to calibrate the FPD according to the parameters encountered in the literature (see Table 3.1) and subsequently characterise the levels or intensities of FPI.

Table 3.1. Indicates the varying definitions of injury severity using a FPI device.

Author/Year	**Intensity/Severity**	**Intra-cranial pressure (atm)**
McIntosh *et al* (1989)	Low-grade Moderate High-grade	0-1.0 1.5-2.0 2.5-3.6
Prins *et al* (1996)	Mild Moderate Severe	1.35-1.45 2.65-2.75 3.65-3.75
Pierce *et al* (1998)	Severe	2.7-3.1
Knoblach *et al* (1999)	Severe	2.6-2.7
Sanders *et al* (2001)	Mild	1.4-1.5
Raghupathi *et al* (2002)	Mild	1.1-1.3
Hicks *et al* (2002)	Moderate	4.5

Once the parameters for the desired severity of the percussive injury were obtained, these reference settings were used to create the same degree of injury repetitively. It was also of importance to produce measurable and comparable levels of FPI as this would directly affect the findings of the study, especially since we are interested in the efficacy of VCP at a particular level of injury.

3.2. MATERIALS AND METHODS

3.2.1. COMPONENTS OF THE FLUID PERCUSSION DEVICE

The FPD used in this study was constructed by engineers at the University of Cape Town in the Faculty of Health Sciences mechanical workshop using the specifications detailed in a commercially available FPI device as a guide to construction. Consequently, the FPD used in this study consists of a closed hydraulic system that is activated by a falling pendulum. The pendulum striking force is adjustable either by altering the angle through which the pendulum falls or by adding weights to the pendulum. The applied pressure is accurately measured by a calibrated pressure transducer (mV/psi) coupled to an amplifier and oscilloscope to register the pressure signal. An event trigger switch activated by the swinging pendulum is coupled to a remote triggering device and oscilloscope, which is set to trigger on a positive or rising trigger pulse.

The hydraulic piston is attached by means of hydraulic tubing to the rat via a coupling in the form of an acutely implanted Luer- lock fitting cemented to the rat's skull. A high-pressure stainless steel T-manifold coupler permits transmission of the hydraulic percussion shockwave, to both the animal preparation and to the pressure transducer simultaneously. This arrangement enables direct recording of the actual pressure wave at the coupling to the animal. Once the desired parameters were established these settings for the FP injury were used to create the same degree of injury in the experimental animals used in this study.

3.2.2. PREPARATION AND OPERATION OF THE FLUID PERCUSSION DEVICE

To operate the FPD, the hydraulic cylinder was unlocked and freed from its support post and filled with distilled water, ensuring that no air bubbles were trapped in the cylinder, manifold or high pressure hose. With the stopcock in the open position, the filling procedure began by slowly pushing the piston in and out of the cylinder by hand to dispel any air bubbles trapped in the cylinder. The high pressure hose, with the attached stopcock was

inserted into a beaker of distilled water. The beaker of distilled water was placed at a higher point than the pressure transducer manifold to facilitate escape of any trapped air bubbles. After all the air bubbles were purged, the cylinder was filled to approximately 50 % capacity. The stopcock was then closed before being removed from the beaker of water. The cylinder was placed back into position in the FPD and secured to the support posts. The FPD was thus prepared for each experiment prior to coupling to the rat Fig.3.1.

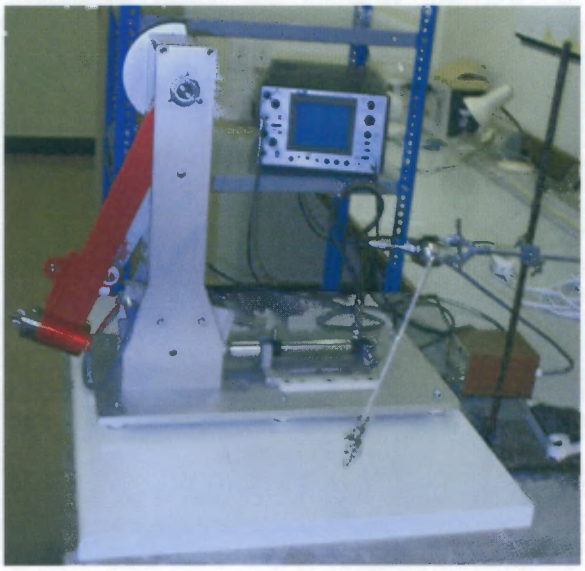


Fig.3.1. This picture shows the FPI device in our surgical laboratory. The lateral view of the FPI device indicates the pendulum in red, pistons and luer attachment assembly.

3.2.3. CALIBRATION OF THE FLUID PERCUSSION DEVICE

The pressure transducer converts hydraulic pressure in the closed hydraulic system into a voltage signal which is displayed on the oscilloscope. The triggering level was set by releasing the pendulum forward past the magnetic sensor trigger switch. The pulse wave is displayed as a continuous waveform on the oscilloscope from which the amplitude and pulse duration can be calculated.

The FPD was calibrated by incrementally adjusting the height of the pendulum swing, in order to quantify the percussion pressure. The angle at which the pendulum strikes the saline-filled piston varied from 26 to 40 degrees. The volts per division readings on the y-axis, displayed on the oscilloscope were recorded for each angle at which the pendulum was

dropped and subsequently the atmospheric pressure was calculated on the basis of a 5V output from the amplifier being equivalent to 300 pounds per square inch (psi). In order to convert the voltage and psi reading to atmospheres, the relationship of 1 atmosphere being equivalent to14.7 pounds per square inch was used.

3.2.4. STEREOTAXIC SURGERY

Rats were deeply anesthetised with Equithesin (100mg/kg) (intra-peritoneal) as indicated by a lack of corneal reflex and the absence of footpad-pinch withdrawal response. All surgical procedures were done under semi-sterile conditions were approved by the University of Cape Town Animal Ethics Committee. When fully surgically anaesthetised, the rats were immobilised in a stereotaxic frame.

A burr-hole (5mm dia.) was constructed in the cranium, centered 3 mm lateral to the sagittal suture and 4.5 mm posterior to bregma in the A-P plane, according to the rat stereotaxic atlas (Paxinos and Watson, 1998). The underlying dura was left intact. A Luer-loc hub was centralised over the exposed dura, and then cemented to the skull with dental cement. Following the hardening of the dental cement, the Luer attachment was then coupled to a calibrated FP device via the high-pressure hydraulic tubing pre-filled with isotonic saline.

A percussion shock was then applied via the FP device. Shortly before the percussion shock or injury, rats were still deeply anesthetized. After the percussion shock, the dental cement was carefully removed and wounds were carefully sutured. The animals were allowed to recover from the effects of surgery in separate cages, while being frequently checked.

3.2.5. HISTOLOGY

Rats were once again anesthetised with Equithesin before being transcaridially perfused with physiological saline and then fixed with 10% buffered formalin (appendix B8 and B15). The fixed brain was retrieved by placing the skull in the stereotaxic frame. The scalpel blade was placed between the cerebral and cerebellar cortices so that the cut completely

transected the brain at the posterior level and then anterior level. By visual inspection, it was ensured that the injection cannula was within the boundaries of the two cuts. The brain was stored in 10% buffered formalin and was sent for tissue processing. The brain tissue was sectioned and stained with Haemotoxylin and Eosin (H&E).

3.3. RESULTS

3.3.1. EVALUATION OF SURVIVAL FOLLOWING FLUID PERCUSSION INJURY

Since the FPD was used for the first time in our laboratory with the intention of measuring therapeutic effects of VCP, it was important to evaluate the percussion shock administered in relation to the survival of the animal. We chose to commence with a high-grade or severe percussion and therefore set the pendulum to the upper limits of tolerance.

Ten rats were used in this preliminary study. Four rats were first subjected to a most severe percussion at the pendulum angle of 40° (which correspond to 4 atmospheres) before the strike-angle was reduced to 33°, 30° and 26° angles, respectively (see Table 3.2. for corresponding pressure values in atmospheres). Four of the rats that were subjected to percussion greater than 3.0 atmospheres died immediately after impact of fluid on the brain. All the other six rats that were subjected to FPI equal to or less than 3 atmospheres survived. It was therefore evident that the most severe injury that could be caused by the FPD without fatality was at 3.0 atmospheres.

Table 3.2 Oscilloscope readings in millivolts (mV) and the corresponding intracranial pressure measured in atmospheres

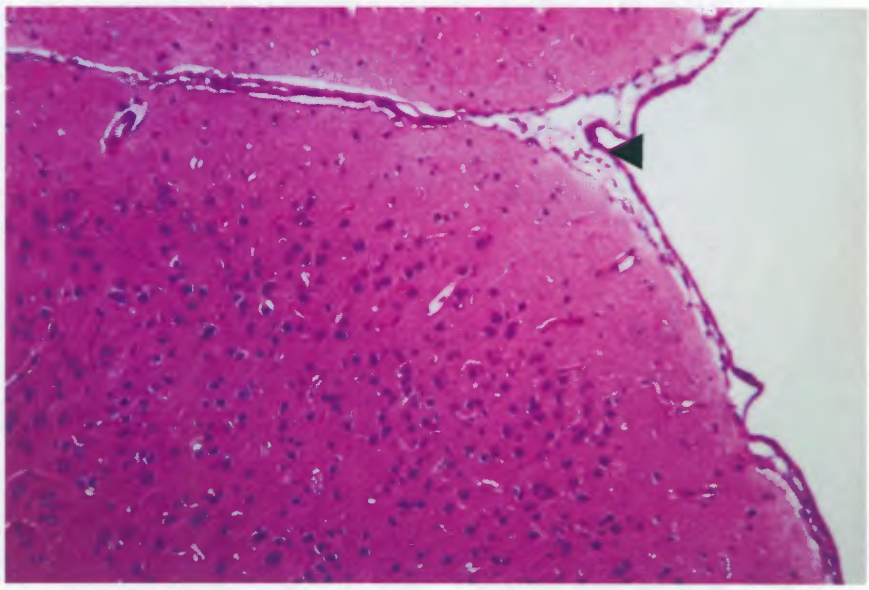
Angle of Strike	Oscilloscope reading (mV)	Atmospheres (atm)
26°	250	1.1
	400	1.8
30°	500	2.2
	600	2.7
33°	650	2.9
	700	3.0
	800	3.5
40°	900	4.0
	1000	4.4
	1200	5.3

3.3.2. HISTOLOGICAL EVALUATION OF INJURY

The brains of rats receiving various levels of injury intensities were removed after fixation by means of transcardial perfusion and cut serially at a 3 micron thickness. Sections were stained with H&E.

At varied injury intensities, striking differences in rat cortices were evident as shown in Fig. 3.2. Microscopic examination also revealed neutrophils in only the injured cortex after a percussion shock of 1.1 atm. In percussion injury of 3.0 atm, gliosis, macrophages, neovascularisation and oedema were evident on the ipsilateral cortex Fig 3.3.

A



B

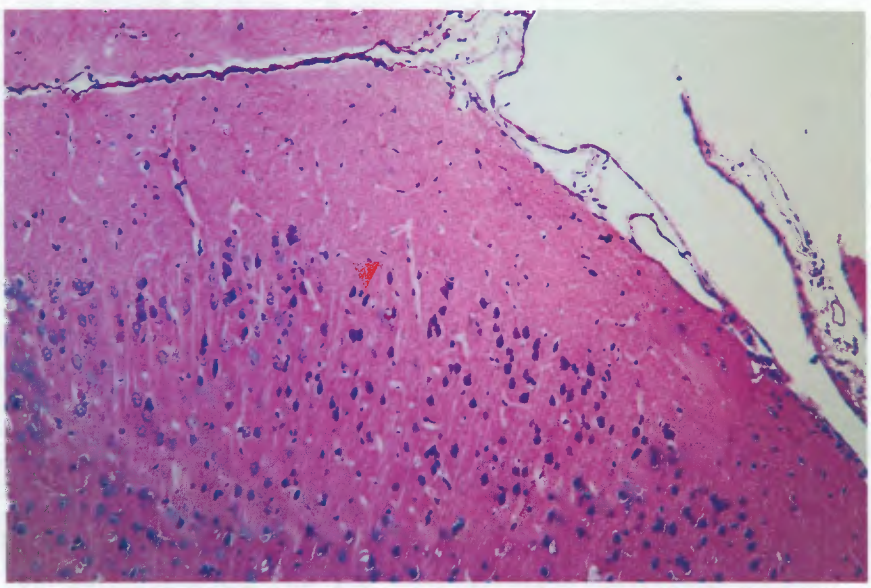
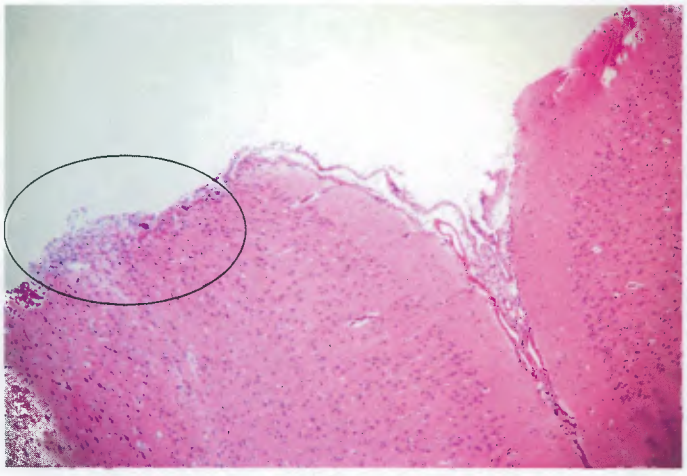


Fig 3.2.Coronal brain sections of Haematoxylin and Eosin Staining of rat cortices at 10X magnification.
Panel A: Cortex of uninjured rat showing unaffected or normal histology of the brain.
Panel B: FP-injured rat cortex at 1.1 atm (mild) and few neutrophils evident in view.

A



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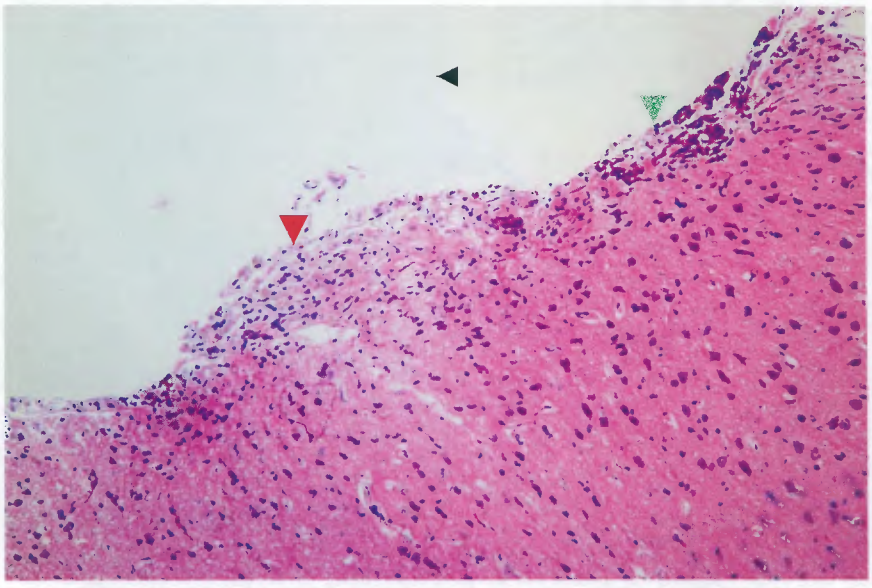


Fig 3.3. Haematoxylin and Eosin Staining of rat cortices. Panels A: FP-injured rat cortex at 3.0 atm (severe) magnification at 4X.
Panel B: At higher magnification (10X) gliosis, macrophages, neovascularisation and oedema are evident. Red arrow indicates oedema. Green arrow indicates blood vessel formation.

3.4. DISCUSSION

It was shown that with increasing intensity of percussion shock a rise in mortality occurred in Wistar rats. 40% of the rats that received an injury greater than 3.0 atm. died immediately after percussion. 60% of rats that received percussion less than 3.0 atm survived for the duration of the experiment (2 weeks). Rats that received a percussion of 1.1 atm survived to a pre-determined experimental time of 3 months.

Other research groups also established that mortality rates rised with increasing intensity of percussion (Prins et al., 1996; McIntosh et al., 1989). Prins and co-workers showed that in adult Sprague – Dawley rats the mortality rate increased from 0-55% in mild to severe injury. Rats that underwent a percussion of less than 1atm survived over a 4 week post-injury period (McIntosh et al., 1989). 10% of animals died within 24h of injury after receiving a percussion shock of 1.5-2.0 atm. 60% of animals that had undergone a percussion of 2.5-3.6 atm had died within one week post-injury. This has important implications for the interpretation and comparison of results from various studies. In this study, the severity of FP–induced injury compares to that first described by McIntosh and colleagues (1989), where they classified low-grade injury as being 1.0 atm; moderate as 1.5-2.0 atm and severe injury as 2.5-3.6 atm.

Post-injury the cortical damage caused by the locally produced FPD was confined to the ipsilateral cortex. Furthermore, mild TBI showed neutrophil influx and a severe TBI showed gliosis, macrophage infiltration, neovascularisation and oedema formation as shown in Fig 3.3. Similarly, McIntosh and colleagues found that haemorrhage, cavitation and vascular damage were greater at a higher magnitude of injury.

A CNS injury of 1.4-1.5 atm showed mild swelling of the cortical axons and the damage caused by percussion was limited to the injury site (Sanders et al, 2001). With lateral percussion injury of 1.1 - 1.3 atm, apoptotic cell death was observed in the cortex and sub-cortical white matter ipisilateral to the site of impact (Raghupathi, 2002).

For the purposes of this study the following gradings of severity will be used mild injury 0 - 1.1 atm; moderate injury 1.8 - 2.2 atm and severe injury as 2.7 - 3.0 atm.

CHAPTER 4
ADMINISTRATION OF VACCINIA VIRUS COMPLEMENT CONTROL PROTEIN IN A MILD HEAD INJURY MODEL

4.1. MILD HEAD INJURY

Mild traumatic brain injury (TBI) is one of the most common neurological disorders (Alexander, 1995). The vast majority of head injuries (75-90%) are clinically classified as mild (Langfitt and Gennarelli, 1982; Kraus and Nourjah, 1988). Ten to fifteen percent of patients remain symptomatic with postconcussive symptoms enduring for a year or longer (Chamelian and Feinstein, 2004). These symptoms include cognitive deficits, emotional distress, behavioral disturbances and dizziness (Chamelian and Feinstein, 2004; Zohar et al., 2003; Hicks et al., 1999). Due to the subtle symptoms following mild TBI, rates of hospital admission are low and little is known of the short-term behavioral and cognitive deficits that may arise and persist. Animal models are often used to mimic cognitive and behavioral events produced by human traumatic head injuries, however, mimicking of mild TBI poses a challenge since cognitive changes are subtle. Cellular changes are more easily distinguishable. Studies have shown that after mild TBI in rats, a breakdown of the blood-brain barrier (Tanno et al., 1992); microglia and macrophage activation (Aihara et al., 1992); apoptotic cell death (Raghupathi et al., 2002) and alterations in neutrophil expression in the hippocampus (Hicks et al., 1999) are evident. Complement components contribute to inflammatory injury in a variety of tissues, including the central nervous system (CNS). Evidence of elevated levels of complement components in the cerebrospinal fluid (CSF) and accumulation of C3 at the site of TBI in patients may implicate the contribution of complement to secondary damage caused by inflammation (Kossmann et al., 1997). More recent evidence demonstrates the critical role that C3 and C5 play in TBI when secondary damage following acute TBI in C3 and C5 deficient mice is significantly reduced (Sewell et al., 2004). This supports the role of a C3 inhibitor as a candidate for modulation of inflammation and hence the use of VCP.

4.2. MATERIALS AND METHODS

4.2.1. Subjects

A total of 24 Wistar rats (250g-275g) were each randomly assigned to one of four groups: FPI + VCP treated (n=6), sham + VCP treated (n=6), FPI+ saline treated (n=6) and sham +saline treated (n=6). All animals were subject to testing in the Morris Water Maze (MWM) and a battery of neurological tests.

4.2.2. The Morris Water Maze

The Morris Water Maze (MWM) consists of a circular pool filled with water. It is made of translucent white plastic and is 1.67 metres in diameter and 67 centimetres in height. The pool is placed on and supported by a steel stand that is 60cm above the ground. The water was made opaque with tempera paint so that the platform was hidden. The water temperature was between 21°C and 24°C. If the water temperature was below 21°C a heating element was placed in the pool until the temperature rose to 21°C.

The colourless perspex platform is hidden from view by being submerged in opaque water that covers the 30 cm long platform. The platform was placed 0.5m from the side of the pool and the position was fixed for each animal. A round platform (15cm in diameter) was used so that there was no preferred direction of approach by the animal. These dimensions of the platform were used so that the animals were able to rest on it.

4.2.3. The Extra Morris Water Maze Environment

Visible cues were positioned so that the rat had landmarks to orientate itself with respect to the platform and its relative position in the maze.

Four different posters were fixed onto the middle of the 4 room walls well above the level of the rim of the tank to serve as visual cues. The posters displayed various geometric shapes and objects, and all were visually distinct from each other. The room was illuminated by fluorescent lights. The camera was mounted on a lighting balance directly above the MWM.

The field of view was kept constant throughout the experiments. White fabric sheets were positioned on the ceiling, to act as diffusers, so that the reflected light did not interfere with video-recording. The video recordings on 8mm tapes were transferred onto VHS tapes. Once the MWM was visualised on the screen, it was divided into the respective quadrants (as indicated in Fig 4.1). The goal latency times were determined by tracking the animal in slow motion and scoring the number of seconds the animal spent in each quadrant (Smith et al., 1991; Keeling et al., 2000). Swim speeds and swim patterns were not recorded due to the unavailability of the tracking software package needed to determine sensorimotor function and random and spatial strategies. However, other means of determining sensorimotor function were used as fully described in section 4.2.9.

4.2.4. Cognitive Testing

In this study, spatial learning was evaluated using the Morris Water Maze hidden platform version (Morris, 1984). The animals were placed randomly at each of the four starting points (north, south, east, and west) into the water to avoid habituating the animal to a particular area of the pool as shown in Fig. 4.1.

The animals were released at these variable positions and were required to find the hidden platform with the aid of visual and spatial cues (markings on posters) positioned on the walls outside the maze.

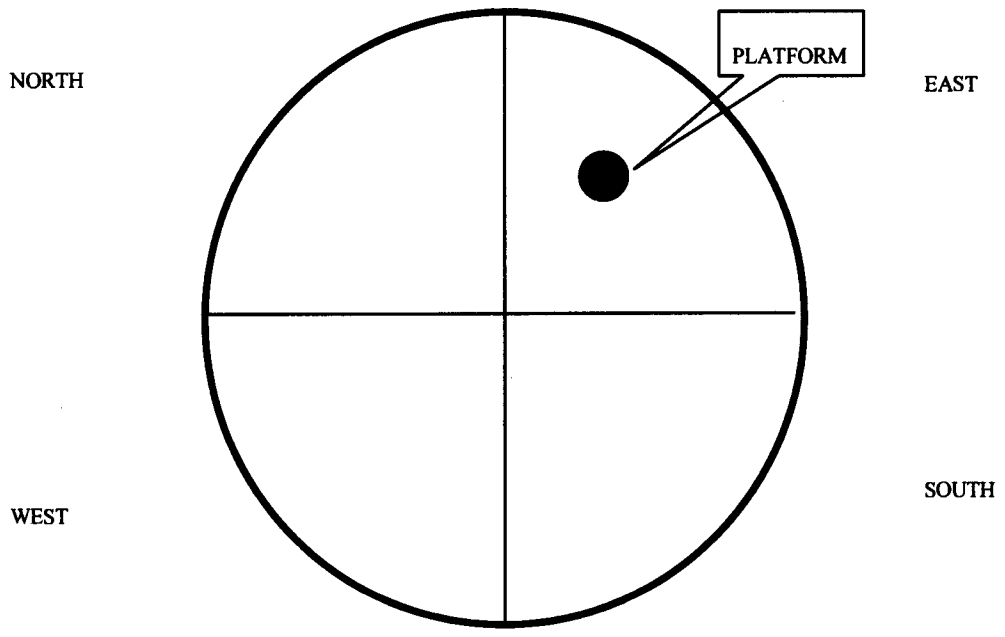


Fig 4.1. Diagrammatic representation of quadrant locations and platform position (black dot) in the Morris water maze.

4.2.5. Pre-injury Testing

Pre-injury training trials started 2 days before surgery. The training began in the afternoon of day 1 and then continued on the morning of day 2. Each rat was given eight trials in total with a 4 minute interval per trial each day. 120 seconds were allotted to find the hidden platform in trial 1 and a maximum of 60 seconds in subsequent trials. The time taken for the rat to reach the platform (goal latency) was measured. After finding the platform, the animal was allowed to remain on the platform for 10 seconds and then lifted out of the MWM. A rat that failed to find the platform within 120 seconds in trial 1 or 60 seconds in subsequent trials was classified as having the maximum goal latency for that trial. These rats were then placed on the platform and allowed to stay there for 15 seconds so that they may visualise the extra-maze posters that served as cues for orientation. After each acquisition trial, the rat was returned to a holding cage for 4 minutes and then re-introduced to the MWM.

At the end of the training session, the rat was dried and then returned to the home cage for 2 hours before undergoing surgery (Hicks et al., 2002).

4.2.6. Post-injury Testing

The injured rats were tested for memory retention 2 weeks post-injury. Eight acquisition trials were undertaken as described in the pre-training trial, using the same platform location as before. After memory retention testing, the platform was removed and a probe trial was performed. This probe trial that lasted for 60 seconds, was used to assess whether the animal exhibits a strong preference for the platform location or was using random search patterns to locate the hidden platform. This performance is determined by the amount of time in seconds, from the moment of release, the rat spends in all quadrants. The probe test is scored as the percentage of time rats spent in each of the four quadrants in a 60 second period. This tests the difference in spatial retention amongst the various groups of rats.

4.2.7. Data Analysis

The goal latencies were averaged for trials 1-8 on day 1 and day 2. The probe test was analysed with a one-way analysis of variance (ANOVA) to determine if there were differences between the groups. When significant differences were obtained, a post-hoc multiple comparison was done, using the Bonferroni test, to determine which groups were significantly different from each other (refer to appendix C3). Non-parametric analysis of the data was also undertaken in order to confirm the results revealed by parametric data analysis (that which assume normality of distribution). Non-parametric alternatives to the one-way ANOVA were performed. The Kruskal- Wallis ANOVA and Mann-Whitney U are similar to that of the one-way ANOVA except that they are based on ranks rather than means.

4.2.8. Surgical preparation and Fluid Percussion

The rats were anesthetised with Equithesin (100mg/kg) (intra-peritoneal). All surgical procedures were done under semi-sterile conditions. When fully surgically anaesthetised, the rats were immobilised in a stereotaxic frame. The dorsum of the head was shaved. A burr-hole (5mm diam) was constructed in the cranium, centered 3mm lateral to the sagittal suture and 4.5mm posterior to bregma in the A-P plane, leaving the underlying dura intact (Paxinos and Watson, 1998). A Luer-loc hub was centralized over the exposed dura, and then cemented to the skull with dental cement. Following the hardening of the dental cement, the Luer attachment was then coupled to a calibrated FP device via the high-pressure hydraulic tubing pre-filled with isotonic saline (as described in chapter 3). A Hamilton syringe containing VCP (10μl, 1.7mg/ml) was attached to a stereotaxic-injection frame and delivered 6.8mm into the rat cortex at the site of injury. Rats were then removed from the stereotaxic device; wounds carefully sutured and allowed to recover in separate cages from the effects of surgery. Animals were frequently checked. The control animals were treated identically, except they

received a sham-injury, defined as being attached to the fluid percussion injury device (as previously described above) but without the percussion. Fourteen days post surgery the animals were tested for spatial learning and memory by measuring goal latency in the probe test using a Morris Water Maze (MWM). These animals remained in separate cages for the entire duration of recovery. Food and water were freely available and cages were kept in a 12h light and 12h dark cycle.

4.2.9. Neurological evaluations

A battery of sensorimotor tests were performed by a trained observer who was blind to the injury status of the animals. Motor function was assessed by measuring the following tasks (1) resistance to lateral pulsion; (2) forelimb flexion response during suspension by the tail and (3) hind limb flexion, a response of the hind limb and toes when raised by the tail. Animals were evaluated daily for 14 consecutive days after injury for lateral pulsion (left and right); hind paw grip (left and right), forelimb and hind limb extension; visual placing and tactile placing. These animals were also evaluated for their ability to stand for 5 seconds on an inclined board, at 7 angles ranging from 20° to 50°. The injury status of the animal was scored using a modified scaling system (McIntosh et al., 1989). Animals were scored as follows: 20 (normal function), 15 (slightly impaired), 10 (moderately impaired) and 5 (severely impaired). To test for impaired ambulation, the animals were released from the center of the inner circle on a flat surface and were required to move a full body length to the outer circle within a minute. Animals were graded according to the time taken to leave the inner circle as follows 0-10s (normal), 11-25s (slightly impaired), 26-40s (moderately impaired) and 41-60s (severely impaired).

4.3. RESULTS

In the first trial of pre-training sessions, rats located the platform in an average time of 92 seconds on day 1 (appendix C1). The goal latency of the first trial on day 2 was 54 seconds. By the last trial on day 2 animals were able to find the platform in an average of 11 seconds. The overall shorter goal latencies on day 2 than on day 1 were taken to mean that rats were trained Fig. 4.2.

The rats were then tested in the MWM 14 days post-FPI and treatment to evaluate differences between groups (appendix C2). A one-way ANOVA was performed to evaluate differences between groups in each of the four quadrants (appendix C3). A significant difference [$F(3,23)= 3.91$, ($p<0.05$)] between groups was found in the amount of time spent in one of the incorrect quadrants Fig.4.3. Post hoc analysis revealed the FPI+VCP and FPI+ saline groups contributed to the significant effect in MWM testing [$F(1,10)=11.37, p<0.05$] (appendix C3). FPI+VCP group spent 9% of the time in one of the incorrect quadrants compared to the FPI+ saline group that spent 20% of the time in the incorrect quadrant Fig.4.4.

On the basis of neurological scores obtained, the majority of tests on motor function (left and right lateral pulsion and hindpaw grip) were statistically not significant (appendix C4 and C5), with the exception of the ambulatory and inclined plane gripping test Fig. 4.5 and Fig. 4.8. The ambulation test was performed daily for 14 consecutive days (appendix C6). From day 9 to day 14 significant differences [$F(3,20)=4.26, p=0.02$] were found between all groups as demonstrated in Fig.4.8a. More specifically, VCP-treated injured rats scored significantly better [$F(1,10)=5.8, p=0.04$] than saline-treated injured rats after day 9 as shown in Fig.4.8b. Twenty-four hours following FPI, the saline group showed a higher frequency of sliding from the inclined plane than did the VCP-injected group (appendix C7).

Analysis showed that the FPI+ saline group turned more frequently than did other groups Fig. 4.6. Injured rats showed more freezing behaviour than did sham rats. The FPI +saline group demonstrated more freezing behaviour than the FPI+VCP group (Fig.4.7).

A one-way ANOVA revealed a significant difference [F(1,10)=5.8,p=0.04] in ambulation between FPI + saline and FPI+VCP groups from day 9 to day 14 as shown in Fig. 4.8 (appendix C6).

4.3.1. Pre-injury goal Latencies

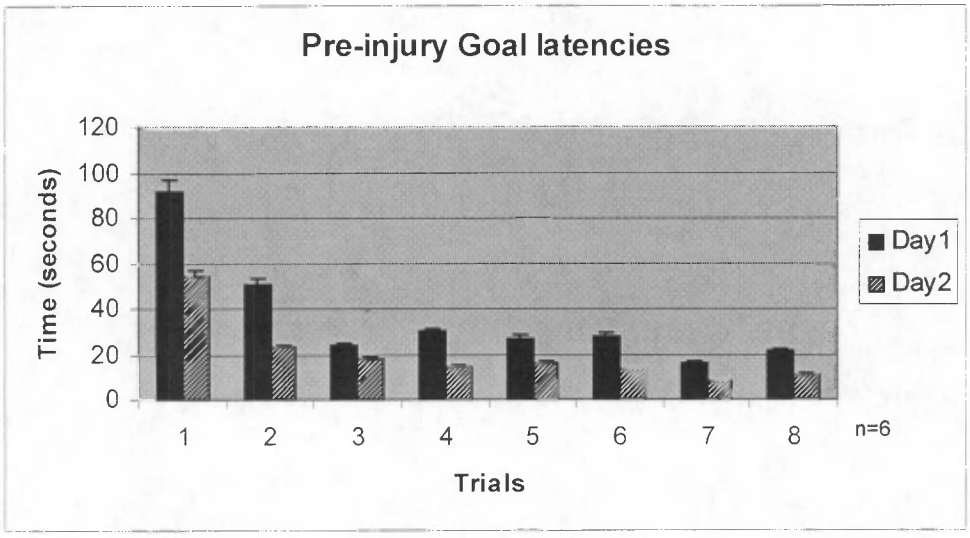


Fig.4.2. Animals were randomly assigned to groups after two days of training. Pre-training goal latencies on day 1, ranged from 92 to 21 seconds (i.e. from first trial to trial number 8) and similarly from 54 to 11 seconds on day 2. Error bars indicate SEM.

4.3.2. Probe Test Analysis

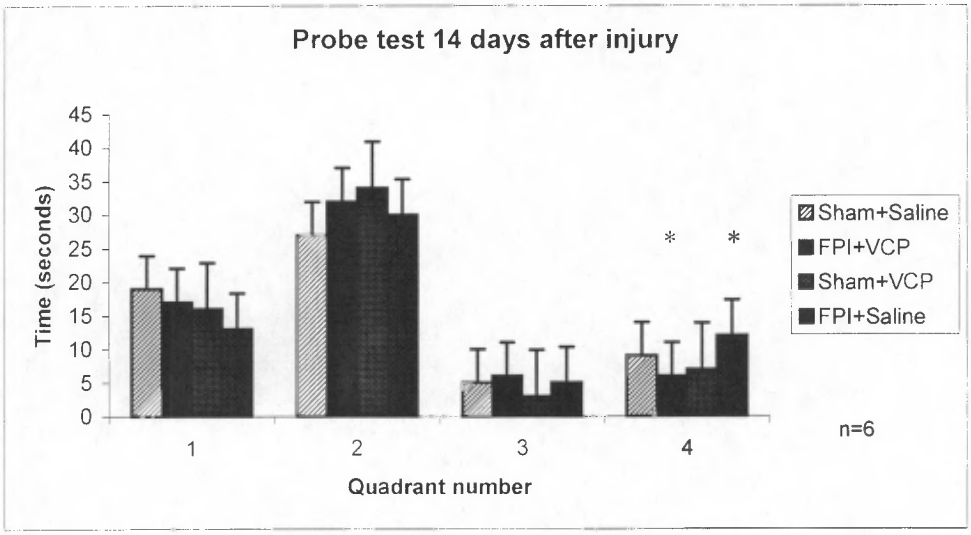


Fig.4.3. A one-way ANOVA demonstrated a significant difference (*p<0.05) in the amount of time the FPI+ saline compared to the FPI+VCP group, spent in the incorrect quadrant number 4 in a probe trial test 14 days post-injury. There were no significant differences in other quadrants. Error bars indicate SEM.

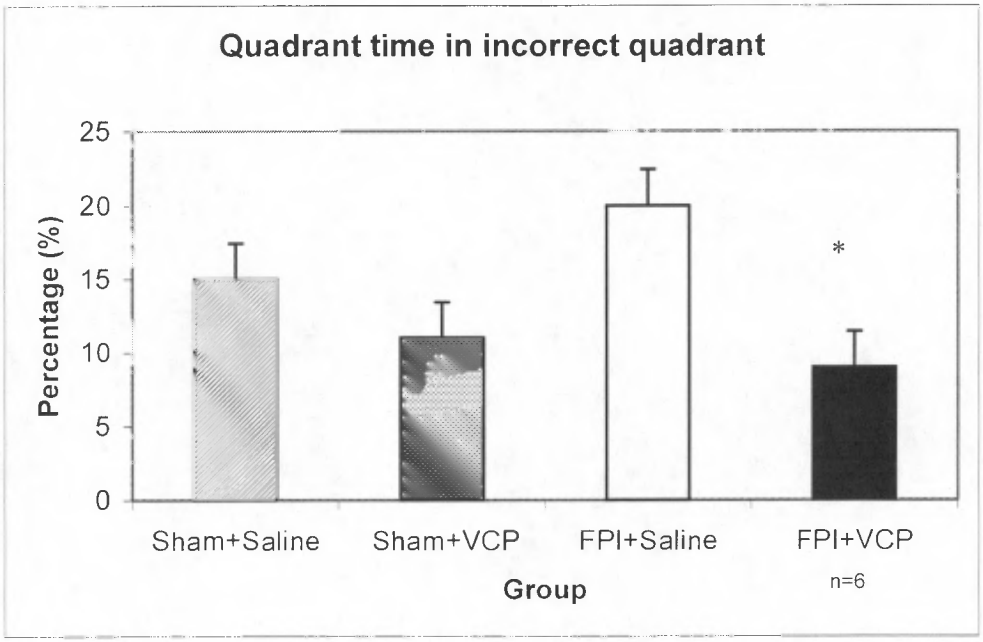


Fig.4.4. Fourteen days post-injury testing revealed that the FPI+ saline groups spent 20% of time in the incorrect quadrant (4). The FPI+VCP groups spent 9% of time in this quadrant (*p<0.05). Error bars indicate SEM.

4.3.3. Neurological Evaluations

When rats were placed on the angle-board, the number of times a particular behaviour such as sliding, turns and freezing were noted as shown in Fig.4.5-4.7. (other behaviours are noted in appendix C7). Since sliding, turns and freezing were the most frequent behaviours observed among groups, bar graphs were used to represent these data. No standard deviations or standard error means were indicated on the graphs as these data represent counts and not means.

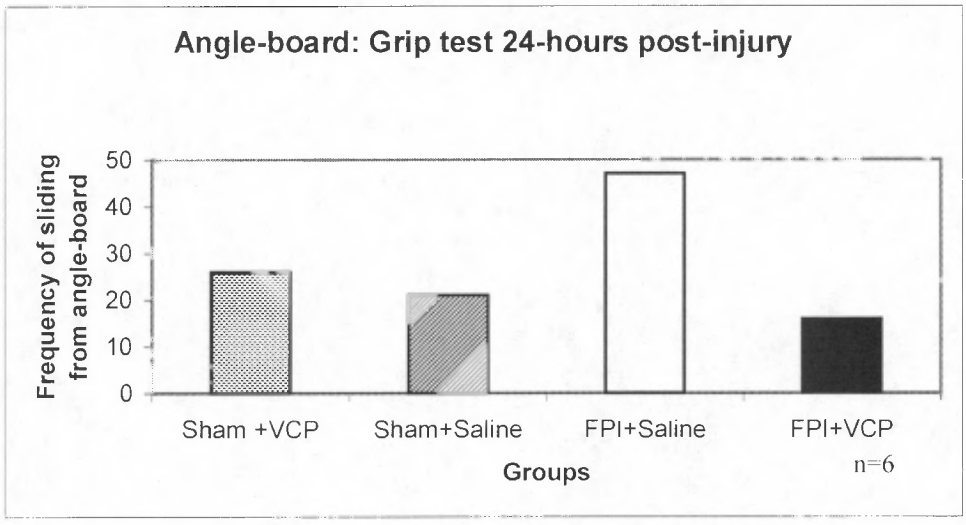


Fig.4.5. Sensorimotor impairment in grip was revealed by the rats' ability to stand on an inclined plane. The FPI+ saline group showed greater inability to grip than other FPI and sham groups.

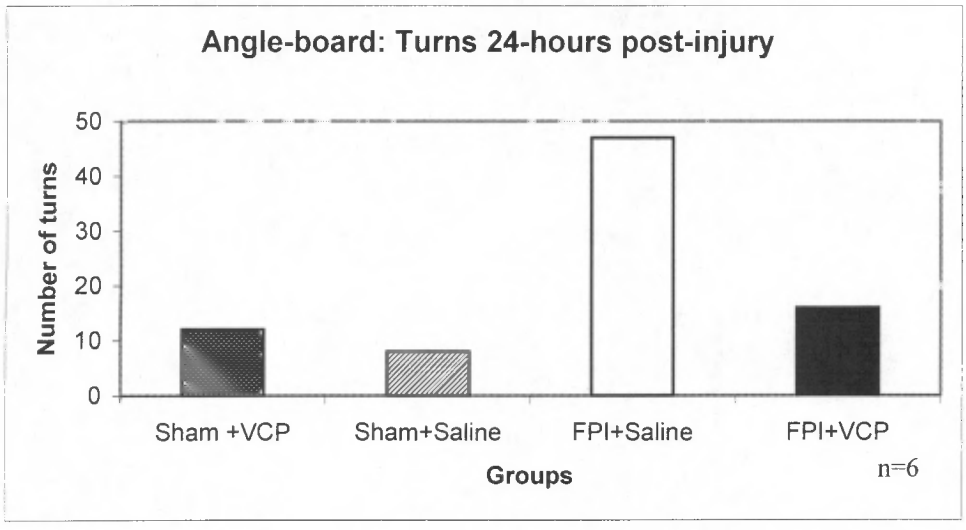


Fig.4.6. 24 hours post-injury, the sham groups and the FPI+ VCP group showed no significant differences in the frequency of turns. However, the FPI+ saline group turned more frequently on the inclined plane.

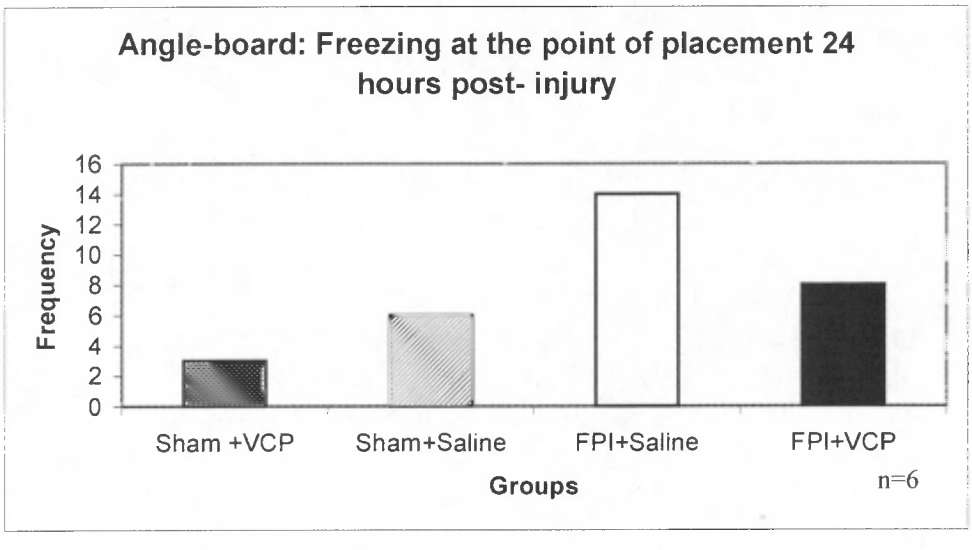


Fig.4.7.The effect of VCP on freezing behaviour at the point of placement demonstrated differences between sham and FP-injured rats. Freezing behaviour was more frequent in FP-injured animals than other groups.

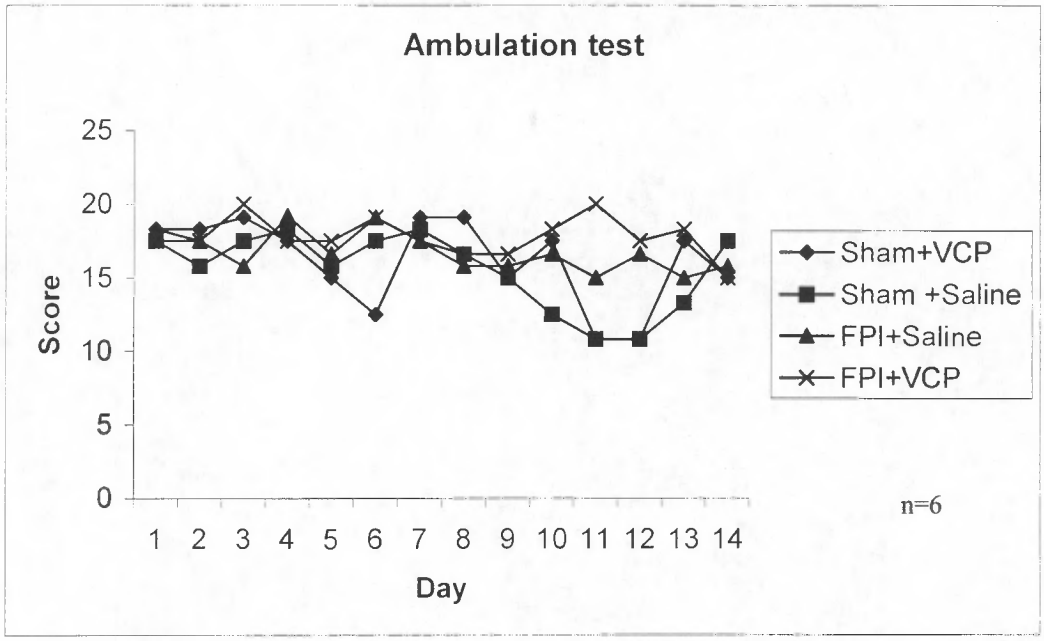


Fig.4.8 a. To test for the effect of VCP on ambulation the animals were released from the center of the inner circle on a flat surface and were required to move a full body length to the outer circle within a minute. Daily scores were recorded for fourteen consecutive days. A one-way ANOVA revealed significant differences ($p<0.05$) between groups after day 9.

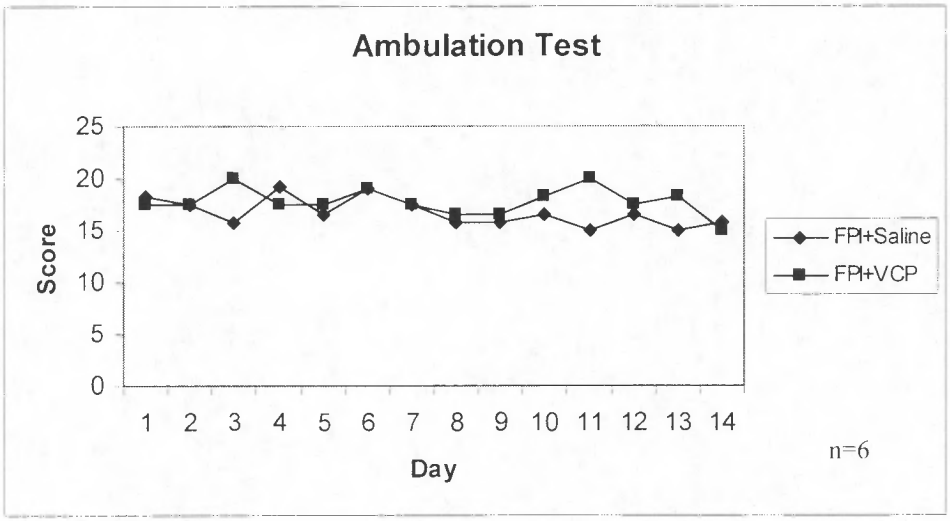


Fig 4.8.b A one-way ANOVA showed significant differences between saline-treated and VCP-treated injured rats. VCP-treated rats showed improvement in ambulation from day 9 to day 14 ($p<0.04$).

DISCUSSION

In this study we have evaluated the effect of VCP administration following mild (1.0 -1.1 atm) TBI in Wistar rats. Cognitive evaluation, using the Morris Water Maze (MWM), and sensorimotor function, using neurological testing, in the acute phase of recovery were measured. This mild FPI model posed to be challenging in the interpretation of the results since in mild TBI, only subtle changes in cognition are expected to occur and are therefore not easily distinguishable from sham injuries. The time spent by all of the four groups in the first 3 quadrants was not significantly different except for quadrant 4 as shown in Fig.4.3. All the groups spent a similar amount of time in the correct quadrant and this suggests that the strategy used was not based on random search patterns but spatial learning. In view of the fact that VCP injured rats spent significantly less time in the incorrect quadrant 4 than saline-treated injured rats, means that the VCP-treated rats are more orientated to the retention of spatial location of the platform. This suggests that VCP-treated injured rats used a spatial strategy and not a random strategy in finding the platform location. This we believe is the subtle change in cognition in a mild FPI model that was improved by VCP as shown in Fig.4.4. In a previous study using Sprague-Dawley rats, the administration of VCP in a moderate injury (4.5 atm) model revealed that spatial learning in the MWM after VCP injection was not enhanced over saline treated injured controls (Hicks et al., 2002). It is however, difficult to directly compare the results because of differences in the severity of injury, rat strain and administered dose of VCP.

In this mild head injury model, administration of VCP significantly influences sensorimotor function. An indication of sensorimotor impairment in grip was revealed by the rats' ability to stand on an inclined plane. As shown in the grip test (Fig. 4.5) the FPI saline-treated injured rats show greater inability to grip than the FPI VCP-treated injured group. FPI saline-treated rats turned more frequently on the angle-board than did the sham and FPI VCP-treated groups. The direction of turning behaviour was not noted; however, the frequency of turns in the saline-treated injured rats was three times higher than the VCP treated injured rats as a result of motor weakness. The injury

on one side will result in contralateral weakness and consequently affect contralateral turning of the animal.

In this study FPI rats that were given VCP demonstrated less freezing behaviour than FPI saline groups on the angle-board as shown in Fig. 4.7 Freezing is defined as the cessation of all body movement other than respiratory movements (Power and McGaugh, 2002). Altered cholinergic input of the basolateral amygdala in rats has been shown to influence freezing behaviour in an open field test (Power and McGaugh, 2002). VCP may offer protection of brain circuits involved in the expression of freezing behaviour.

In the ambulation test, the VCP-treated FPI group scored higher than the saline-treated FPI group after day 9 until day 14, which suggests that VCP may have a beneficial effect on motor control following a mild brain injury as shown in Fig.4.8a and 4.9b.

Taken together, these data show that in a mild head injury model, VCP has a positive influence neurological outcome and may offer some improvement in spatial learning and memory.

CHAPTER 5
ACUTE LATERAL VENTRICULAR ADMINISTRATION OF VACCINIA VIRUS COMPLEMENT CONTROL PROTEIN

5.1. THE BLOOD-BRAIN BARRIER AND HEAD INJURY

The blood-brain barrier (BBB) serves to prevent most substances of the bloodstream from entering the brain and spinal cord in order to maintain an ideal environment for optimal brain functioning. Transport of substances are anatomically prevented from entering the brain, by the tight junctions located between the cells, with the exceptions of essential lipid soluble substances such as oxygen, carbon dioxide, alcohol and steroid hormones (Sherwood, 2004). Other nutrients, such as glucose and amino acids enter the brain via membrane-bound carriers. Transport of larger molecules including insulin, leptin and iron transferrin through the cells is physiologically restricted by receptor-mediated endocytosis (Pardridge et al., 1985; Zhang and Pardridge, 2001). Because of the stringent selectivity of substances that cross the BBB, many drugs are unable to penetrate the barrier making treatment of CNS injuries and other brain disorders a challenging task.

The integrity of the BBB has been shown to disrupt in FP-injured rats (Tanno et al., 1992; Baldwin et al., 1996; Cernak et al., 2004). Furthermore, in patients with severe head injury, an increase of C3 and factor B levels in the cerebrospinal fluid (CSF) was found and proposed to be due to leakage across the BBB (Kossmann et al., 1997). Since the breach of the BBB facilitates the crossing of C3 into the CSF, this may provide an opportunity to block complement components in the CSF via VCP administration directly into the third ventricle (a brain region in which CSF is compartmentalised). According to a literature survey, it appears that this is the first time that the effect on cognitive processes (as measured by the MWM) is evaluated following an intraventricular VCP injection in head-injured rats.

5.2. MATERIALS AND METHODS

5.2.1. Subjects

Twelve rats were randomly selected and divided into two treatment groups (VCP or saline) after 2 days of training in the MWM (for detailed descriptions see chapter 4, sections 4.2.4-4.2.6). All 12 Wistar rats (250g-300g) were subjected to a severe head injury via a FPD.

5.2.2. Fluid percussion injury and VCP administration

All surgical preparations were carried out in a manner identical to those procedures previously described (in chapter 4, section 4.2.8). A severe percussion (2.7-3.0 atm) was then applied to all test animals, via the FPD (see description in chapter 3, section 3.2.2). Standard procedures were followed to ensure that all the animals were deeply surgically anaesthetised for the duration of the experiment. After the percussion shock, the dental cement, holding the luer attachment, was carefully removed. Immediately thereafter a second burr-hole was constructed at the following co-ordinates, 1.5mm lateral to the sagittal suture and 8.0mm posterior to bregma in the A-P plane according to the rat stereotaxic atlas as indicated in Fig.5.1 (Paxinos and Watson, 1998). An injection cannula consisting of a 29 gauge needle attached to plastic tubing was inserted in the lateral ventricle to withdraw CSF. Once inside the lateral ventricle a gentle suction was applied in order to withdraw CSF into the plastic tube to verify correct placement of the injection cannula. VCP (10μl, 1.7mg/ml) or saline was then administered using a Hamilton syringe attached to a stereotaxic injection device. Rats were then removed from the stereotaxic device and wounds were carefully sutured and checked regularly until they recovered from the effects of anesthesia. Animals were housed in separate cages. Food and water were freely available and rats were kept in a 12h light and 12h dark cycle. Two days post-surgery rats were tested for spatial learning and memory in the MWM.

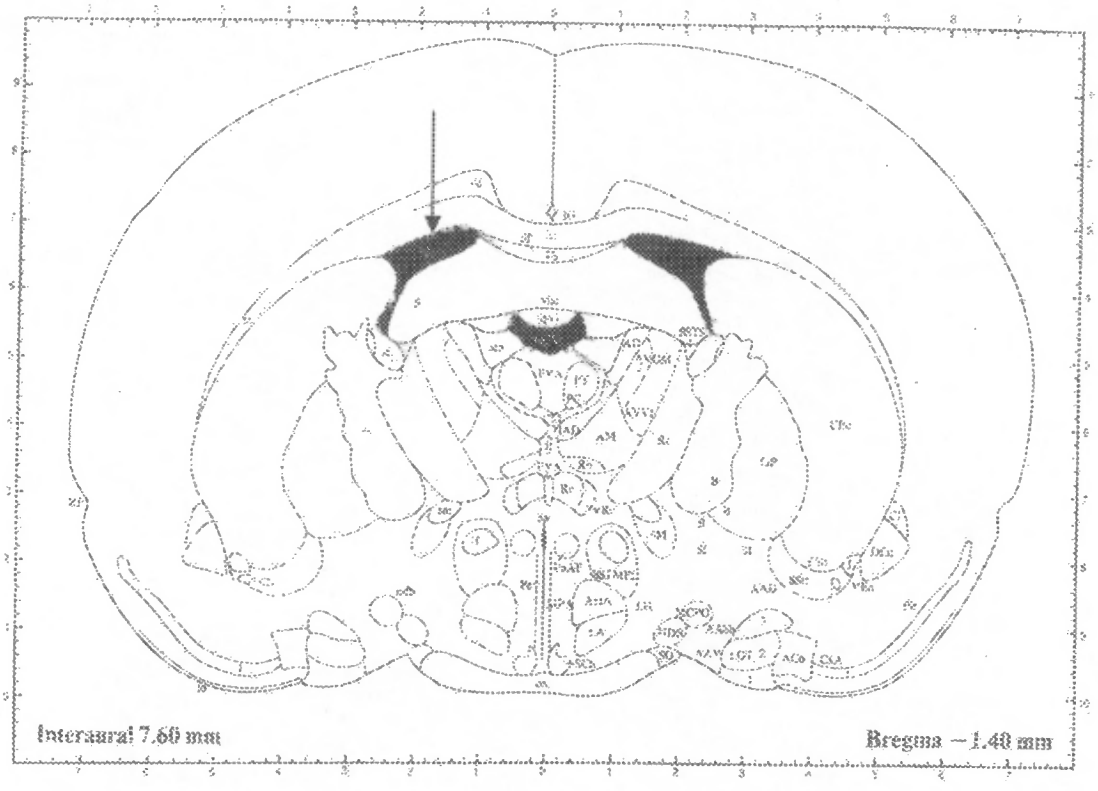


Fig.5.1. Stereotaxic map of lateral ventricular VCP injection cannula (arrow) at stereotaxic co-ordinates 1.5mm lateral to the sagittal suture and 8.0mm posterior to bregma in the A-P plane (Paxinos and Watson, 1998.)

5.2.3. Harvesting of brain tissue

Forty-eight hours following post-injury testing, rats from each group were deeply anesthetised before decapitation. The brain was removed from the skull and each whole brain was then dissected in the midline into the injured cortex and opposite (uninjured) cortex and stored in labelled vials at -80°C. The average time taken from the point of decapitation to the snap-freezing of the brain tissue in liquid nitrogen was 3.5 min (Keeling et al., 2000).

5.2.4. Homogenisation

Brain tissue of three saline-treated injured rats and three VCP- treated injured rats from each group of six were randomly selected for homogenisation. The contralateral uninjured hemispheres of the VCP treated and the saline-treated injured rats were used as negative controls. The injured hemispheres were analysed for the presence of VCP.

Each rat brain hemisphere was individually homogenised in Tris-saline containing leupeptin (0.1mM), EDTA-Na^{2+} (1mM), pepstatin A (0.1mM) and 4-(2-aminoethyl) benzene sulfonyl fluoride (0.25mM). The homogenates were centrifuged twice at 13 000 rev/min for 15 min. The supernatants were used as the test samples (Keeling, 2000).

5.2.5. Western blotting

Western blotting is used for the identification and quantitation of specific proteins in complex mixtures usually by using antibodies as probes. The test samples were first solubilised with detergents and reducing agents before being resolved on a SDS-PAGE gel by electrophoresis (as described in chapter 2, section 2.2.2). The Hybond-ECL nitrocellulose membrane (Amersham Biosciences, UK), 2 sheets of 3 mm Whatmann paper (cut to the equivalent size of the gel) and 2 sponges were pre-soaked for 10 min in cold Western blot transfer buffer (48 mM Tris-base, 39 mM Glycine and 20% methanol made up to 1 litre of distilled water) in order to equilibrate before the assembly of the sandwich. Flanking the gel and blot were the filter papers and sponges creating a sandwich. The Western blot apparatus (Bio-Rad, USA) was kept cool with an ice pack and by circulating the buffer on a magnetic stirrer. The proteins were transferred from the gel onto the nitrocellulose membrane electrophoretically at 100V for one hour. The blot was stained with Ponceau S (0.1% (w/v) Ponceau S, 1% glacial acetic acid) for 2 min on an orbital shaker SO3 (Stuart Scientific, UK) in order to confirm the successful transfer of proteins. The blot was subsequently, washed with 1%TBS-T (Tween-20 and TBS) before it was blocked overnight in 5% blocking solution (low-fat milk powder and TBS) to prevent non-specific antibody binding. The primary antiVCP – rabbit antibody was diluted 1 in 1000 in 0.05% blocking solution before incubation for 1 hour at room temperature on the orbital shaker SO3 (Stuart Scientific, UK). Following primary antibody incubation, the blot was washed twice, each in 30 ml 0.1% TBS-T for 10 mins with shaking. Thereafter, the blot was washed twice in 0.5% blocking solution. The secondary antibody, IgG-horseradish peroxidase labelled anti-mouse/rabbit

(Roche, USA), was diluted to a concentration of 40mU/ml in 0.5% blocking solution and was followed by shaker-incubation of 30 min at room temperature.

The blot was briefly rinsed in TBS-T and washed for 15 min. The washes were repeated four times before detection in the dark room commenced. The blot was incubated in a 100:1 detection reagent mix (solution A, luminescence substrate and solution B, starting solution) for 60 secs. Excess detection reagent was drained off the blot before it was exposed with a sheet of X-ray film in a developing cassette. Following exposure, the X-ray films were briefly incubated in developer solution, 2% acetic acid stop solution and finally fixing solution. After rinsing in distilled water, the X-ray film was allowed to dry.

5.3. RESULTS

Rats were randomly placed in treatment (n=6) and saline groups (n=6) following pre-injury training sessions (raw data shown in appendix D1). In general, the pre-injury training sessions showed that the goal latency had decreased on subsequent trials and days Fig.5.2. Probe test results revealed that there were no significant differences in the amount of time treatment and saline groups spent in the target quadrant Fig.5.3 (for raw data appendix D2 and D3).

Six rats were randomly chosen for Western blot analysis as shown in Fig 5.4. The Western blot analysis was done on brains of all these rats to determine if VCP was present in the VCP-treated injured rat brain and to check if VCP uptake had an effect on the spatial learning and memory task in the probe test. No VCP was detectable by the blot, showing absence of VCP in the brain tissue.

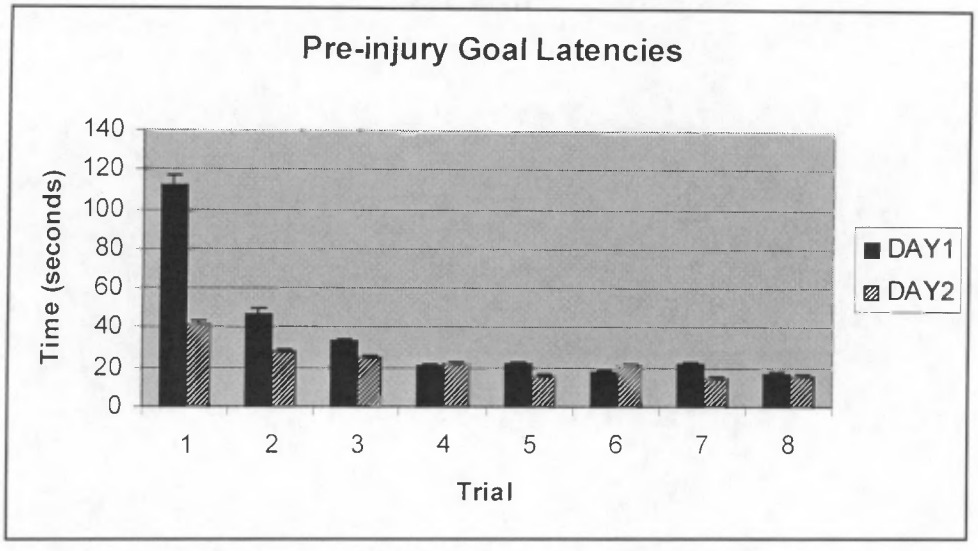


Fig.5.2. Animals were randomly assigned to treatment and saline groups after following a 2-day training procedure to find the platform. A decrease in goal latency is indicative of adequate training before FPI. Error bars indicate SEM.

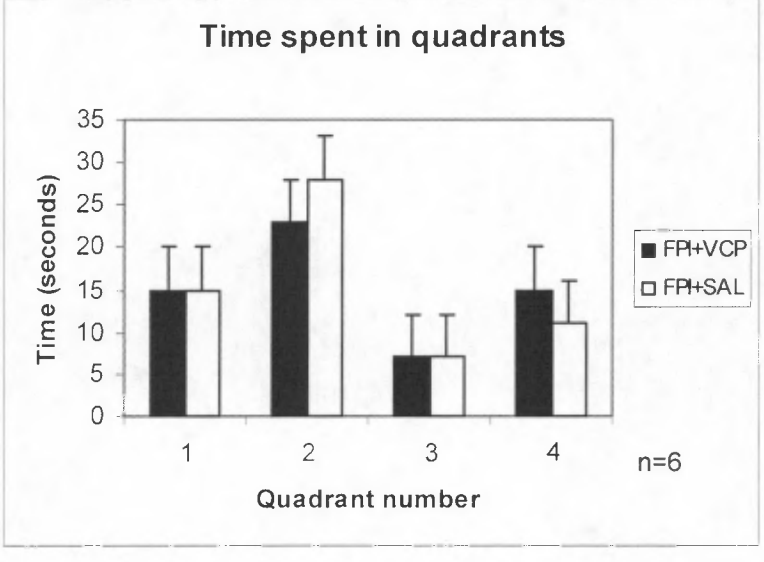


Fig.5.3. Forty-eight hours post injury testing after VCP administration in severe-FP injured rats revealed no significant differences between treatment and saline-injected groups. Error bars indicate SEM.

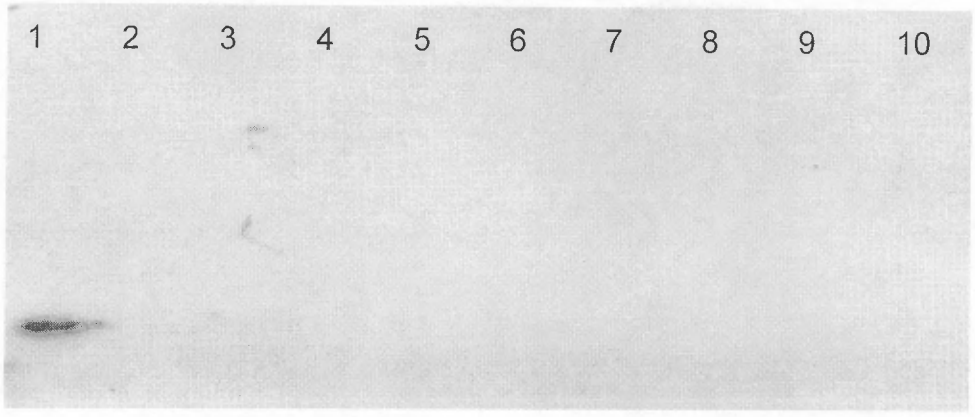


Fig.5.4. Six rats were randomly chosen for Western blot analysis. VCP [1.7mg/ml] was used as a positive control (Lane 1). Saline- treated rats (Lanes 2-4) and rats with contralateral uninjured cortices (Lanes 6, 8, 10) were chosen as the negative control. VCP was absent in the brain tissue of VCP treated injured rats (Lanes 5, 7, 9).

5.4. DISCUSSION

To the best of our knowledge, these data are the first reported to investigate the VCP administration route via the lateral ventricle of the brain. Previously, it has been shown that complement fragments (C3) are present within the injured rat cortex following FPI (Keeling et al., 2000) and that VCP administered at the injured site improved spatial learning and memory in mild and moderate head injury models (Hicks et al., 2002; Pillay et al., 2005). This study showed that under these experimental conditions, VCP administered in the third ventricle did not improve spatial learning and memory, since no significant differences were found in saline and VCP-treated groups. A sham group was not included in this part of the study as we were confident that the intensity of graded injury severity was fully characterised (as described in chapter 3). Furthermore, we were convinced that the cognitive deficits observed in this part of the study was due to a severe head injury because during the recovery-hours (following surgery), most injured rats demonstrated seizure-like activity. This was not observed in the mildly-head injured rats described in chapter 4. Another study demonstrated that only a single episode of severe FPI (3.75 - 4.0 atm) could cause seizures or post-epilepsy in the rat (Ambrosio et al., 2004).

One of the reasons the treatment may not have been as effective as the subdural injection route was because the VCP dose may not have been

adequate for this severity of injury and this route of delivery. The administration of 1.7µg/µl of VCP after mild TBI showed cognitive improvement in rats (Pillay et al., 2005). Besides the concentration not being adequate, there is no evidence that VCP crossed the BBB despite injury to the cortex.

One of the possible limitations of this experiment is that the entire volume of CSF would have been cleared many times before the detection of VCP by Western blot analysis. However, since this study was designed to assess cognitive outcome, the soonest possible time for post-injury testing was a 48-hour period. Furthermore, in another lateral FPI study cognitive testing was evaluated 48 hours post-injury because some extinction of tasks was reported if tested at later time-point (Smith et al., 1991).

CHAPTER 6
ADMINISTRATION OF VACCINIA VIRUS COMPLEMENT CONTROL PROTEIN IN A SEVERE HEAD INJURY MODEL

6.1. SEVERE HEAD INJURY

Survivors of severe TBI may suffer from mild symptoms of post-traumatic headaches (Walker et al., 2005) to a severe persistent vegetative state (Finfer and Cohen, 2001). Children below the age of 4 years have a lower chance of full recovery after severe TBI (Noppens and Brambrink, 2004). However, the overall neurological recovery following severe TBI in children is better than in adults.

Furthermore, in an epidemiology study that included men who were World War II navy and marine veterans, it was found that only those who sustained moderate or severe head injuries were at an increased risk of developing AD (Plassman et al., 2000). A pioneering neuropathological study revealed that patients who sustained severe head injuries had significant Aβ protein deposition within a short time of 6-18 days following trauma (Roberts and Gentleman, 1991). The increase in expression of sAPP-β in the acute phase after neuronal damage in the human brain leads to the accumulation and deposition of Aβ that is believed to initiate the progression of AD (Roberts and Gentleman, 1991; Roberts et al., 1991).

An experimental study using rats that were severely injured by FPI were evaluated for up to one year (Pierce et al., 1998). Researchers found that head-injured rats showed spatial learning and sensorimotor impairments that persisted for a year. In addition, progressive neurodegenerative changes occurred and APP and/or APP-like protein accumulation in damaged axons were evident.

The following study investigates the efficacy of VCP at a lower dose (1.7μg/μl) than previously reported (2.25μg/μl) (Hicks et al., 2002). The latter study reported moderate head injury induced at a level of 4.5 atmospheres, however, according to the classification scheme presented by McIntosh (McIntosh et al., 1989) this level of injury will be classified as severe. Since we are adopting this classification (as described in chapter 3), the current value of the fluid percussion injury (2.7-3.0 atm) is within the severe range of injuries. The aim of this study is therefore to test and compare the efficacy of VCP at a lower dose.

6.2. MATERIALS AND METHODS

6.2.1. Subjects

A total of 12 Wistar rats (250g-275g) were each randomly assigned to one of two groups: FPI + VCP treated (n=6) and FPI+ saline treated (n=6). All animals were also subjected to a battery of neurological tests that were scored by an independent evaluator blinded to the injury status of the rats for 10 consecutive days.

6.2.2. Experimental Procedures

Briefly , training in a MWM consisted of a total of 16 trials over a 2 day period before animals were anesthetized and subjected to a severe (2.7-3.0 atm) lateral FPI, 3.0 mm lateral to the sagittal suture and 4.5 mm posterior to bregma. 10 μl of VCP (1.7mg/ml) was injected into the injury site immediately after FPI. Two weeks post-FPI the animals were assessed in the MWM for spatial learning and memory. Neurological motor function tests were carried out after FPI for 10 consecutive days. Experimental procedures are outlined in Fig.6.1. For detailed descriptions of methods see Chapter 4 sections 4.2.2-4.2.9.

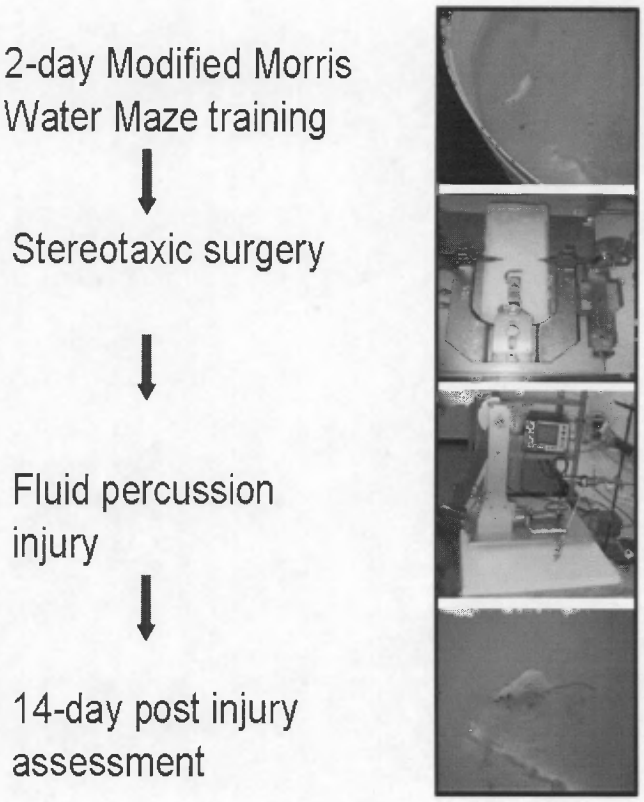


Fig.6.1. Summary diagram of experimental procedures. Training commenced 2 days prior to surgery. Two weeks later the cognitive outcome was assessed using the Morris Water Maze.

6.3. RESULTS

On the first trial of day 1 of training, rats located the platform in an average time of 106 seconds, whereas on the second day of the first trial a 50% reduction in goal latency time was evident as shown in Fig.6.2. By the last trial of day 2, rats were able to find the platform in 21 seconds (for details see appendix E1 for pre-injury training scores). The overall decrease in goal latency was taken to indicate that rats were efficiently trained to swim to the platform. Following a severe head injury rats were then tested in the MWM 14 days post-injury after been given VCP or saline treatment. In the first trial 14 days post injury, FPI-saline treated rats took twice as long to reach the platform than FPI-VCP treated rats, however, in subsequent trials the goal latencies were not significantly different as indicated in Fig.6.3. No significant differences in the amount of time spent in the target quadrant 2 were found between the VCP and saline treated groups in the probe trial as shown in Fig.6.4 (for details see appendices E2 and E3 for post-injury scores and data analysis).

Motor function, as tested on the angle- board, showed that there was a significant difference [$F(4, 9) = 5.19$ ($p=0.019$) between the FPI-VCP and FPI-saline treated groups (for details see appendix E4 for angle-board data). Rats in both groups were able to proceed immediately to climb up or down the board, from the point of placement i.e. when placed either nose up or nose down on the angle-board. VCP treated rats (15%) were also more likely to climb off the board than saline-treated rats (9%). However, a larger proportion of FPI-saline treated rats (27%) tended to slide down immediately when placed on the angle-board than did FPI-VCP treated rats (13%) as shown in Fig. 6.5 A and B.

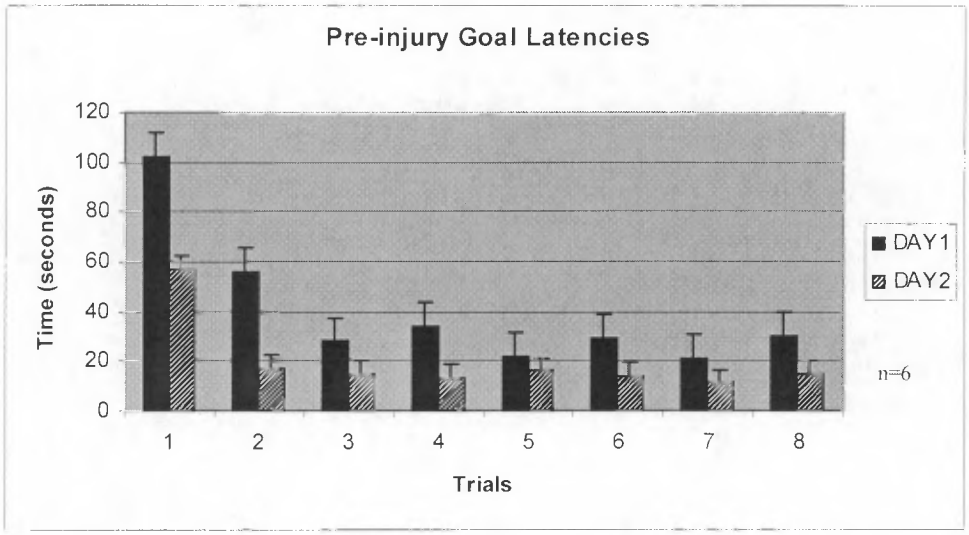


Fig.6.2. On day 1 of the first trial a 50% reduction in goal latency was evident. Pre-injury latencies ranged from 106 to 25 seconds on day 1 and on day 2 from 61 to 21 seconds. Error bars indicate SEM.

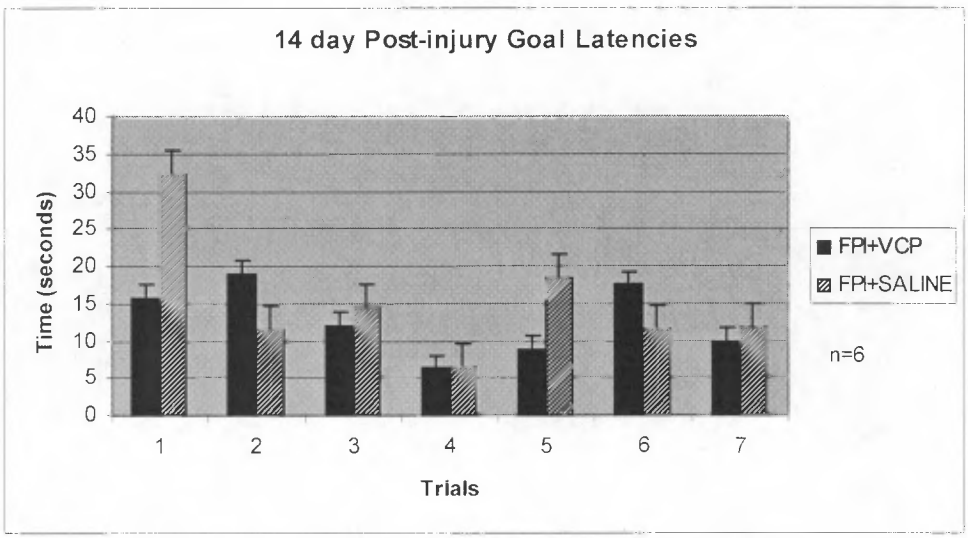


Fig.6.3. In the first trial two weeks post-injury saline-treated rats took twice as long to reach the platform than VCP-treated rats. There were no significant differences in subsequent trials. Error bars indicate SEM.

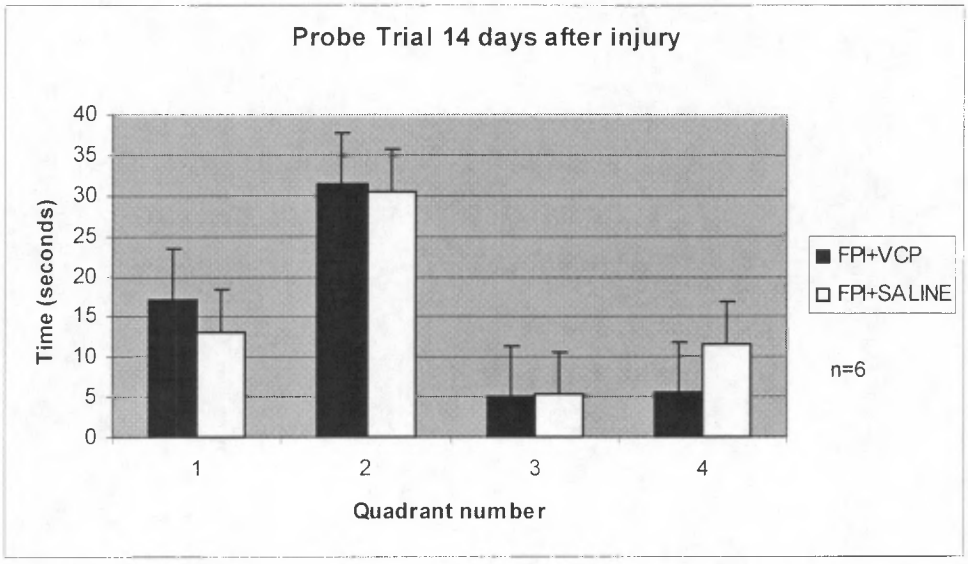


Fig.6.4. Bar graph showing the amount of time VCP and saline-treated rats spent in each quadrant during the probe trial (eighth trial when platform is removed). No significant differences were shown between the two groups. Error bars indicate SEM.

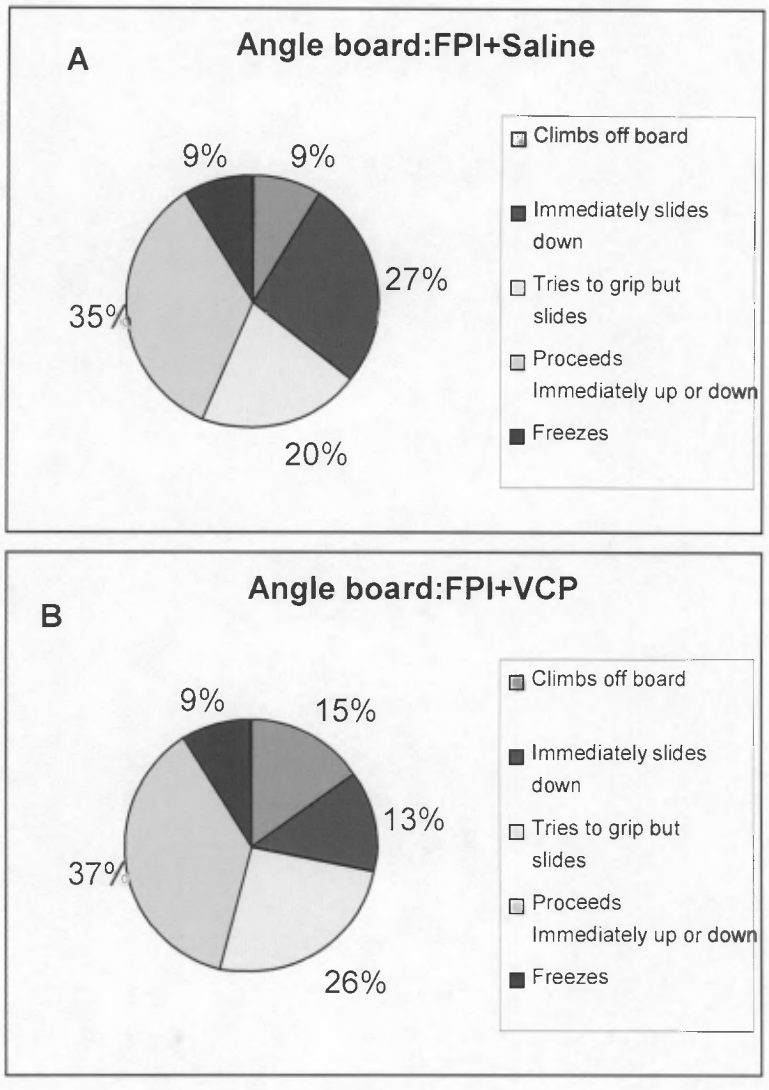


Fig.6.5. Pie Charts to show the percentage of motor indicators when injured rats were placed on the angle board.

A and B: A one-way ANOVA showed that there were overall significant differences between the saline-treated and VCP-treated injured groups. A greater proportion (27%) of saline-treated rats immediately slide down from the angle-board compared to VCP treated rats (13%) (*$p<0.05$). The VCP-treated rats climbed off the board 6% more often than the saline-treated rats.

The ambulation test showed that there were no significant differences between the two groups (Mann-Whitney U test) (for details see appendix E5 for ambulation scores and non-parametric analysis). The hind paw grip test, forelimb and hind limb extension and visual placing tests all showed no significant differences (for details of raw data see appendix E6). However, both tactile placing and left and right lateral pulsion tests were statistically significant (for details of raw data see appendices E7 and E8). More specifically, a significant difference [F (1, 9) =5.32 (p=0.002)] was found in tactile placing between the two groups on the first five days of testing as shown in Fig.6.6. Lateral left pulsion [F(1,7)=5.98 (p=0.001)] in the first five days and right pulsion [F(1,5)=7.71(p=0.007)] in the first four days of testing were significantly better in VCP treated rats than in saline treated rats as confirmed by Mann-Whitney U and Kruskal-Wallis ANOVA tests and shown in Fig.6.7.A and B. Thus, irrespective of been unilaterally lesioned, pulsion to left and right sides were similarly affected. No significant differences were found in subsequent days.

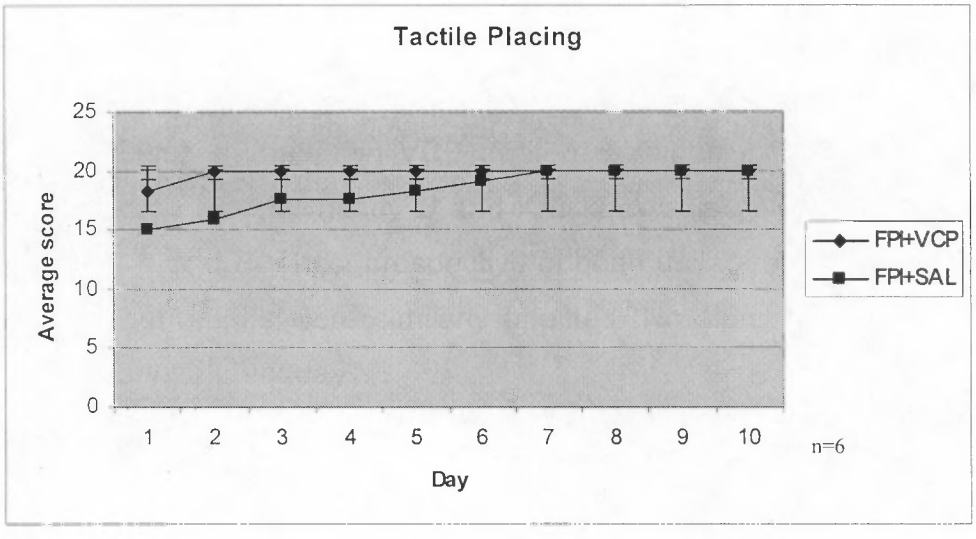
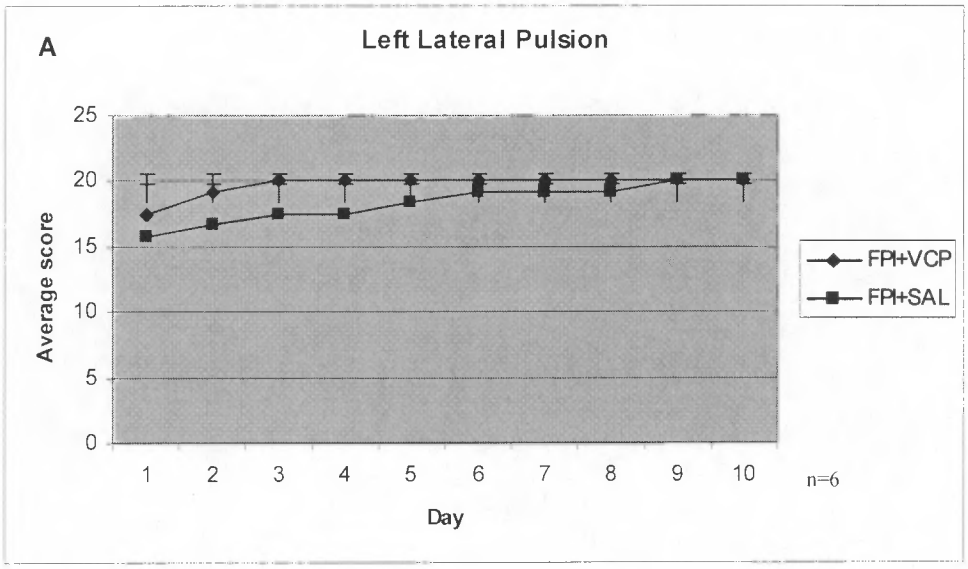


Fig.6.6. Mann-Whitney U test showed that VCP-treated rats performed significantly better than saline-treated rats in the first five days of testing (*p<0.05). Error bars indicate ±SD.



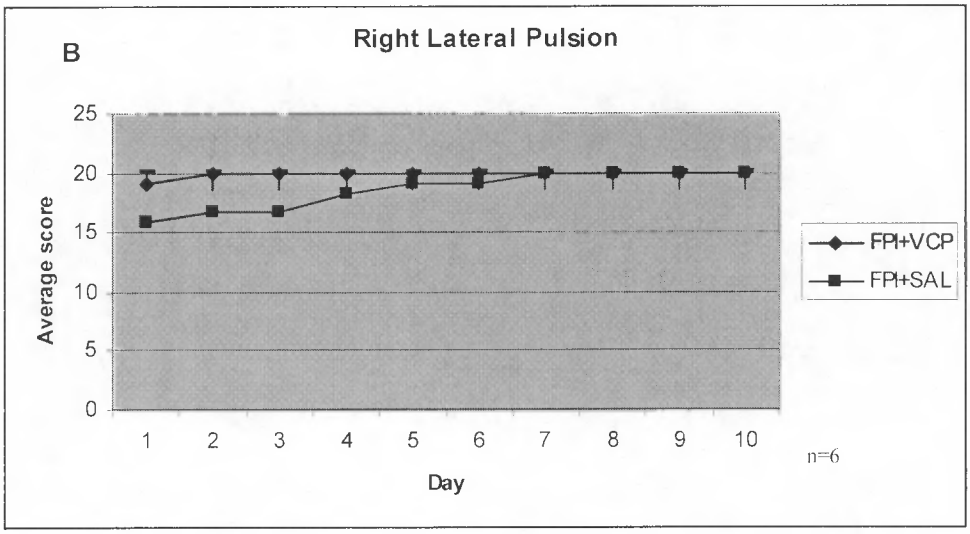


Fig.6.7. Saline- treated rats showed a greater resistance to lateral pulsion on both left and right sides.

A and B: A significant difference (*$p<0.05$) was found on the first five days and four days of left and right lateral pulsion testing, respectively as shown by Mann-Whitney U and Kruskal Wallis ANOVA tests. Error bars indicate ±SD.

6.4. DISCUSSION

This study shows that VCP administration significantly influenced sensorimotor function recovery, but did not significantly improve the cognitive outcome after severe head trauma in Wistar rats. Previous studies that used VCP as a therapeutic agent for head trauma in mild and moderate injury respectively showed cognitive improvements, as measured by the use of a MWM (Pillay et al., 2005; Hicks et al., 2002). In these studies more specifically during the probe trial, saline-treated rats that were mildly-injured (1.0-1.1.atm) spent a larger amount of time (20%) in the incorrect quadrant than VCP–treated rats (9%) (Pillay et al., 2005). It was also reported by Hicks et al. (2002), that after a moderate head injury, saline-treated rats spent less time in the quadrant of the pool where the platform was previously located than VCP-treated rats. In the current study, both VCP and saline treated rats spent about 50% in the quadrant of the pool where the platform was previously located and no significant differences were found.

These findings may be accounted for in terms of the different VCP injection dosages administered in these head trauma models. Concentration of VCP at the site of injection influences recovery outcome and may therefore be a critical factor in influencing the overall recovery. Moderately-injured rats received 2.25µg/µl of VCP (Hicks et al., 2002) whereas both mildly (Pillay et al., 2005) and severely head injured rats received 1.7µg/µl of VCP.

In spite of the non-significant effect on cognition with this dosage of VCP, sensorimotor function recovery was significantly improved after severe lateral fluid percussion injury as measured by a battery of tests.

A study performed by Petullo and co-workers (1999) showed that when normal rats were placed on the angle-board they would usually turn 180 degrees and proceed up the board. However, injured rats cannot make this turn to proceed up the board (Petullo et al., 1999). A different behaviour was noted in this study since severely head-injured rats receiving saline or VCP tended to climb off the angle-board 9% or 15% of the time respectively; behaviour not observed in mildly- head injured rats or normal rats (Pillay et al., 2005; Petullo et al., 1999). This behaviour is possibly an indication of the extent of neurological impairment caused by a high intensity of injury. It was

evident in this study that severely head-injured rats treated with saline (27%), tended to slide down the angle board twice as much as the VCP- treated rats (13%) as indicated in Fig. 6.5. A greater frequency of sliding down the angle board was also evident in saline-treated than VCP-treated rats that sustained a mild head injury (Pillay et al., 2005). These findings demonstrate that VCP is able to exert a favourable influence on the motor system of treated rats since there is a marked improvement in grip function. Freezing in rats, as tested in an open field, is a fear-motivated behaviour and is associated with the basolateral amygdala of the brain (Power and McGaugh, 2002). In the mild- head injury model saline-treated rats showed more frequent freezing behaviour than VCP-treated rats (Pillay et al., 2005). However, after severe traumatic brain injury, no significant differences were found. This finding may possibly indicate that with this dosage (1.7µg/µl) and higher intensity of injury, VCP may not have an effect on freezing behaviour, and thus a deeper lying structure such as the amygdala do not respond to the effects of VCP injected cortically.

Lateral pulsion and tactile placing tests are good indicators of motor function. Normal rats would generally show equal resistance when pushed to the left or right sides (Petullo et al., 1999). During the first five days after severe head injury, saline-treated rats demonstrated a greater weakness in lateral pulsion than VCP-treated rats ($p<0.05$). During tactile placing tests, saline-treated injured rats were less able to extend their forelimbs toward the edge of the table when lowered from above it, than VCP-treated rats.

Taken together, these findings show that a VCP dose of 1.7µg /µl significantly contributed to the recovery of the overall sensorimotor function in severely injured rats but had minimal effect on cognitive outcome. Future challenges would be to resolve how best to deliver functional VCP to the brain and also to produce a more potent VCP in order to minimise the amount required for *in vivo* studies with greater effects.

CHAPTER 7
ADMINISTRATION OF VACCINIA VIRUS COMPLEMENT CONTROL PROTEIN IN Mo/Hu APPswe PS1dE9 MICE

7.1. TRANSGENIC AD MOUSE MODELS

A good mouse AD model should have at least one of the following pathological hallmarks: plaques, tangles or loss of cholinergic neurons (as described fully in chapter 1). Over the years several AD transgenic mouse models have been developed (Games et al., 1995; Hsiao et al., 1996; Johnson-Wood et al., 1997; Borchelt et al., 1997). Two main types of mutations are associated with the different expression patterns of amyloid peptide accumulation in APP transgenic mice models. The 'Swedish' mutation K670N/M671L is associated with the overproduction of Aβ via the stimulation of the β-secretase pathway that results in an accumulation of predominantly $A\beta_{1-40}$ (Thinakaran et al., 1996). The V717I and 'Indiana' (V717F) mutations are associated with γ-secretase mediated cleavage resulting in the generation of predominantly $A\beta_{1-42}$ accumulation (Maruyama et al., 1996; Suzuki et al., 1994).

Three main types of promoters have been predominantly used in the development of transgenic mouse models. The platelet-derived growth factor β-chain (PDGFβ) promoter was used to drive the expression of APP cDNA that encompassed all the human APP splice variants of 695,751 and 775 amino acid residues in Swiss-Webster x C57B6/DBA mice, and these types were named the PDAPP mice (Games et al., 1995; Johnson-Wood et al., 1997). The mammalian prion protein (PrP) gene was identified as a vehicle to express foreign proteins in the central and peripheral nervous systems (Scott et al., 1992; Hsiao et al., 1995). Using the PrP gene-derived vector called cos.Tet (Scott et al., 1992), Hsiao and her co-workers (1995) developed mice expressing high levels of APP but also exhibited a high frequency of spontaneous death (Hsiao et al., 1995). Borchelt and his co-workers (1996) later characterised an expression plasmid using a mouse prion protein, (MoPrP.Xho), that offered protein expression levels relatively proportional to the number of integrated transgene copies that was useful for use in the brains and hearts of transgenic mice (Borchelt et al., 1996).

Other APP mouse models were developed using the Thy-1 promoter (Sturchler-Pierrat et al., 1997). C57 mice carrying the double-mutant allele

(Swedish plus V717I) with promoter human Thy-1, developed plaques at 18 months, whereas mice carrying the Swedish mutation alone with a mouse Thy-1, developed plaques at 6 months. Amyloid plaque deposition varied and was found not to be dependent on the different promoters used but rather dependent on the trangenes (Sturchler-Pierrat et al., 1997).

Using these types of mice which fulfil the pathological hallmarks of AD, it is important that behavioural tests are sufficiently robust so that cognitive deficits or changes can be evaluated. Behavioural tests are represented by the open-field test (Hall, 1934), the Morris water maze (MWM) (Morris, 1984) and the Y- or T-maze tests (Douglas, 1966). The open-field test assesses spontaneous exploratory activity in a novel environment. Spatial learning and memory is usually evaluated through the MWM and the Y- or T-maze tests.

In selecting the appropriate behavioural test the natural development and habitat of the animals should be also considered. Mice are naturally found in dry fields and rats in wetlands (Gerlai and Clayton, 1999), so this may have fundamental effects on behaviour. When trained in wet conditions and similar performance requirements for learning, mice needed about five times more trials than rats. These differences in performance and habitat may be attributed to specie differences in task suitability rather than memory ability (Gerlai and Clayton, 1999). So taking these factors into consideration, a novel approach was used in testing the spatial learning and memory in mice by using a modified version of the cheeseboard maze (Kirwan et al., 2005).

The expression of the two transgenes, mutant human presenilin 1 (DeltaE9) and a chimeric mouse/human amyloid precursor protein (APPSwe) are directed by the mouse prion protein promoter. The early-onset AD results from a deletion of exon 9 (DeltaE9) mutation of the human presenilin 1 gene. This part of the study investigates the effect of VCP on memory processes in the Mo/Hu APPswe PS1dE9 transgenic mouse that corresponds to a form of early-onset AD.

7.2. MATERIALS AND METHODS

7.2.1. Breeding of mice

Two breeding pairs of AD transgenic mice (stock number:004462) and control mice were ordered from Jackson's mice Laboratory (USA) and were bred in the quarantine facilities at the Research Animal Facilities at The University of Cape Town. Mice were mated when they were sexually mature (6 to 8 weeks old). Since there were more female mice than male mice available for breeding, male mice were rotated through the female cages in order to optimise the reproduction of the transgenic mice population. When females were pregnant the males were removed from the mating cage. Soon after a female mouse gave birth a male mouse was re-introduced into the cages to mate again. Subsequent offspring were used in this study as shown in Fig.7.3 and Fig.7.4. Offspring were randomly chosen for genotyping (for details see appendix F1).

7.2.2. Genotyping

Genotyping was done to check for the presence of the two transgenes and prion promoter in the Mo/Hu APPswe PS1dE9mice.

DNA Extraction from Mice Tail Biopsies

In order to extract DNA for genotyping, mice tail biopsies were taken and were allowed to digest overnight at 55°C in lysis buffer (see appendix B) and 24µl Proteinase K (10mg/ml). The tails were then centrifuged at 13 200 rev/min in a bench-top centrifuge (Velp Scientifica, Italy) for 15 min. The supernatant was added to newly labelled tubes with 500µl of isopropanol. The tubes were inverted until a visible precipitate was formed. The precipitate formed a pellet when centrifuged at 13 200 rev/min for 20 sec and the supernatant was removed before 1ml of 75% ethanol was added. The pellet was then resuspended on a RX^3 vortex (Eppendorf, Hamburg). The resuspended pellet was then centrifuged for a further 10 min at 13 200 rev/min. The supernatant was removed and the pellet was dried for at least one hour in order for complete evaporation of the ethanol. Finally, the pellet was resuspended in 200µl Tris-EDTA (pH8) and incubated at 55°C until the DNA dissolved.

Sample Preparation for Electrophoresis

The DNA samples were prepared by adding 6ul DNA to 2ul of 6X Gel loading buffer. The gel loading buffer consists of a tracking dye, bromophenol blue in 30% glycerol (appendix B9). When added to the samples, its density increases so that it falls into the wells of the gel, thereby providing a visible marker to monitor the movement of the samples in the gel. A molecular size standard was also prepared by mixing 5ul of the Benchtop 1kb DNA Ladder (Promega, USA) to 1ul of the loading buffer. This ladder serves as a reference that enables determination of double-stranded DNA from 250-10 000 base pairs.

Preparation of DNA Agarose Gels

Agarose is used to analyse and isolate DNA fragments up to 25 kb (Sambrook and Russell, 2001).The agarose solution was dissolved by heating 1% or 1.5% agarose in 50ml of 1x Tris Boric acid EDTA (TBE) (appendix B 21) in a microwave. The agarose solution was cooled to approximately 55°C before ethidium bromide, a fluoresecent dye that binds to DNA, was added to a final concentration of 0.5µg/ml. The agarose solution was then poured into a casting tray and the 10-well gel comb was promptly inserted. After the gel had solidified, the comb was removed and the DNA ladder and samples were loaded. The buffer reservoir of the gel box was filled with TBE buffer and the samples were electrophoresed by an applied voltage of 90V for 45 minutes. Ethidium bromide stained gels are visualised using a UV light. Photographs of the DNA in the gels were taken using an integrated system (Alpha Innotech Corporation, California) that transmits gel images directly to the computer monitor and is visualised in real time (see Fig 7.5-7.7).

Polymerase Chain Reaction (PCR)

Genotyping was done by the polymerase chain reaction (PCR) method. Firstly, double-stranded DNA templates were denatured by heat, then the temperature was appropriately lowered so that oligonucleotide primers anneal to single-stranded target sequences, this was then followed by an extension stage that was carried out at an optimal temperature for DNA

synthesis when catalysed by *Taq* polymerase. A number of cycles were followed so that primers were able to amplify regions of the transgenes and the promoter region of interest, and by so doing the presence or absence of the transgenes could be assessed.

Primers used

Primers that were used for the genotyping were manufactured at Integrated DNA Technologies (USA) according to the specifications on the JAX® mice data sheet (see appendix F2 for primer details). Primers GK1 and GK2 amplified a 750bp from the prion protein promoter gene for an internal control. Primers GK3 and GK4 amplified a 608bp product from the presenilin1 transgene. A 350bp product from the transgene APP 695 was amplified when primers GK5 and GK6 were used.

Tg(APPswe)

The PCR reaction components (Promega, USA) were placed on ice. Each reaction tube consisted of a final concentration of 0.8X reaction buffer, 2.0mM $MgCl_2$, 0.2mM dNTP, 1.0μM GK5 primer, 1.0μM GK6 primer, 0.025u/μl *Taq* DNA Polymerase, 13.3μl dH_2O and ~20ng DNA. The tubes were immediately placed in the PCR Sprint thermal cycler (Thermo Hybaid, UK) under the cycling conditions (see appendix F2 and F3 for details).

Tg(PSEN1)

Similar procedures were followed as described above, except primers GK1 and GK2 were used under cycling conditions A and primers GK3 and GK4 were used under cycling conditions B (see F3 for details).

7.2.3. The Cheeseboard maze

This consisted of a circular board made of wood and painted white, and placed in the middle of the testing room. It was positioned on a table at an elevation of 72 cm above floor level. The surface of the board comprised 156 wells arranged in parallel rows as depicted in Figure 1. Each well was 1 cm in diameter and 10 mm deep, and a white plastic cup could be fitted into each well. The animals were maintained on a food deprivation schedule throughout the experiment, and their body weight was monitored daily, and compared to that obtained on the day before food deprivation began. The animals were maintained at 80% of their *ab lib* weight. On each day, the animals were allowed access to food for 1-2 hrs and were allowed access to water *ad libitum*. They were also familiarised with the odourless reward pellets (to be used as the reinforcement in the experiment) in their home cages.

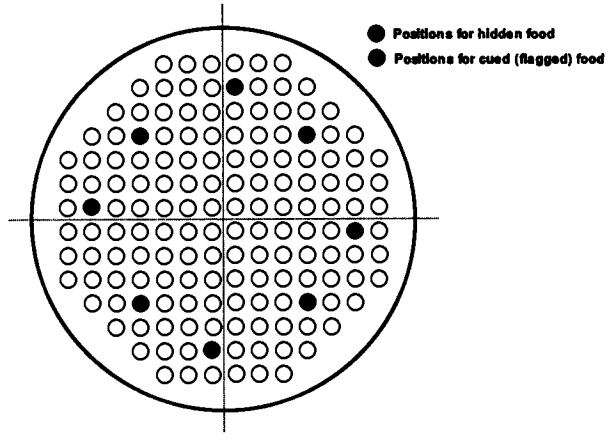


Fig.7.1. Diagrammatic representation of Cheeseboard Maze indicating positions of hidden food and flagged food.

7.2.4. The Extra-Maze Environment

The maze was kept in a well-lit room with one door, no windows, bench-top equipment, a chair and three posters on the wall. The posters depicted three distinctly different shapes. Mice were maintained on a 12h light/dark cycle. All testing was performed during the light phase of the cycle.

7.2.5. Habituation

Habituation lasted for 10 days. On each habituation session, the animals were individual placed in the centre of the cheeseboard, with one 20mg Noyes Precision odourless pellet (Research diets Inc, USA) baited at each of the 156 food wells. They were allowed to freely explore for a maximum of 5 min. At the end of habituation, all animals freely explored across all areas of the cheeseboard and readily consumed the food pellets.

7.2.6. Pre-training

This phase of behavioural training familiarises the animals to the visual cues to be employed in the next phase (cued task) and that not all food wells would be baited with reward pellets. Four food wells, as specified in Fig. 7.1, were marked by a distinct visual cue comprising a vertical wooden bar (17 cm high) with a paper flag attached to its tip. Four reward pellets were baited in each of these four specified food wells. The animals were placed at the centre of the board and allowed to freely explore for a maximum of 5 minutes or when all reward pellets were collected.

7.2.7. Cued task

In the cued task, 4 trials were performed with the flag placed randomly in either centre, NE, SW, SE or NW positions (i.e. 5 locations). Four reward pellets were placed at the flag. All the mice were placed at the same starting position i.e. south. A maximum time of 60 seconds was allotted for the animal to find the flag and the reward pellets at one of the 5 locations. If the mice did not find the flag within this time in the first trial, they were placed at the flag for a further 60 second period in order to re-acquaint themselves with the

not find the flag within this time in the first trial, they were placed at the flag for a further 60 second period in order to re-acquaint themselves with the reward at the flag. This task requires incremental learning from a constant starting position over 8 days of training and results in the formation of long-term reference memory (Savonenko et al., 2005).

7.2.8. Spatial acquisition task

The spatial acquisition task consisted of 4 trials each with different starting positions i.e. N, S, E and W. A cup consisting of 4 pellets was hidden at one of the positions SE, SW, NE or NW as indicated by the shaded dot in Fig. 7.1. The animal was given a maximum of 60 seconds to find the pellets. This task relied on the animal's ability to form memory traces of unique events in time (change of flag position and starting positions).

7.2.9. Intracranial injections

The mice (n=6) were deeply anesthetised after an i.p. injection of ketamine/ 2% xylazine (1.2ml ketamine, 0.8ml xylazine,8ml 0.9% saline) mixture. The animals were then injected via a transcranial route using a 20 gauge needle. This was placed over the parietal cortex. Injections were either 5µl saline or 5µl VCP (1.7 µg/µl). The mice were regularly monitored during the recovery period, until fully recovered from the effects of anaesthetic administration and regularly on subsequent days.

7.3.0. Probe Phase: Probe Task and Reverse Task

Fourteen days after the administration of VCP or saline, the spatial learning and memory of the mice were tested in the probe and reverse tasks. The probe task was done on two consecutive days and consisted of 4 trials with intervals of 30 seconds. After being released from the starting point at south, the mice were given a maximum 60 second latency period to find the flag. During the first trial, the flag was not baited. The flags (moved to a new location at each trial) were then baited with reward pellets on the second and third trials, but the pellets were removed in the last trial. The probe task evaluates the long-term reference memory of the mice as previously trained

The reversal task was also done on two consecutive days and consisted consisted of 4 trials with a maximum goal latency of 60 seconds. The flag was positioned at one of the following locations centre, SE, SW, NE and NW on the cheeseboard. The flags were baited with reward pellets on the second and third trials only. Mice were released randomly from different positions (N, S, E and W) on each of the 4 trials. The reverse task evaluates the episodic memory of the mice as previously trained in the spatial acquisition task (i.e. different flag and starting positions) (Savonenko et al., 2005).

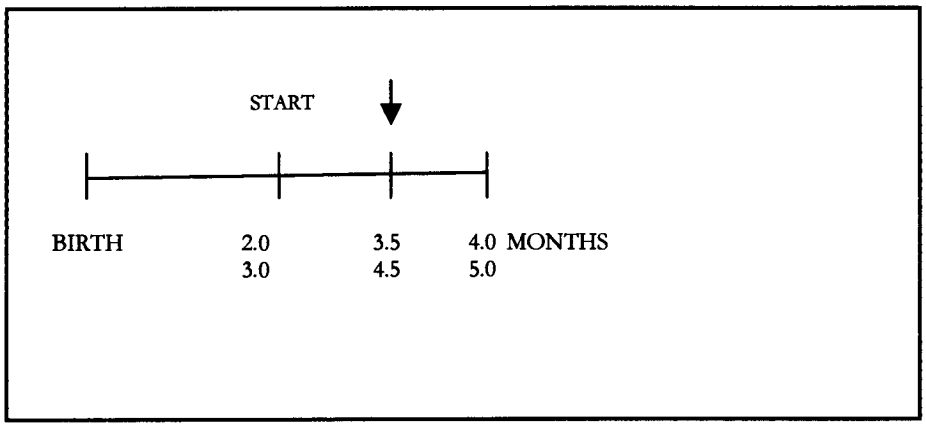


Fig.7.2. Timeline for behavioural study in months. The arrow indicates the intracranial injection time.

7.3.1. Data Analysis

The habituation, pre-training and spatial task phases were analysed by calculating the average goal latency and/or the number of pellets eaten daily in transgenic and wildtype mice and are represented in graphs. Probe trials were calculated by using the combined daily scores (day 1 and day 2) and calculating the overall average scores. A one-way analysis of variance (ANOVA) was used to determine if there were statistically significant differences between groups (for analysis of probe phase see appendix G9).

7.3. RESULTS

7.3.1.1. Breeding results

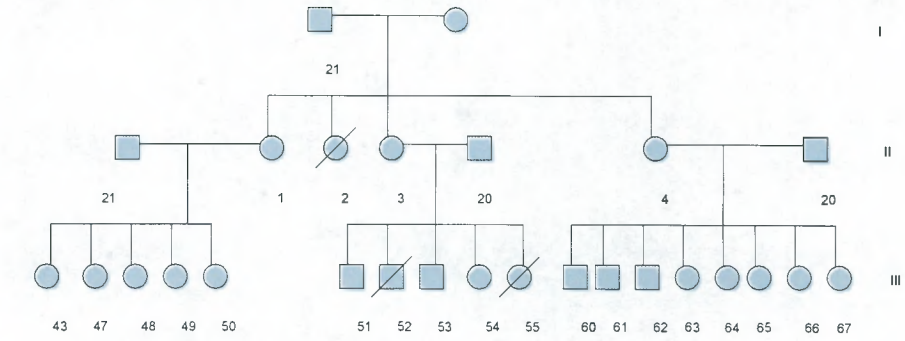
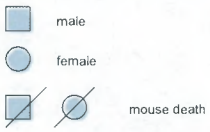


Fig. 7.3. Pedigree chart of transgenic mice Mo/Hu APPswe PS1dE9 after three generations of breeding, Generation II was born one month after generation I had mated. Generation III mice from female 1 were born three months later. Females 3 and 4 gave birth to their offspring after 4 months of mating. Mouse 2 was eliminated from the breeding as it lacked the APP 695 gene.



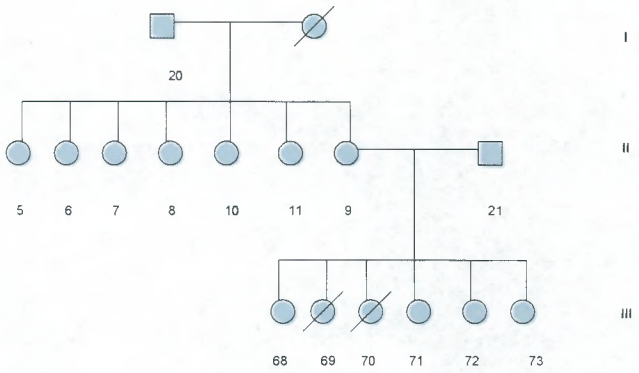


Fig. 7.4. Pedigree chart of transgenic mice Mo/Hu APPswe PS1dE9 after three generations of breeding (second breeding pair). Generation II was born two months after generation I had mated. Subsequently, mouse 9 was mated with mouse 21 and produced 6 offspring. (see appendix F1 for further details)

7.3.1.2. DNA extraction and Genotyping

The presence of the presenilin transgene (Fig. 7.5) and APP695 transgene (Fig. 7.6) and the mouse prion protein promoter (Fig. 7.7) were confirmed by genotyping the tails by the PCR method.

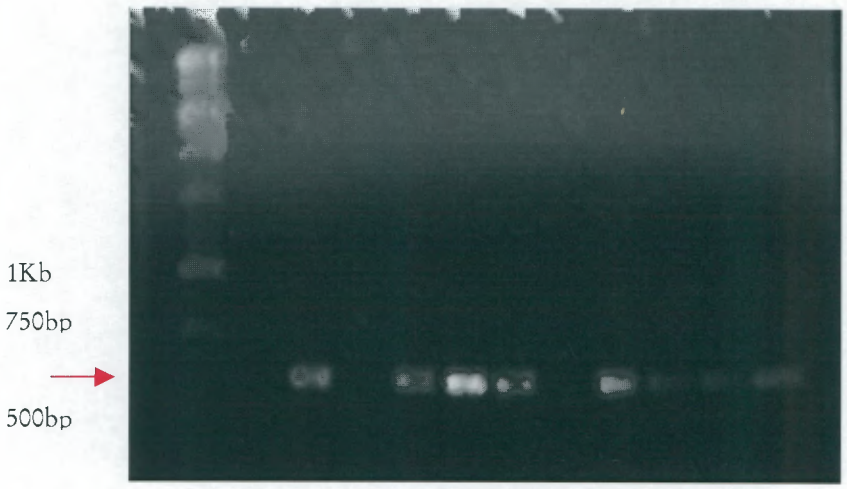


Fig.7.5. Primers (GK3 and GK4) were used by the PCR method to check for the presence of a 608 bp (red arrow) amplified region in the presenilin transgene.

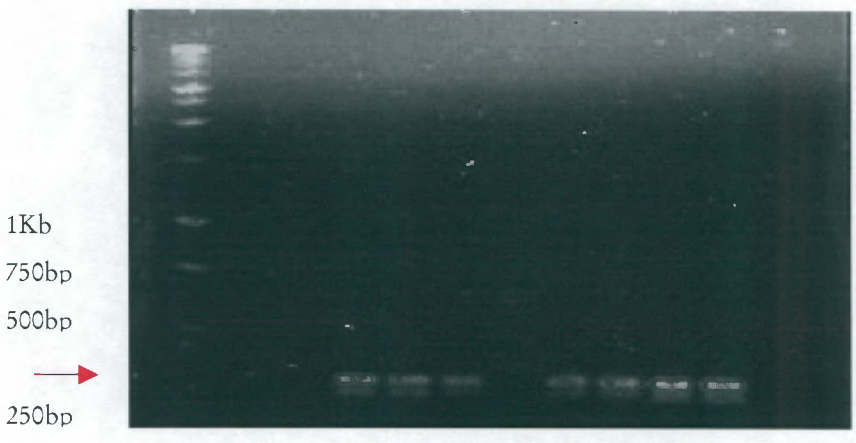


Fig.7.6. 350 bp products (red arrow) from the transgene (APP695) were present following PCR analysis of the genomic DNA extracted from mice tails.

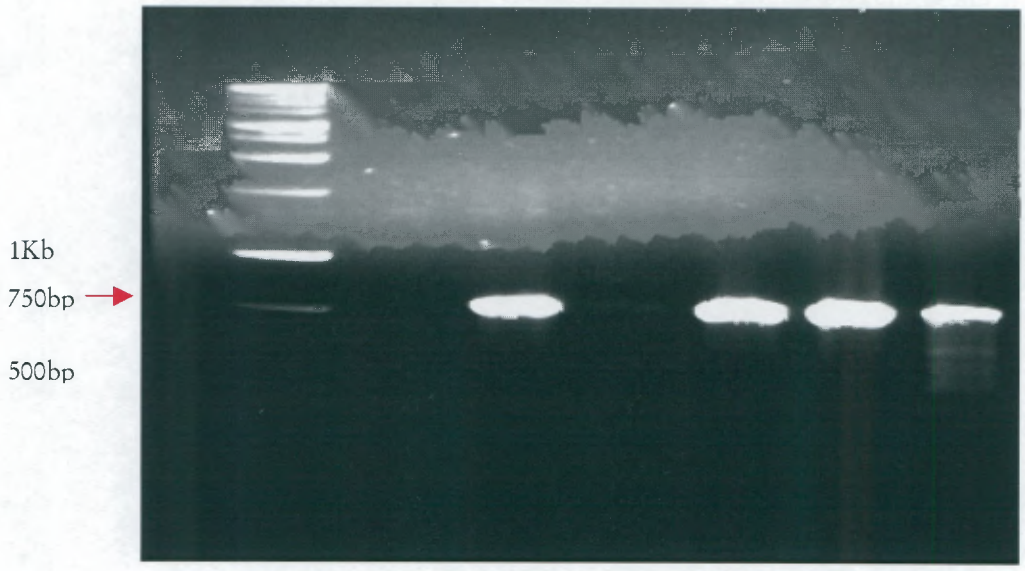


Fig.7.7. With primers (GK1 and GK2) a 750bp (red arrow) product from the mouse prion promoter gene was present.

7.3.1.3. Training results

The training of the mice was done in four phases, namely, the habituation phase, pre-training phase (to locate 4 flags), cued and spatial acquisition task phases (see appendix G1 –G7 for data collection and analysis).

A one-way ANOVA showed that there was a significant difference [$F(1,16)=7.08(p=0.017)$] between transgenic and wildtype mice in the number of pellets eaten during the habituation phase as shown in Fig.7.8.

Pre-training results showed that there were no significant differences between the wildtype and transgenic mice in the time spent in locating flags and the number of pellets eaten as shown in figures (for graph analysis see appendix G6).

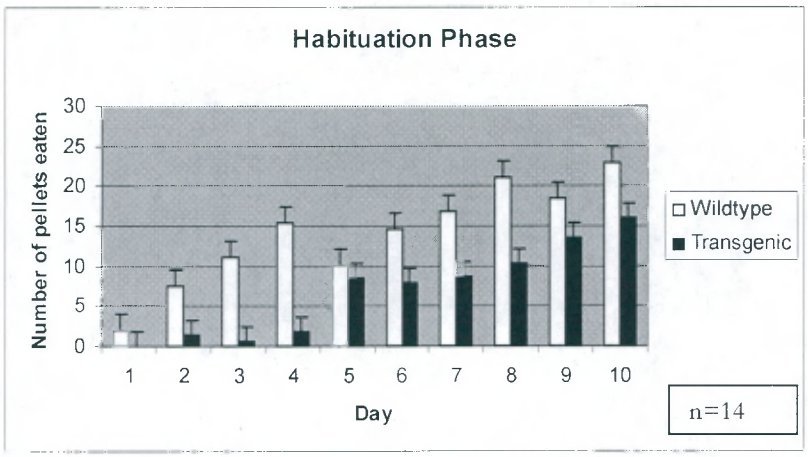


Fig.7.8. Bar graphs showing the number of pellets eaten over a 10-day period in transgenic and wildtype mice ($p<0.05$). Error bars indicate SEM.

Cued task results showed that the time taken to find the flag over an eight day period steadily decreased after each day of training in wildtype mice as depicted in Fig.7.9. After the fourth day of training the rate of learning the flagged food positions stayed constant in transgenic mice as indicated in Fig.7.10. Significant differences ($p<0.05$) were found in each of the four trials over the 8 days of training between transgenic and wildtype mice (for precise p-values see table in appendix G7). The spatial acquisition task showed no differences between the wildtype and transgenic mice in locating the flagged food when release from different positions for all 3 days of training (for graphic analysis see appendix G8).

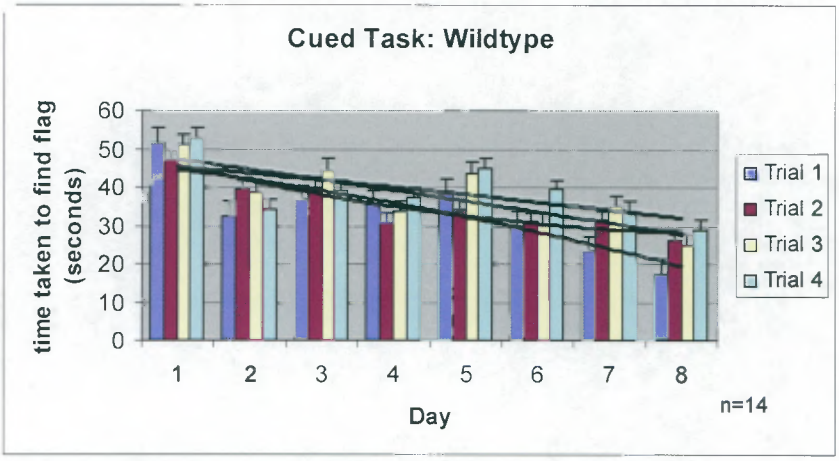


Fig.7.9. On each day of training, the time taken to find the flagged food in each trial steadily decreased in wildtype mice. The solid lines represent the trends for each of the trials on 8 consecutive days. Error bars indicate SEM.

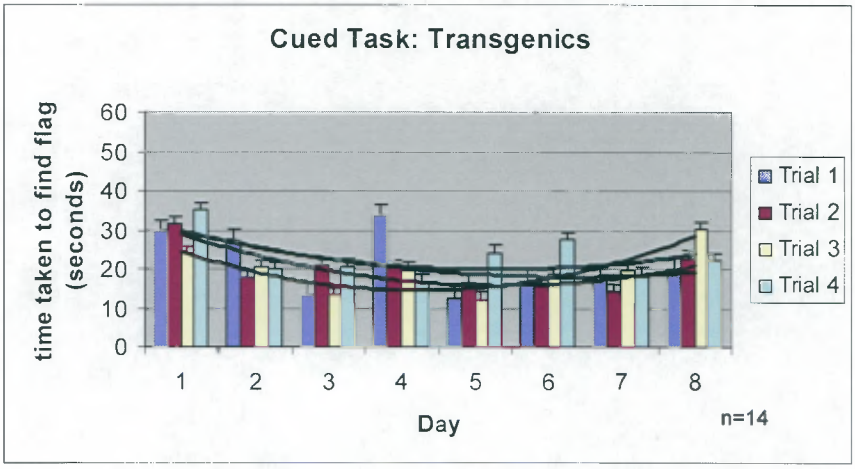


Fig.7.10. Transgenic mice showed no marked improvement in the time taken to find the flagged food as indicated by the trends (solid lines) from day 1 to day 8. Error bars indicate SEM.

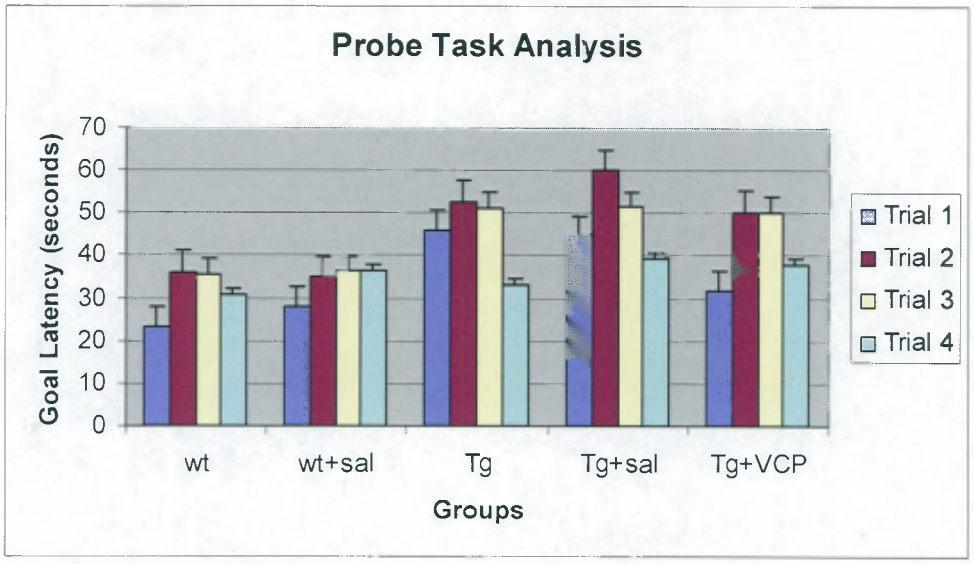
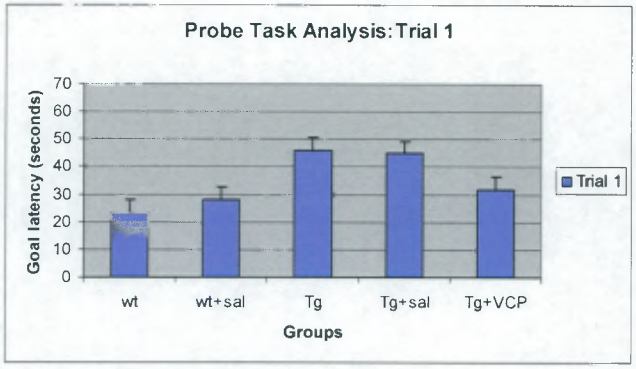
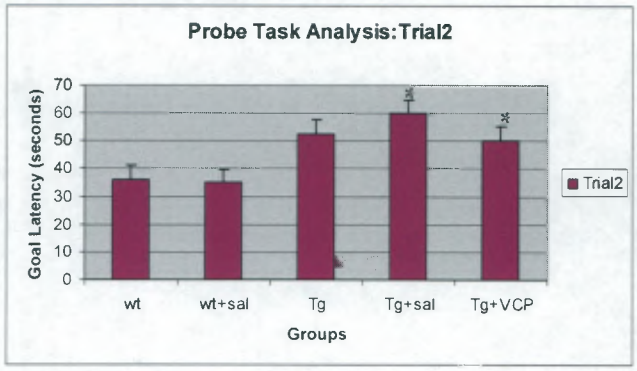
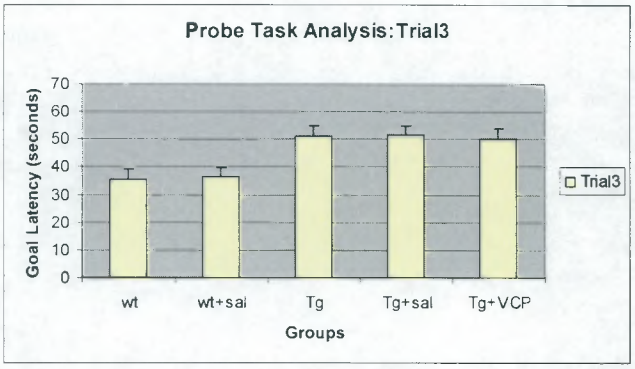


Fig.7.11. Food was removed at the beginning and end of the sessions on each of the two days of training (trials 1 and 4). A one-way ANOVA revealed significant differences within groups in the first three trials where wt= wildtype; wt+sal= saline-injected wildtype; Tg= transgenic; Tg+sal= saline-injected transgenic; Tg+VCP= VCP-injected transgenic. These abbreviations apply to all following graphs. Error bars indicate SEM.

The probe task analysis revealed significant differences within groups in trial 1 [$F(4,55)=3.89$ ($p=0.007$)], trial 2 [$F(4,55)= 5.53$ ($p=0.0008$)] and trial 3 [$F(4,55)=2.72$ ($p=0.038$)] as shown in Fig 7.11 and 7.12. Although, there was no significant difference between VCP-injected and saline injected mice in trial 1, VCP-injected mice showed a 29% improvement in goal latency scores than saline-injected transgenic mice. On further analysis of trial 2, there was a significant difference [$F(1,22) =5.47$ ($p=0.028$)] between saline-injected and VCP-injected transgenic mice.







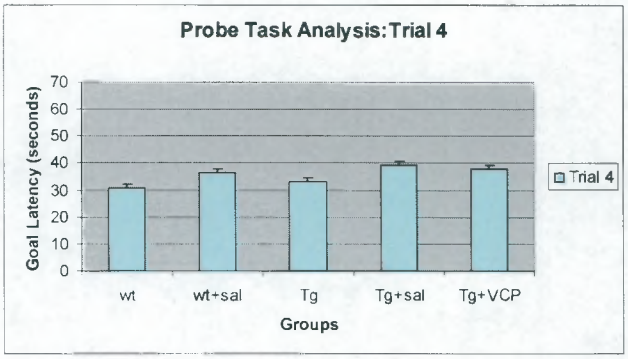


Fig.7.12. A one-way ANOVA showed that there are significant differences within groups in the first three trials. In trial 2, a significant difference was found between saline-injected and VCP-injected transgenic mice (*$p<0.05$). There was an overall decrease in goal latency by trial 4 in all transgenic groups. Error bars indicate SEM.

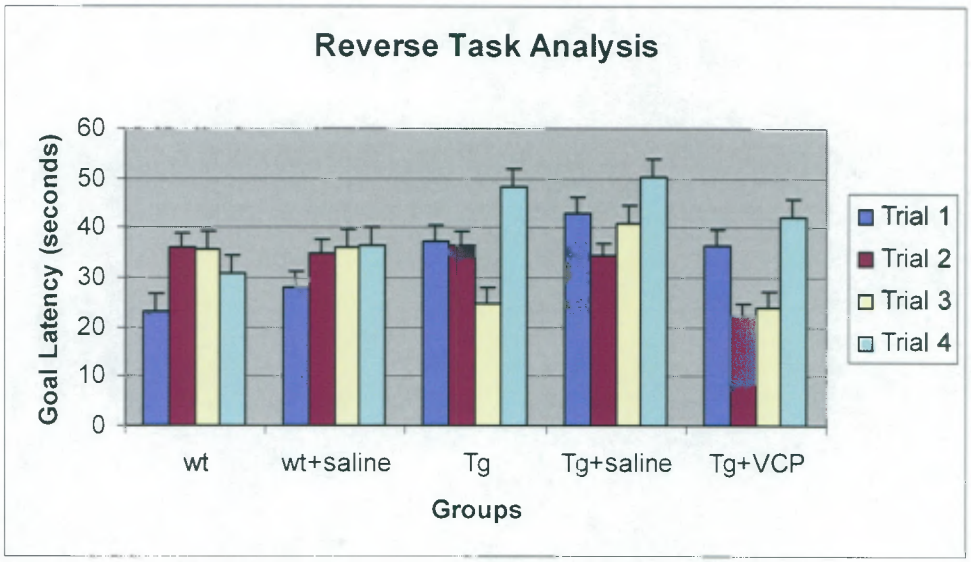
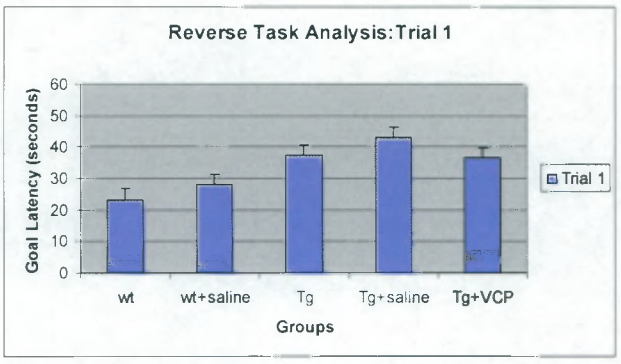
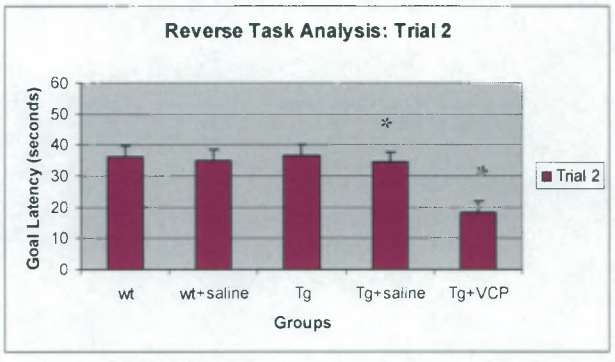
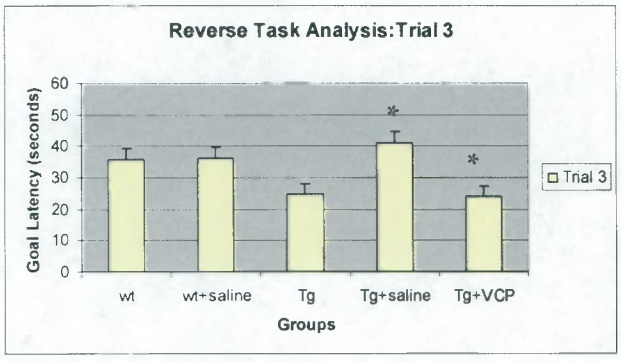


Fig.7.13. Reverse task analysis showed significant differences in trials 1 and 4 (food was removed). Error bars indicate SEM.

The reverse task analysis revealed significant differences between groups in trial 1 [F(4,55)=2,23 (p=0.077)] and trial 4 [F(4,55)=2.66 (p=0.04)] as shown in Fig. 7.13 and 7.14. On further analysis of trials, trial 2 [F(1,22)=7.76 (p=0.01)] and trial 3 [F(1,22)=4.35 (p=0.05)] showed significant differences between saline-injected and VCP-injected transgenic mice as indicated in Fig. 7.14.







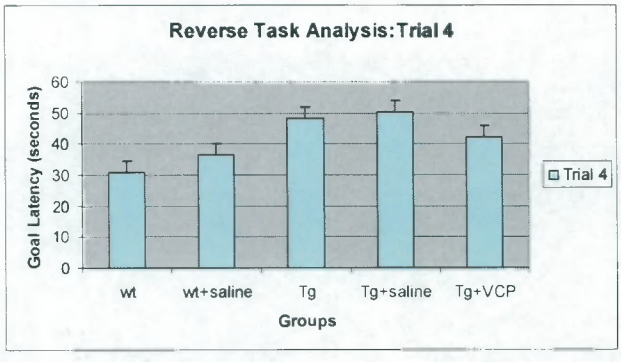


Fig.7.14. A one-way ANOVA revealed significant differences between saline-treated and VCP-injected mice in trials 2 and 3 (*$p<0.05$). Error bars indicate SEM.

7.4. DISCUSSION

In this part of the study transgenic mice progenies were bred from two breeding pairs as shown in Fig.7.3 and 7.4. The presence of transgenes APPswe/PS1De9 and the prion promoter were confirmed by genotyping as shown in representative gels in Fig.7.5 -7.7. This study examines episodic-like and reference memory using a modified version of a cheeseboard maze. At the initial stage of the experimental procedure (habituation phase) the use of a cheeseboard maze (a dry maze) was able to distinguish activity differences between wildtype and APPswe/PS1De9 transgenic mice. In this phase of training, 2 – 3 month old transgenic group and an age-matched wildtype mice group were required to find and eat as many food reward pellets baited in all 156 wells of the cheeseboard. In the initial phase of the task (first four days), transgenic mice showed a delay in habituating to the task procedure. Over the 10-day task of habituation a significant difference [$F(1,16)=7.08(p=0.017)$] was evident between the wildtype and transgenic groups. In the pre-training tasks no significant differences between the wildtype and transgenic groups were found in the time taken to find the flag. However, there was an overall decrease in goal latency and an increase in the number of pellets eaten over 5 days of training. This suggested that the mice had learnt that at each flag was baited with food reward pellets. The cued task required incremental learning over 8 days. Mice were released from a constant starting position in order to form a long-term reference memory. A steady decrease in the time taken to find the flag was evident in wildtype but not in transgenic mice as indicated by the trend lines in Fig. 7.9 and Fig. 7.10. Noteworthy, is that the transgenic mice in the cued task phase display a much more rapid acquisition of the food than the wildtype mice. This may be due to the transgenic mice being more active than the wildtype mice, but this aspect of the observation was not pursued and would require further investigation by video tracking.

After spatial acquisition training, that entailed learning of "new" events (i.e. different flag and starting positions) maintaining the same extra-maze environment, no significant differences were found between transgenic and wildtype groups. After the training phases, mice were randomly divided into

treatment groups and either received no injections or intracranial injections of VCP or saline. The probe task consisted of four trials in total each day and was carried out two weeks following administration of treatment in order to assess the effect of treatment on memory between groups. The probe trial was performed at the beginning and end of the trial block. This block of trials relies on the use of reference memory. Goal latencies of trial 1 (probe) [$F(4,55)=3.89$ ($p=0.007$)] and food trials, trial 2 [$F(4,55)= 5.53$ ($p=0.0008$)] and trial 3 [$F(4,55)=2.72$ ($p=0.038$)]) demonstrated significant differences between groups. VCP-injected APPswe/PS1De9 mice did significantly better [$F(1,22) =5.47$ ($p=0.028$)] at finding the flag than saline-injected APPswe/PS1De9 mice. In the first trial, VCP-treated mice also performed 29% better at finding the flag than saline-treated mice. These findings perhaps imply that to an extent a trace of reference memory may still be intact in the VCP-treated mice enabling a better performance in the task. The reversal task trial block assesses the ability of mice to form new memory of unique events in time (episodic memory) and in so doing suppress memories of old food locations (Savonenko et al., 2005). In food trials 2 and 3, VCP-treated mice performed significantly better than saline-treated mice. This observation implies that early treatment with VCP offers improvement in episodic memory. This is an important finding because in AD patients episodic memory is the first to deteriorate in the initial phases of disease progression (as discussed in chapter 1, see the nature of memory processes). Although, this behavioural paradigm took 34 days in total training and testing time, it is evident that it is a robust approach in detecting early changes in cognition as early as in 2-month old mice. A previous study using the Morris water maze to assess reference and episodic-like memory in APPswe/PS1De9 mice could not distinguish cognitive measures between wildtype and 6- month old transgenic mice (Savonenko et al.,2005). Taking all factors into consideration, the current study suggests that our modified-version of the cheeseboard is effective in distinguishing cognitive measures (i.e. episodic and reference memory) and that VCP is a promises to be a possible therapeutic agent for early-onset AD.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Over the years the elucidation of the pathophysiology of TBI and AD has given researchers clues on therapeutics targets and interventions.

However, diseases of the brain present a challenge for therapeutic interventions not only because the brain is an anatomically and functionally complex structure but also because few molecules are able to cross the blood-brain barrier. Viral vectors used in gene therapy have become alternative options to pharmacological intervention in neurological diseases; however these therapies are not without obstacles of their own. There are a number of factors that have to be taken into account such as the specificity and efficiency with which the vector infects the target cells, the duration of expression, determining the number of cells that must be transduced to examine the effect of change and regulating the levels of toxicity (Smith et al., 1991; Lundstrom, 2003). Most vectors used in gene therapy originate from viral pathogens commonly found in humans (Davidson and Breakefield, 2003). Their ability to introduce their genetic material into recipient cells has made viruses good choices for gene delivery systems. These vectors have the ability to infect many cell types besides those involved in their own normal life cycle, therefore offering a broad cellular host range.

Inflammation mediated by the complement system plays a major contributory role in disease progression and tissue destruction. Since TBI and AD affect brain functioning, patients suffer from cognitive deficits that manifest in behavioural, psychological and emotional problems that not only affect the victims but also the family or caregivers (van der Sluis et al., 1998; Farias et al., 2003).

This project used animal models that closely resemble the human condition of mild and severe head trauma and a transgenic AD mouse model to test a strategy targeted at attenuating the complement system by use of VCP.

The data presented in this study, together with the limited information on the efficacy of VCP in severity-dependent brain injury, contributes to conceptualising the therapeutic and neuroprotective effects of VCP.

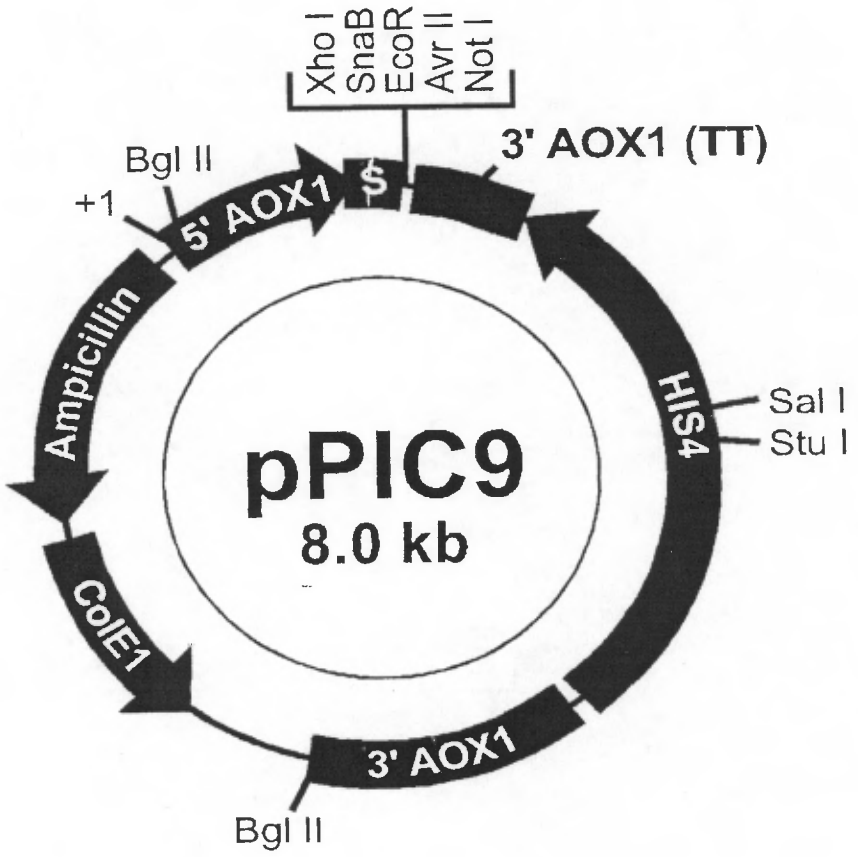
We conclude from the results of the neurological tests that in the FPI rats that VCP, at a dose lower than previously reported positively influences neurological outcome in both mild and severe TBI and may offer some improvement in spatial memory retention in mild TBI. VCP also shows promise as a therapeutic agent at early intervention in AD, since behavioural tests show significantly improved episodic-like memory in the APPswe/PS1dE9 mouse model. These transgenic mice have been reported to show plaque deposition at 6 months of age. According to Savonenko and co-workers (2005), an increase in amyloid burden correlates with episodic-like memory impairment in APPswe/PS1dE9 mice between 6 and 18 months of age. Although the brains of the mice in our study were not examined for plaque deposition, it may be that VCP prevents or at least reduces the formation of β-amyloid and/or blocks the action of β-amyloid at this early stage (2-3months). Further plaque deposition may be arrested and hence may prevent the deterioration of episodic-like memory.

Patients of mild and severe head injury are likely to benefit from VCP treatment because of the significant sensorimotor improvement evident. VCP treatment may also be beneficial to patients in the initial phase of AD, when episodic-like memory usually deteriorates.

Future work should be directed at developing a more potent VCP to minimise the quantity required for *in vivo* studies; and most importantly, a more effective and practical means of VCP administration to the brain, other than direct injection should be investigated. We have investigated the administration of VCP via the cerebrospinal fluid route and although the data suggest that this route of administration is not effective, other factors need to be tested. It seems evident that the dose may not be optimal and/or the chemical structure of VCP does not readily lend itself to uptake across the blood-brain barrier. Other means, like gene-therapy using a vector that is stable in gene expression, able to transduce both dividing and non-dividing cells and most importantly safe to use should be investigated.

The effect of VCP administration on later stages of AD should also be further investigated in establishing the most effective stage [2-3 months (early), 6 months or 12-18 months (late)] of VCP administration in AD. This would be useful information for treatment regimens.

APPENDIX A: VECTOR MAP



Taken from the Invitrogen *Pichia Pastoris* Protein Expression Manual

APPENDIX B: BUFFERS, MEDIA AND SOLUTIONS

B1: 30% Acrylamide-bisacrylamide

Acrylamide	29.2g
N,N'-methylene bisacrylamide	0.8g

Dissolved in 60ml of warm dH_2O. Made up to 100ml with dH_2O and filter sterilized through a 0.4µm filter.
Stored at 4°C in a dark bottle.

B2: 0.02% Biotin

Biotin	0.02g

Made up to 100ml with dH_2O.Filter sterilized through a 0.22µm filter-driven syringe device and stored at 4°C.

B3: 5% Blocking solution

Non-fat milk powder	5g

Made up to 100ml with TBS.

B4: Buffered Glycerol-complex medium (BMGY)

Yeast extract	10g
Peptone	20g

Made up to 700ml with dH_2O.
Autoclaved at 121 °C for 20 minutes.
Allowed to cool at room temperature and added the following:

Potassium phosphate buffer (pH6)	100ml
10% glycerol	100ml
13.4% YNB	100ml
0.02% biotin	2ml

Stored at 4°C.

B5: Buffered Methanol-complex medium (BMMY)

Same as BMGY expect replaced 10% glycerol with 100ml of filter sterilized 5% methanol. Stored at 4°C.

B6: Coomassie Brilliant Blue Stain

Coomassie Blue R-250	0.25g
Methanol	45ml
dH_2O	45ml
Glacial acetic acid	10ml

Reagents were mixed together before glacial acetic acid was added. The solution was filtered through 0.4µm filter paper.

B7: Destain

Methanol	454ml
Glacial acetic acid	75ml

Made up to 1litre with dH_2O.

B8: 10% Buffered Formalin

Formaldehyde	200ml
$Na_2HPO_4.H_2O$	15g
$NaH_2PO_4.2H_2O$	8g

Made up to 2 litre with dH_2O.

B9: Gel loading buffer (6X)

Bromophenol blue powder	0.025g
Glycerol	3ml

Made up to 10ml with dH_2O.

B10: 10% Glycerol

Glycerol	100ml

Made up to 1 litre with dH_2O.
Autoclaved at 121°C for 20 minutes.

B11: 0.4% Histidine

Histidine	0.04g

Made up to 10ml with dH_2O.

B12: Lysis Buffer for Mouse Tail DNA extraction

100mM NaCl	20ml
10mM Tris-Cl	5ml
25mM EDTA	50ml
0.5% SDS	12.5ml

Made up to 500ml with dH_2O.

B13: 5% Methanol

Methanol	50ml

Made up to 1 litre with dH_2O.
Filter sterilized through a 0.22µm filter-driven syringe device. Stored at room temperature.

B14: Minimal Methanol Histidine (MMH)

Agar	15g

Made up to 800ml with dH_2O.
Autoclaved at 121 °C for 20 minutes. Kept in the water-bath at 60°C before adding the following:

5% methanol	100ml

0.02% biotin	2ml
0.4% histidine	10ml
10% YNB	100ml

Plates were poured immediately. Once set, plates were stored at 4°C.

B15: Physiological Saline

NaCl	9g

Made up to 1 litre with dH_2O.

B16: Ponceau S Stain

Ponceau S	0.1%(w/v)

Made up to 100ml with 1% glacial acetic acid.
Stored at room temperature.

B17: 0.1M Potassium Phosphate Buffer (pH6)

1M K_2HPO_4	132ml
1M KH_2PO_4	868ml

Adjusted to pH 6 with KOH and autoclaved at 121°C for 20 minutes.

B18: 12%Resolving gel

dH_2O	1.6ml
30% acrylamide mix	2ml
1.5M Tris (pH8.8)	1.3ml
10% SDS solution	500µl
10% Ammonium persulphate	500µl
TEMED	2µl

B19: 5%Stacking gel

dH_2O	2.1ml
30% acrylamide mix	500µl
1M Tris (pH6.8)	380 µl
10% SDS solution	30 µl
10% Ammonium persulphate	30 µl
TEMED	3 µl

Ammonium persulphate followed by TEMED were added last to initiate polymerization.

B20: 1%TBS-T

Tween-20	1ml
TBS	999ml

B21: TBE (5X)

Tris	5.4g
Boric Acid	2.75g
EDTA	0.292g

Made up to 100ml with dH_2O.

B22: Tris-Buffered Saline (TBS)

Tris-base	50mM
NaCl	150mM
dH_2O	800ml

Adjusted pH to 7.5 and then added dH_2O to a final volume of 1 litre.

B23: TE (10mM Tris-Cl pH8, 1mM EDTA)

1M Tris-Cl (pH8)	1ml
500mM EDTA	200µl

Made up to 100ml with dH_2O.
Autoclaved at 121°C for 20 minutes.

B24: Western Blot Transfer Buffer

Tris-base	48mM
Glycine	39mM
Methanol	20%

Made up to 1 litre with dH_2O.

B25: Yeast Nitrogen Base (YNB)
(with ammonium sulphate, without amino acids)

YNB	134g

Made up to 1 litre with dH_2O.Filter-sterilised and stored at 4°C in a darkened bottle.

B26: Yeast Peptone Dextrose (YPD) Media

Yeast extract	10g
Bactopeptone	20g
Dextrose	20g

Made up to 1 litre with dH_2O. Autoclaved at 121°C for 20 minutes.

APPENDIX C:
MILD HEAD INJURY DATA COLLECTION AND ANALYSIS

C1: PRE-INJURY TRAINING

	DAY 1 PRE-INJURY TESTING							
Rat number	Time (secs)							
	Tr1	Tr2	Tr3	Tr4	Tr5	Tr6	Tr7	Tr8
2	81	62	38	81	34	7	3	19
12	113	33	39	4	5	26	3	5
13	60	52	34	26	8	7	26	1
40	120	15	13	59	30	8	9	3
55	120	30	24	18	13	4	18	2
62	72	43	6	1	13	43	14	2
1	78	57	15	120	5	85	39	34
10	80	101	20	32	11	120	6	120
14	81	120	12	8	23	16	18	32
21	120	7	8	32	41	7	4	11
31	120	120	80	64	22	31	38	123
41	120	33	44	37	23	16	17	84
3	96	98	28	2	8	33	59	24
20	36	7	56	2	42	27	25	3
24	107	37	32	2	8	18	9	2
25	69	16	5	15	86	41	26	11
44	120	45	8	13	43	16	11	1
45	27	86	10	5	25	6	13	2
5	120	40	15	3	21	16	5	1
15	120	51	3	16	9	7	7	2
17	72	13	26	7	17	10	9	8
28	48	27	13	35	27	11	5	9
35	102	115	7	115	120	101	14	4
58	120	13	42	14	6	7	10	1
Average	92	51	24	30	27	28	16	21
SEM	6	7	4	7	5	6	3	7

	DAY 2 PRE-INJURY TESTING							
Rat number	Time (secs)							
	Tr1	Tr2	Tr3	Tr4	Tr5	Tr6	Tr7	Tr8
2	69	8	4	1	5	7	3	1
12	65	5	2	22	10	10	9	5
13	4	8	5	3	6	9	2	5
40	10	28	2	22	6	20	16	11
55	10	9	6	26	17	14	18	20
62	66	25	9	3	19	12	13	3
1	80	25	75	120	50	15	23	23
10	65	15	5	3	4	4	3	4
14	108	5	37	12	7	6	2	8
21	41	5	25	1	8	8	6	17
31	107	6	5	10	5	9	7	2
41	15	14	13	1	22	9	7	3
3	16	120	47	7	16	48	5	21
20	120	6	9	9	43	15	7	3
24	80	11	10	3	4	14	4	4
25	15	10	30	2	33	4	6	26
44	12	13	11	6	42	10	5	12
45	102	7	7	5	5	4	11	5
5	2	67	31	3	26	28	4	1
15	65	6	4	6	5	5	4	2
17	13	5	7	16	2	10	1	15
28	48	10	5	7	23	12	2	1
35	120	74	60	39	16	8	21	19
58	71	77	25	16	6	27	2	47
Average	54	23	18	14	16	13	8	11
SEM	8	6	4	5	3	2	1	2

C2: 14-DAY POST-INJURY TESTING

	14-day post-injury test			
Rat number	Time (secs)			
Sham+Saline	Quad 1	Target quad2	Quad 3	Quad 4
2	24	16	9	11
12	24	22	5	9
13	19	27	5	9
40	15	35	4	6
55	15	33	6	6
62	18	29	2	11
	19	27	5	9
STDEV	1.7	2.9	0.9	0.9
FPI+VCP				
5	19	22	10	9
15	18	30	6	6
17	19	34	1	4
28	12	41	3	5
35	23	26	8	3
58	12	36	6	6
	17	32	6	6
STDEV	1.8	2.8	1.3	0.8
Sham+VCP				
1	23	33	1	3
10	19	33	2	6
14	4	37	10	9
21	11	34	1	14
31	18	35	4	3
41	22	32	0	6
	16	34	3	7
STDEV	3.0	0.7	1.5	1.7
FPI+Saline				
3	8	31	7	14
20	14	26	3	17
24	17	26	5	12
25	15	30	6	9
44	11	32	6	11
45	13	37	4	6
	13	30	5	12
STDEV	1.3	1.7	0.6	1.6

C3: PARAMETRIC RESULTS AND POST-HOC ANALYSIS

ANOVA: Time spent by groups in all quadrants during the probe trial.

	Univariate Tests of Significance for Var2 (Spreadsheet2 Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	21570.01	1	21570.01	172.6658	0.000000
"Var1"	0.03	3	0.01	0.0001	0.999999
Error	11492.96	92	124.92		

ANOVA: Time spent by groups in each quadrant and post-hoc comparisons.

Quadrant 1

	Univariate Tests of Significance for Var2 (Spreadsheet2 Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	6435.375	1	6435.375	260.2781	0.000000
"Var1"	119.125	3	39.708	1.6060	0.219490
Error	494.500	20	24.725		

	Bonferroni test; variable Var2 (Spreadsheet2) Probabilities for Post Hoc Tests Error: Between MS = 24.725, df = 20.000				
Cell No.	Var1	{1} 19.167	{2} 17.167	{3} 16.167	{4} 13.000
1	1		1.000000	1.000000	0.264862
2	2	1.000000		1.000000	0.973080
3	3	1.000000	1.000000		1.000000
4	4	0.264862	0.973080	1.000000	

Quadrant 2

Effect	Univariate Tests of Significance for Var2 (Spreadsheet2 Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	22632.04	1	22632.04	766.1058	0.000000
"Var1"	152.12	3	50.71	1.7165	0.195777
Error	590.83	20	29.54		

Cell No.	Bonferroni test; variable Var2 (Spreadsheet2) Probabilities for Post Hoc Tests Error: Between MS = 29.542, df = 20.000				
	Var1	{1} 27.000	{2} 31.500	{3} 34.000	{4} 30.333
1	1		1.000000	0.223879	1.000000
2	2	1.000000		1.000000	1.000000
3	3	0.223879	1.000000		1.000000
4	4	1.000000	1.000000	1.000000	

Quadrant 3

Effect	Univariate Tests of Significance for Var2 (Spreadsheet2 Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	541.5000	1	541.5000	68.11321	0.000000
"Var1"	25.5000	3	8.5000	1.06918	0.384459
Error	159.0000	20	7.9500		

Cell No.	Bonferroni test; variable Var2 (Spreadsheet2) Probabilities for Post Hoc Tests Error: Between MS = 7.9500, df = 20.000				
	Var1	{1} 5.1667	{2} 5.6667	{3} 3.0000	{4} 5.1667
1	1		1.000000	1.000000	1.000000
2	2	1.000000		0.702205	1.000000
3	3	1.000000	0.702205		1.000000
4	4	1.000000	1.000000	1.000000	

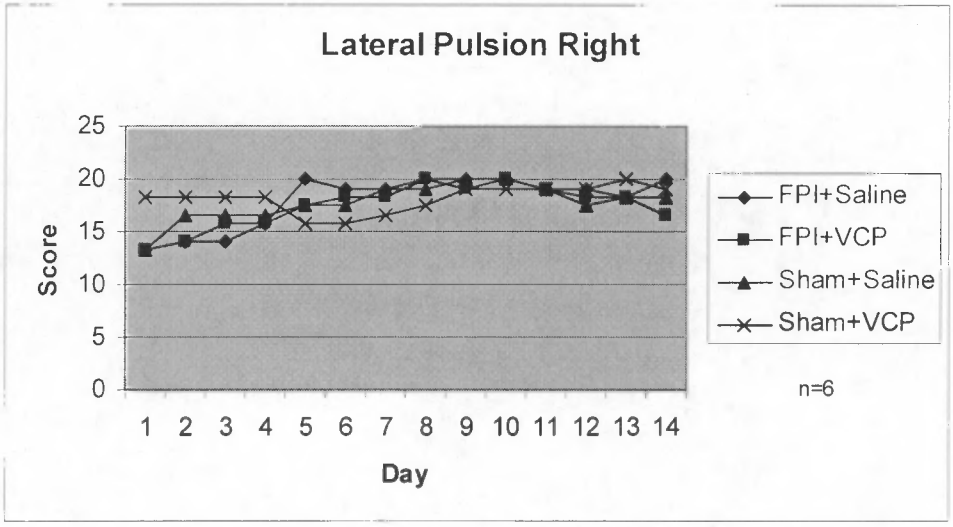
Quadrant 4

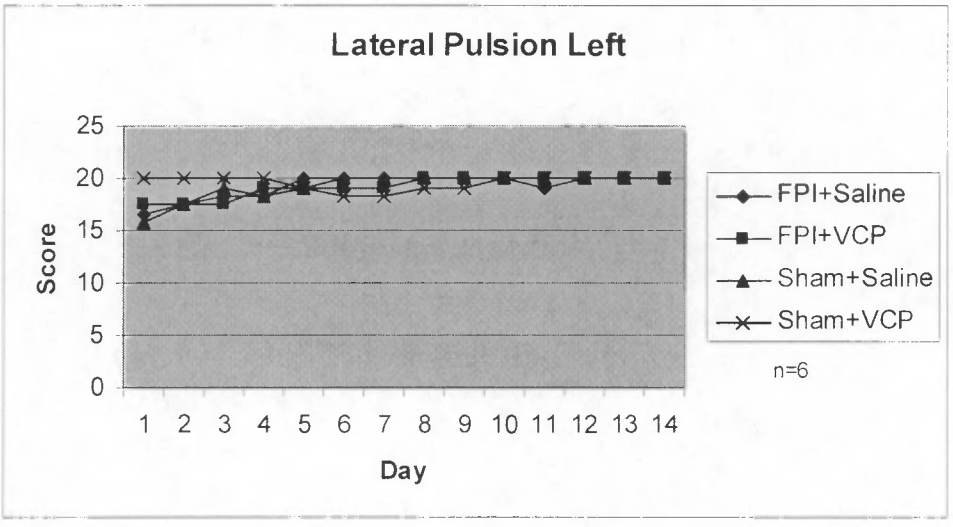
Effect	Univariate Tests of Significance for Var2 (Spreadsheet2 Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	1584.375	1	1584.375	152.9566	0.000000
"Var1"	121.458	3	40.486	3.9086	0.023923
Error	207.167	20	10.358		

Cell No.	Bonferroni test; variable Var2 (Spreadsheet2) Probabilities for Post Hoc Tests Error: Between MS = 10.358, df = 20.000				
	Var1	{1} 8.6667	{2} 5.5000	{3} 6.8333	{4} 11.500
1	1		0.623001	1.000000	0.857800
2	2	0.623001		1.000000	0.025249
3	3	1.000000	1.000000		0.124325
4	4	0.857800	0.025249	0.124325	

C4: LATERAL PULSION

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Lateral Pulsion Right														
FPI+Saline	13.3	14.1	14.1	15.8	20	19.1	19.1	20	20	20	19.1	19.1	18.3	20
FPI+VCP	13.3	14.1	15.8	15.8	17.5	18.3	18.3	20	19.1	20	19.1	18.3	18.3	16.6
Sham+Saline	13.3	16.6	16.6	16.6	17.5	17.5	19.1	19.1	20	20	19.1	17.5	18.3	18.3
Sham+VCP	18.3	18.3	18.3	18.3	15.8	15.8	16.6	17.5	19.1	19.1	19.1	19.1	20	19.1
Lateral Pulsion Left														
FPI+Saline	16.6	17.5	18.3	18.3	20	20	20	20	20	20	19.1	20	20	20
FPI+VCP	17.5	17.5	17.5	19.1	19.1	19.1	19.1	20	20	20	20	20	20	20
Sham+Saline	15.8	17.5	19.1	18.3	19.1	20	20	20	20	20	20	20	20	20
Sham+VCP	20	20	20	20	19.1	18.3	18.3	19.1	19.1	20	20	20	20	20


Lateral Pulsion Right
Score
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day
FPI+Saline
FPI+VCP
Sham+Saline
Sham+VCP
n=6


Lateral Pulsion Left
Score
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day
FPI+Saline
FPI+VCP
Sham+Saline
Sham+VCP
n=6

C5: HINDPAW GRIP TEST

Hindgrip Paw test														
Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
FPI+Saline														
Hindpaw grip left	16.6	17.5	17.5	19.1	20	20	20	20	20	20	20	20	20	20
Hindpaw grip right	10.8	12.5	14.1	13.3	16.6	17.5	18.3	19.1	18.3	19.1	17.5	19.1	18.3	19.1
FPI +VCP														
Hindpaw grip left	17.5	20	17.5	19.1	18.3	19.1	18.3	19.1	20	20	20	20	20	20
Hindpaw grip right	11.6	13.3	17.5	16.6	18.3	18.3	18.3	17.5	19.1	18.3	19.1	18.3	17.5	18
Sham + Saline														
Hindpaw grip left	15.8	17.5	18.3	17.5	19.1	20	19.1	20	19.1	20	19.1	20	20	20
Hindpaw grip right	13.3	15.8	16.6	14.1	15.8	15	16.6	19.1	19.1	18.3	17.5	17.5	18.3	18.3
Sham + VCP														
Hindpaw grip left	18.3	19.1	20	20	18.3	18.3	19.1	19.1	20	20	20	20	20	20
Hindpaw grip right	17.5	16.6	15	16.6	13.3	16.6	15.8	16.6	16.6	19.1	18.3	19.1	20	19.1

HINDPAW GRIP						
SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**		
FPI+VCP	14	241.7	17.26428571	4.696319		
FPI+Saline	14	233.6	16.68571429	7.905934		
Sham+VCP	14	240.2	17.15714286	3.365714		
Sham+Saline	14	235.3	16.80714286	3.320714		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	3.2014286	3	1.067142857	0.221299	**0.881175**	2.78259904
Within Groups	250.75286	52	4.82217033			
Total	253.95429	55				

C6: AMBULATION TEST:ANOVA

Sham+VCP	Sham+Saline	FPI+Saline	FPI+VCP		Anova: Single Factor		ALL				
18.3	17.5	18.3	17.5								
18.3	15.8	17.5	17.5		SUMMARY						
19.1	17.5	15.8	20		*Groups*	*Count*	*Sum*	*Average*	*Variance*		
17.5	18.3	19.2	17.5		Sham+VCP	14	225.5	16.10714	8.871484		
15	15.8	16.6	17.5		Sham+Saline	14	217.2	15.51429	7.025934		
12.5	17.5	19.1	19.1		FPI+Saline	14	234.6	16.75714	1.911868		
19.1	18.3	17.5	17.5		FPI+VCP	14	248.9	17.77857	1.778736		
19.1	16.6	15.8	16.6								
15	15	15.8	16.6								
17.5	12.5	16.6	18.3		ANOVA						
10.8	10.8	15	20		*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
10.8	10.8	16.6	17.5		Between Groups	39.48929	3	13.1631	2.687989	0.055854	2.782599
17.5	13.3	15	18.3		Within Groups	254.6443	52	4.897005			
15	17.5	15.8	15								
					Total	294.1336	55				

Anova: Single Factor	FROM DAY 9					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Sham+VCP	6	86.6	14.43333	9.170667		
Sham+Saline	6	79.9	13.31667	6.733667		
FPI+Saline	6	94.8	15.8	0.512		
FPI+VCP	6	105.7	17.61667	2.901667		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	61.80833	3	20.60278	4.266027	0.017534	3.098393
Within Groups	96.59	20	4.8295			
Total	158.3983	23				

Anova: Single Factor	FROM DAY 9					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
FPI+Saline	6	94.8	15.8	0.512		
FPI+VCP	6	105.7	17.61667	2.901667		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.900833	1	9.900833	5.800703	0.03678	4.964591
Within Groups	17.06833	10	1.706833			
Total	26.96917	11				

C7: ANGLE-BOARD DATA

Group	**Sham+ Saline**							
Time	**24-hours post-injury**							
Angles		**20**	**25**	**30**	**35**	**40**	**45**	**50**
immediately slides down	Nose up				2	4	4	4
tries to grip but slides down	Nose up						1	
turn 45 and freezes	Nose up							
turn 180 and freezes	Nose up							
turn 180 and proceed to top	Nose up							
turns and proceeds to bottom	Nose up	1	1	1				
Walks backward on from pop	Nose up							
Freezes at pop	Nose up							
Immediately proceeds from point	Nose up	3	3	3	1	1		
of orientation								
immediately slides down	Nose down				1	1	1	4
tries to grip but slides down	Nose down					1	1	1
turn 45 and freezes	Nose down							
turn 180 and freezes	Nose down							
turn 180 and proceed to top	Nose down	1	1	1	1	1		
turns and proceeds to bottom	Nose down							

Walks backward on from pop	Nose down	1						
Freezes at pop	Nose down		1	1	1	1	1	
Immediately proceeds from point	Nose down	2	2	2	1			
of orientation								
Group	**Sham +VCP**							
Time	**24-hours post-injury**							
Angles		**20**	**25**	**30**	**35**	**40**	**45**	**50**
immediately slides down	Nose up			1	4	3	3	4
tries to grip but slides down	Nose up					1	1	
turn 45 and freezes	Nose up							
turn 180 and freezes	Nose up							
turn 180 and proceed to top	Nose up							
turns and proceeds to bottom	Nose up	1	2	1	1			
Walks backward on from pop	Nose up							
Freezes at pop	Nose up	1	1					
Immediately proceeds from point	Nose up	3	2	3				
of orientation								
immediately slides down	Nose down	1	1	1	2	2	2	2
tries to grip but slides down	Nose down					1	1	1
turn 45 and freezes	Nose down							
turn 180 and freezes	Nose down							
turn 180 and proceed to top	Nose down	2	1	2	1			
turns and proceeds to bottom	Nose down		1					

Walks backward on from pop	Nose down								
Freezes at pop	Nose down	1							
Immediately proceeds from point	Nose down	1	2	2	2	2	2	2	
of orientation									
Group	**FPI +VCP**								
Time	**24-hours post-injury**								
Angles		**20**	**25**	**30**	**35**	**40**	**45**	**50**	
immediately slides down	Nose up					2	4	3	
tries to grip but slides down	Nose up								
turn 45 and freezes	Nose up			1					
turn 180 and freezes	Nose up								
turn 180 and proceed to top	Nose up				1				
turns and proceeds to bottom	Nose up	2	1	1	1				
Walks backward on from pop	Nose up								
Freezes at pop	Nose up	1	1			1		1	
Immediately proceeds from point	Nose up		1	1	1				
of orientation									
immediately slides down	Nose down					3	2	2	
tries to grip but slides down	Nose down					1	1	2	
turn 45 and freezes	Nose down								
turn 180 and freezes	Nose down	1	1	1	1				
turn 180 and proceed to top	Nose down	1							
turns and proceeds to bottom	Nose down	2	1	1					
Walks backward on from pop	Nose down								

Freezes at pop	Nose down	1	1			1	1		
Immediately proceeds from point	Nose down		1	1	1				
of orientation									
Group	**FPI+ Saline**								
Time	**24-hours post-injury**								
Angles		**20**	**25**	**30**	**35**	**40**	**45**	**50**	
immediately slides down	Nose up		1	3	3	5	5	5	
tries to grip but slides down	Nose up								
turn 45 and freezes	Nose up								
turn 180 and freezes	Nose up								
turn 180 and proceed to top	Nose up			1	1				
turns and proceeds to bottom	Nose up								
Walks backward on from pop	Nose up					1	1	1	
Freezes at pop	Nose up	3	4	1	1				
Immediately proceeds from point	Nose up	3	1	1	1				
of orientation									
immediately slides down	Nose down	1	2	3	4	5	5	5	
tries to grip but slides down	Nose down								
turn 45 and freezes	Nose down								
turn 180 and freezes	Nose down								
turn 180 and proceed to top	Nose down	1							
turns and proceeds to bottom	Nose down	1	2	2	1				
Walks backward on from pop	Nose down		1						
Freezes at pop	Nose down	3	1	1					

Immediately proceeds from point			Nose down							
of orientation										

APPENDIX D:
THIRD VENTRICULAR ADMINISTRATION OF VCP: DATA COLLECTION AND ANALYSIS

D1: Pre-injury Training Scores

	DAY 1 PRE-INJURY TESTING							
	Time (secs)							
Rat number	Tr1	Tr2	Tr3	Tr4	Tr5	Tr6	Tr7	Tr8
3	120	60	15	11	21	21	11	11
8	115	49	37	17	11	8	9	32
2	120	60	60	60	60	50	48	20
25	120	12	25	13	18	60	2	14
16	107	64	60	50	8	2	9	5
18	120	27	60	3	11	8	40	54
70	120	26	17	9	56	4	15	39
35	105	20	20	22	38	10	26	3
60	55	120	60	50	8	20	60	5
9	120	20	5	7	15	17	6	20
33	120	48	16	3	7	12	22	2
11	120	57	23	3	7	7	11	3
	112	47	33	21	22	18	22	17

	DAY 2 PRE-INJURY TESTING							
	Time (secs)							
Rat number	Tr1	Tr2	Tr3	Tr4	Tr5	Tr6	Tr7	Tr8
3	43	24	8	9	4	57	18	13
8	60	17	14	53	14	21	5	15
2	60	60	60	13	55	31	12	22
25	54	31	6	6	15	37	12	2
16	33	16	31	12	10	12	4	13
18	44	20	3	16	7	7	5	8
70	21	14	9	30	12	21	7	9
35	29	23	60	19	13	3	43	41
60	60	31	58	48	14	12	13	15
9	17	37	10	32	13	15	26	43
33	18	5	8	5	2	11	37	8
11	57	60	37	16	28	27	3	2
	41	28	25	22	16	21	15	16

D2: Post-injury Probe Trial Scores

	14-days post-injury probe test			
Rat number	Time (secs)			
FPI+VCP				
3	14	31	3	12
8	35	22	1	2
2	8	8	14	30
25	12	27	1	20
16	16	17	17	10
18	5	35	4	16
	15	23	7	15
FPI+Saline				
70	25	19	10	6
35	14	31	9	6
60	17	28	5	10
9	11	34	0	15
33	18	28	6	8
11	5	26	11	18
	15	28	7	11

D3: Parametric Analysis of Probe Trial

	Univariate Tests of Significance for Var2 (Spreadsheet Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	6864.083	1	6864.083	104.8218	0.000001
"Var1"	234.083	1	234.083	3.5747	0.087962
Error	654.833	10	65.483		

APPENDIX E:
SEVERE HEAD INJURY DATA COLLECTION AND ANALYSIS

E1: Pre-training

	DAY 1 PRE-INJURY TESTING							
Rat number	Time (secs)							
	Tr1	Tr2	Tr3	Tr4	Tr5	Tr6	Tr7	Tr8
6	120	43	23	8	20	13	3	43
30	100	60	17	55	11	60	1	4
60	120	60	60	60	60	2	36	27
70	120	48	5	46	3	25	17	20
20	90	120	60	46	26	60	7	32
35	120	60	60	60	60	38	60	60
26	50	60	15	3	11	48	14	16
22	120	46	23	9	10	11	2	8
4	76	60	56	2	10	4	47	4
7	120	34	3	32	2	15	15	17
9	120	28	3	3	33	48	49	48
11	120	60	2	18	60	7	47	15
	106	57	27	29	26	28	25	25

	DAY 2 PRE-INJURY TESTING							
Rat number	Time (secs)							
	Tr1	Tr2	Tr3	Tr4	Tr5	Tr6	Tr7	Tr8
6	19	8	7	3	13	34	5	16
30	120	46	1	4	16	1	12	6
60	54	19	36	2	17	24	2	3
70	73	4	2	2	19	6	8	5
20	23	57	35	3	60	26	7	39
35	120	28	28	5	10	2	9	52
26	51	4	8	17	3	12	7	17
22	60	7	11	10	4	5	12	27
4	42	4	8	5	4	6	11	3
7	10	17	2	14	7	8	10	20
9	120	29	16	2	48	60	60	60
11	45	35	7	32	14	35	5	3
	61	22	13	8	18	18	12	21

E2: Post-injury testing

	14-day post-injury test			
Rat number	Time (secs)			
FPI+VCP				
20	11	29	5	15
35	11	36	4	9
30	7	35	2	16
60	8	39	5	8
70	7	30	3	20
6	3	26	7	23
	17	32	6	6
	1.2	2.0	0.7	2.4
FPI+Saline				
9	5	29	8	18
11	8	40	3	9
22	12	19	12	17
4	0	44	5	11
26	5	17	12	26
7	3	28	5	24
	13	30	5	12
	1.7	4.4	1.6	2.8

E3. Parametric Results and Post-Hoc Analysis of Probe Trial

Effect	Univariate Tests of Significance for Var2 (Spreadsheet Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	11532.00	1	11532.00	162.1941	0.000000
"Var1"	27.00	1	27.00	0.3797	0.551508
Error	711.00	10	71.10		

Cell No.	Bonferroni test; variable Var2 (Spreadsheet Probabilities for Post Hoc Tests Error: Between MS = 71.100, df = 10.000		
	Var1	{1} 32.500	{2} 29.500
1	1		0.551508
2	2	0.551508	

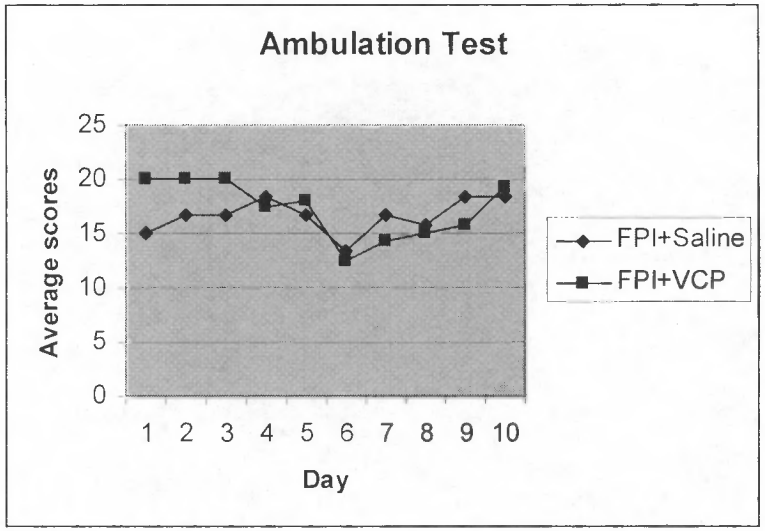
E4: Angle-board Data

Group			FPI +VCP								
Time			24-hours post-injury								
Angles					20	25	30	35	40	45	50
immediately slides down			Nose up								3
tries to grip but slides down			Nose up					1	4	4	3
turn 45 and freezes			Nose up				1	2			
turn 180 and freezes			Nose up				1				
turn 180 and proceed to top			Nose up		6	5	2	1	2	1	
turns and proceeds to bottom			Nose up					1			
Walks backward on from pop			Nose up								
Freezes at pop			Nose up					1			
Immediately proceeds from point			Nose up								
of orientation											
immediately slides down			Nose down							3	4
tries to grip but slides down			Nose down						4	2	2
turn 45 and freezes			Nose down								
turn 180 and freezes			Nose down								
turn 180 and proceed to top			Nose down			2	1	1			
turns and proceeds to bottom			Nose down		3	2	3	2	2		
Walks backward on from pop			Nose down								
Freezes at pop			Nose down					1			
Immediately proceeds from point			Nose down								
of orientation											
Climbs off board					2	3	4	2		1	

Group			FPI+ Saline								
Time			24-hours post-injury								
Angles					20	25	30	35	40	45	50
immediately slides down			Nose up						2	3	6
tries to grip but slides down			Nose up				1	2	2	3	
turn 45 and freezes			Nose up			1			1		
turn 180 and freezes			Nose up				1	1			
turn 180 and proceed to top			Nose up		1						
turns and proceeds to bottom			Nose up			1					
Walks backward on from pop			Nose up				1				
Freezes at pop			Nose up					1	1		
Immediately proceeds from point			Nose up		4	4	2	1			
of orientation											
immediately slides down			Nose down						1	3	6
tries to grip but slides down			Nose down					2	3	3	
turn 45 and freezes			Nose down				1		1		
turn 180 and freezes			Nose down								
turn 180 and proceed to top			Nose down								
turns and proceeds to bottom			Nose down								
Walks backward on from pop			Nose down								
Freezes at pop			Nose down					1			
Immediately proceeds from point			Nose down		4	4	4	3	1		
of orientation											
Climbs off board					3	2	1	1			

E5: Ambulation scores and Non-Parametric Data Analysis

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
FPI+SALINE	15	16.6	16.6	18.3	16.6	13.3	16.6	15.8	18.3	18.3
FPI+VCP	20	20	20	17.5	18	12.5	14.2	15	15.8	19.2



variable	Mann-Whitney U Test (Spreadsheet2) By variable Var1 Marked tests are significant at p <.05000 Rank Sum Group 1	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Group 1	Valid N Group 2	2*1sided exact p
Var2	96.00000	114.0000	41.00000	-0.680336	0.496292	-0.685510	0.493023	10	10	0.528849

Effect	Univariate Tests of Significance for Var2 (Spreadsheet Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	5698.688	1	5698.688	1154.359	0.000000
"Var1"	2.312	1	2.312	0.468	0.502472
Error	88.860	18	4.937		

Cell No.	Bonferroni test; variable Var2 (Spreadsheet Probabilities for Post Hoc Tests Error: Between MS = 4.9367, df = 18.000 Var1	{1} 16.540	{2} 17.220
1	1		0.502472
2	2	0.502472	

E6: Neurological Evaluations

Hindpaw grip Test

Hindpaw grip test										
Day	1	2	3	4	5	6	7	8	9	10
FPI+VCP										
hindpaw grip left	19.1	20	20	20	20	20	20	20	20	20
hindpaw grip right	19.1	20	20	20	20	20	20	20	20	20
FPI+SAL										
hidpaw grip left	16.6	17.5	17.5	17.5	18.3	18.3	18.3	18.3	18.3	18.3
hindpaw grip right	15.8	18.3	20	20	20	20	20	20	20	20

Forelimb Extension

Forelimb extension										
Day	1	2	3	4	5	6	7	8	9	10
FPI+VCP										
forelimb extension left	18.3	20	20	20	20	20	20	20	20	20
forelimb extension right	19.1	20	20	19.1	20	20	20	20	20	20
FPI+SAL										
forelimb extension left	19.1	20	19.1	20	20	20	20	20	20	20
forelimb extension right	19.1	18.3	19.1	19.1	17.5	18.3	20	20	20	20

Hind limb Extension

Hind limb extension											
Day		1	2	3	4	5	6	7	8	9	10
FPI+VCP											
hindlimb extension left		19.1	20	20	20	20	20	20	20	20	20
hindlimb extension right		19.1	20	20	20	20	20	20	20	20	20
FPI+SAL											
hindlimb extension left		19.1	20	20	19.1	18.3	20	20	20	20	20
hindlimb extension right		17.5	19.1	20	19.1	20	20	20	20	20	20

Visual Placing Test

Visual placing											
Day		1	2	3	4	5	6	7	8	9	10
FPI+VCP		19.1	19.1	20	20	20	20	20	20	20	20
FPI+SAL		15.8	17.5	19.1	19.1	19.1	20	20	20	20	20

E7: Tactile Placing Scores and Non-Parametric Data analysis

Tactile placing											
Day		1	2	3	4	5	6	7	8	9	10
FPI+VCP		18.3	20	20	20	20	20	20	20	20	20
FPI+SAL		15	15.8	17.5	17.5	18.3	19.1	20	20	20	20

Mann-Whitney U Test (Spreadsheet7)
By variable Var1
Marked tests are significant at p <.05000

variable	Rank Sum Group 1	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Group 1	Valid N Group 2	2*1sided exact p
Var2	131.5000	78.50000	23.50000	2.003212	0.045155	2.352958	0.018625	10	10	0.043257

Kruskal-Wallis ANOVA by Ranks; Var2 (Spreadsheet7
Independent (grouping) variable: Var1
Kruskal-Wallis test: H (1, N= 20) =5.536411 p =.0186

Depend.: Var2	Code	Valid N	Sum of Ranks
1	1	10	131.5000
2	2	10	78.5000

E8: Lateral Pulsion and Non-Parametric Data Analysis

Pulsion										
Day	1	2	3	4	5	6	7	8	9	10
FPI+VCP										
Pulsion left	17.5	19.1	20	20	20	20	20	20	20	20
Pulsion right	19.1	20	20	20	20	20	20	20	20	20
FPI+SAL										
Pulsion left	15.8	16.7	17.5	17.5	18.3	19.1	19.1	19.1	20	20
Pulsion right	15.8	16.7	16.7	18.3	19.1	19.1	20	20	20	20

Left Pulsion

Mann-Whitney U Test (Spreadsheet7)
By variable Var1
Marked tests are significant at p <.05000

variable	Rank Sum Group 1	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Group 1	Valid N Group 2	2*1sided exact p
Var2	136.5000	73.50000	18.50000	2.381176	0.017258	2.559645	0.010478	10	10	0.014690

Kruskal-Wallis ANOVA by Ranks; Var2 (Spreadsheet7
Independent (grouping) variable: Var1
Kruskal-Wallis test: H (1, N= 20) =6.551781 p =.0105

Depend.: Var2	Code	Valid N	Sum of Ranks
1	1	10	136.5000
2	2	10	73.5000

Right Pulsion

variable	Mann-Whitney U Test (Spreadsheet7) By variable Var1 Marked tests are significant at p <.05000 Rank Sum Group 1	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Group 1	Valid N Group 2	2*1sided exact p
Var2	132.0000	78.00000	23.00000	2.041008	0.041251	2.401092	0.016347	10	10	0.043257

Depend.: Var2	Kruskal-Wallis ANOVA by Ranks; Var2 (Spreadsheet7 Independent (grouping) variable: Var1 Kruskal-Wallis test: H (1, N= 20) =5.765245 p =.0163 Code	Valid N	Sum of Ranks
1	1	10	132.0000
2	2	10	78.0000

APPENDIX F:
MICE DETAILS, PRIMERS AND PCR CYCLYING CONDITIONS

F1. Mice Details

Mice that were randomly selected for genotyping

Mouse Identity Numbers
1-11, 43,48,49,52,61,65-67

Birth dates of mice

Mouse Identity Numbers	Birth date
1-4	20/6/05
5-11	12/7/05
43, 44,48,49,50	26/9/05
51,52,53,54,55	4/10/05
60-73	31/10/05

F2. Primers used

Primers were made according to JAX® Mice Data Sheet

GK1: 5' CCT CTT TGT GAC TAT GTG GAC TGA TGT CGG 3'

GK2: 5' GTG GAT AAAC CCC TCC CCC AGC CTA GAC C 3'

GK3: 5' AAT AGA GAA CGG CAG GAG CA 3'

GK4: 5' GCC ATG AGG GCA CTA ATC AT 3'

GK5: 5' GAC TGA CCA CTC GAC CAG GTT CTG 3'

GK6: CTT GTA AGT TGG ATT CTC ATA TCC G 3'

F3. Cycling conditions

Tg(APPswe):
Cycling Reaction A

Step	Temperature (°C)	Time	
1	94	3 min	
2	94	30 sec	
3	69	1 min	
4	72	1 min	Repeat steps 2-4, 35 cycles
5	72	2 min	
6	10		

Tg(PSEN1):
Cycling Reaction A

Step	Temperature (°C)	Time	
1	94	3 min	
2	94	30 sec	
3	67	1 min	
4	72	1 min	Go to step2, 35 times
5	72	2 min	
6	10		

Tg(PSEN1):
Cycling Reaction B

Step	Temperature (°C)	Time	
1	94	3 min	
2	94	30 sec	
3	70	1 min	
4	72	1 min	Go to step2, 35 times
5	72	2 min	
6	10		

APPENDIX G:
DATA COLLECTION AND ANALYSIS OF CHEESEBOARD MAZE: WILDTYPE AND TRANSGENIC ALZHEIMER'S DISEASE MICE

G1: HABITUATION PHASE DATA AND ANALYSIS

Gender	Genotype	ID	Starting position	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10
male	wildtype	30	Centre	0	7	18	19	16	16	19	21	13	25
male	wildtype	32	Centre	0	9	21	21	19	21	26	29	13	27
male	wildtype	34	Centre	0	2	6	9	5	17	20	16	22	23
male	wildtype	35	Centre	0	2	5	8	5	13	13	23	28	22
male	wildtype	36	Centre	2	3	5	9	7	15	16	32	26	25
male	wildtype	37	Centre	4	13	12	20	15	10	22	20	24	29
male	wildtype	38	Centre	4	8	9	13	10	18	16	22	12	18
female	wildtype	39	Centre	2	8	14	29	8	21	29	27	30	33
female	wildtype	40	Centre	8	8	10	16	9	18	22	24	26	23
female	wildtype	41	Centre	6	11	7	13	14	19	21	23	26	22
female	wildtype	42	Centre	2	4	3	16	6	12	11	15	17	18
female	wildtype	44	Centre	1	15	22	27	18	18	14	34	12	30
female	wildtype	45	Centre	0	5	15	16	9	6	7	2	9	19
female	wildtype	46	Centre	0	9	9	0	0	0	0	8	1	6
				2.1	**7.4**	**11.1**	**15.4**	**10.1**	**14.6**	**16.9**	**21.1**	**18.5**	**22.9**

Gender	Genotype	ID	Starting position	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10
male	transgenic	51	Centre	0	3	2	2	5	7	13	16	16	17
male	transgenic	53	Centre	0	15	4	16	16	4	1	18	23	18
male	transgenic	60	Centre	0	0	0	0	8	3	4	12	10	14
male	transgenic	61	Centre	0	0	0	0	9	11	6	4	10	15
male	transgenic	62	Centre	0	0	0	1	10	16	11	9	10	22
male	transgenic	63	Centre	0	0	0	0	8	8	5	2	9	12
male	transgenic	64	Centre	0	0	0	0	8	12	13	9	21	25
female	transgenic	43	Centre	0	1	0	0	5	5	10	7	17	6
female	transgenic	47	Centre	0	0	0	0	9	7	10	17	14	16
female	transgenic	48	Centre	0	0	0	0	4	3	7	9	11	12
female	transgenic	49	Centre	0	0	0	0	11	9	6	7	8	16
female	transgenic	50	Centre	0	0	0	3	8	11	11	8	9	19
female	transgenic	54	Centre	0	0	2	3	15	11	21	21	24	26
female	transgenic	68	Centre	0	0	0	0	5	4	5	6	8	7
				0	**1.4**	**0.6**	**1.8**	**8.6**	**7.9**	**8.8**	**10.4**	**13.6**	**16.1**

G2: PRE-TRAINING DATA

				Day1		Day2		Day3		Day4		Day5	
Gender	Genotype	ID	Starting position	Pellets	Mins	Pellets	Mins	Pellets	Mins	Pellets	Mins	Pellets	Mins
male	wildtype	30	Centre	12	5	11	3	16	16	16	2	16	1.5
male	wildtype	32	Centre	14	3	12	2	15	15	15	2	16	1.5
male	wildtype	34	Centre	15	3	11	5	16	16	11	5	16	3
male	wildtype	35	Centre	9	5	12	5	15	15	16	4	16	3
male	wildtype	36	Centre	6	5	12	4	12	12	16	5	12	3
male	wildtype	37	Centre	8	5	16	5	16	16	16	3	15	2
male	wildtype	38	Centre	0	5	2	5	12	12	16	5	16	4
female	wildtype	39	Centre	7	5	15	2	16	16	16	1	16	2
female	wildtype	40	Centre	11	5	12	5	16	16	16	2	8	5
female	wildtype	41	Centre	10	5	16	4	15	15	10	5	14	5
female	wildtype	42	Centre	5	5	4	5	13	13	16	4	16	5
female	wildtype	44	Centre	11	5	11	2	5	5	8	5	16	3.5
female	wildtype	45	Centre	6	5	11	5	10	10	10	5	9	4
female	wildtype	46	Centre	8	5	6	5	4	4	4	5	11	4.5
				9	5	11	4	13	13	13	4	14	3

				Day1		Day2		Day3		Day4		Day5	
Gender	Genotype	ID	Starting position	Pellets	Mins	Pellets	Mins	Pellets	Mins	Pellets	Mins	Pellets	Mins
male	transgenic	51	Centre	1	5	1	5	3	5	7	5	7	5
male	transgenic	53	Centre	5	3	8	5	12	5	16	4	16	3.05
male	transgenic	60	Centre	9	5	15	4.29	15	4	14	3.3	15	1.47
male	transgenic	61	Centre	16	4.58	16	2.25	16	3	14	1.55	15	2.27
male	transgenic	62	Centre	16	5	16	2.52	16	3	16	2.05	16	2.2
male	transgenic	63	Centre	16	5	16	3	13	5	14	3.04	8	3.1
male	transgenic	64	Centre	16	2.23	16	3.51	16	2	16	1.48	16	2.07
female	transgenic	43	Centre	15	4.28	14	2.5	16	3.55	16	1.73	16	2.43
female	transgenic	47	Centre	7	5	13	3.5	16	2.06	16	2.16	16	1.57
female	transgenic	48	Centre	12	5	16	2.58	16	2.3	15	2.1	16	1.58
female	transgenic	49	Centre	11	5	16	3.18	16	2.53	16	2.45	16	1.59
female	transgenic	50	Centre	6	5	15	4.25	16	2.49	16	1.49	16	2.42
female	transgenic	54	Centre	0	5	15	4.1	13	2.27	16	3.38	12	5
female	transgenic	68	Centre	3	5	8	5	16	3.39	12	2.07	14	5
				10	**5**	**13**	**4**	**14**	**3**	**15**	**3**	**14**	**3**

G3: CUED TASK DATA

	DAY 1		Trials		
		1	2	3	4
Wildtype		51.7	47.3	51	52.9
Transgenics		28.9	32.8	24.4	36.6

	DAY2		Trials		
		1	2	3	4
Wildtype		32.6	39.4	38.6	34.4
Transgenics		24.7	20	21.4	20

	DAY3		Trials		
		1	2	3	4
Wildtype		36.8	40.2	44.6	39.2
Transgenics		18.3	19.9	15.5	20.6

	DAY4		Trials		
		1	2	3	4
Wildtype		35.4	30.8	33.6	37.4
Transgenics		29.6	20.2	21.9	17.2

	DAY5		Trials		
		1	2	3	4
Wildtype		38.4	34.3	43.5	45
Transgenics		17.9	21.3	14.4	23.2

	DAY6		Trials		
		1	2	3	4
Wildtype		30	31.1	30.2	39.4
Transgenics		15.6	15.5	19.3	27.1

	DAY7		Trials		
		1	2	3	4
Wildtype		23.4	31.6	34.9	33.9
Transgenics		20.9	16.5	26.9	21.1

	DAY8		Trials		
		1	2	3	4
Wildtype		17.5	26.1	25	29.1
Transgenics		23.5	27.9	35.1	25.8

G4: SPATIAL TASK DATA

Day1 **Trials**

Gender	Genotype	ID	Starting position	NE		NW		SW		SE	
male	wildtype	30	N	10	1	30	3	60	4	60	4
male	wildtype	32	S	25	3	39	3	53	2	60	4
male	wildtype	34	N	2	1	9	1	18	3	13	3
male	wildtype	35	S	18	3	3	1	14	3	19	3
male	wildtype	36	E	5	1	5	1	24	3	31	3
male	wildtype	37	W	7	1	15	3	16	3	27	3
male	wildtype	38	N	60	4	48	3	60	4	60	4
female	wildtype	39	N	20	3	23	3	15	1	32	2
female	wildtype	40	S	8	1	60	4	34	3	15	1
female	wildtype	41	E	7	1	34	2	34	2	23	1
female	wildtype	42	W	42	2	7	1	10	1	9	1
female	wildtype	44	N	60	4	41	2	6	1	60	4
female	wildtype	45	S	46	2	25	3	35	3	48	2
female	wildtype	46	E	38	2	60	4	60	4	37	3
				24.9		**28.5**		**31.4**		**35.3**	

Day2 **Trials**

Gender	Genotype	ID	Starting position	NE		NW		SW		SE	
male	wildtype	30	N	12	1	16	1	59	3	60	4
male	wildtype	32	S	21	3	20	1	29	2	43	2
male	wildtype	34	N	1	1	10	3	18	3	7	1
male	wildtype	35	S	6	1	38	3	17	3	3	1
male	wildtype	36	E	8	1	60	1	24	3	50	2
male	wildtype	37	W	30	3	10	3	20	3	20	2
male	wildtype	38	N	39	2	60	2	60	4	11	1
female	wildtype	39	N	18	3	12	1	58	2	60	2
female	wildtype	40	S	10	3	19	3	30	2	16	3
female	wildtype	41	E	6	1	52	2	60	2	60	4
female	wildtype	42	W	18	2	18	3	28	2	19	1
female	wildtype	44	N	22	3	33	2	41	2	55	2
female	wildtype	45	S	23	3	8	1	4	1	28	3
female	wildtype	46	E	22	3	13	1	26	3	31	3
				16.9		**26.4**		**33.9**		**33.1**	

Day3				Trials							
Gender	Genotype	ID	Starting position	NE		NW		SW		SE	
male	wildtype	30	N	10	1	27	3	20	3	60	4
male	wildtype	32	S	12	1	36	3	21	3	45	2
male	wildtype	34	N	19	3	3	4	20	1	9	1
male	wildtype	35	S	9	1	12	1	3	1	2	1
male	wildtype	36	E	60	4	52	2	60	4	29	1
male	wildtype	37	W	8	1	13	3	26	3	17	1
male	wildtype	38	N	60	4	60	4	34	2	42	2
female	wildtype	39	N	12	1	39	2	16	1	28	3
female	wildtype	40	S	60	4	14	1	13	1	14	1
female	wildtype	41	E	22	3	37	2	60	4	48	2
female	wildtype	42	W	30	2	13	1	36	2	57	2
female	wildtype	44	N	11	1	31	3	21	3	60	4
female	wildtype	45	S	26	3	7	1	6	1	35	3
female	wildtype	46	E	39	3	60	4	60	4	60	4
				27.0		28.9		28.3		36.1	

G5: SPATIAL TASK DATA: TRANSGENIC

Day1				Trials							

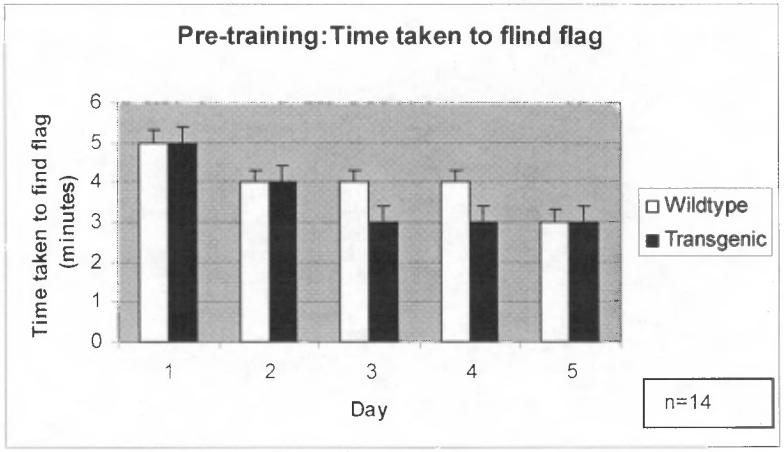
Gender	Genotype	ID	Starting position	NW		NE		SW		SE	
male	transgenic	51	N	6	1	47	2	15	2	44	2
male	transgenic	53	S	11	1	15	2	25	2	4	1
male	transgenic	60	E	10	1	24	2	39	2	8	1
male	transgenic	61	W	36	2	8	1	17	1	4	1
male	transgenic	62	N	20	1	37	2	13	1	31	2
male	transgenic	63	S	19	1	60	4	23	1	29	2
male	transgenic	64	E	3	1	15	1	7	1	5	1
female	transgenic	43	N	8	1	12	1	9	1	9	1
female	transgenic	47	S	3	1	14	1	29	3	3	1
female	transgenic	48	E	49	3	33	2	60	4	60	4
female	transgenic	49	W	12	1	39	2	51	3	42	2
female	transgenic	50	N	3	1	11	1	22	1	3	1
female	transgenic	54	S	54	3	60	4	60	4	60	4
female	transgenic	68	E	41	2	54	3	29	2	5	1
				19.6		**30.6**		**28.5**		**21.9**	

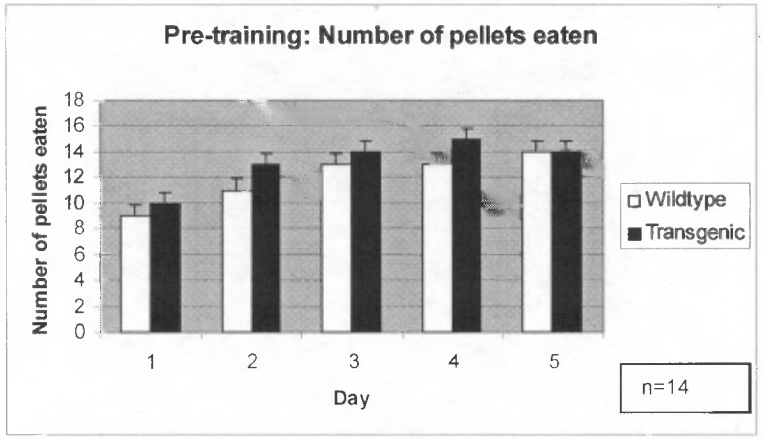
Day2				Trials							
Gender	Genotype	ID	Starting position	NW		NE		SW		SE	
male	transgenic	51	N	3	1	8	1	19	1	23	2
male	transgenic	53	S	6	1	11	1	15	1	11	1
male	transgenic	60	E	18	1	23	1	43	2	50	2
male	transgenic	61	W	12	1	11	1	30	2	9	1
male	transgenic	62	N	60	4	26	1	36	2	24	1
male	transgenic	63	S	58	3	16	1	8	1	15	1
male	transgenic	64	E	2	1	5	1	7	1	7	1
female	transgenic	43	N	3	1	9	1	18	1	20	2
female	transgenic	47	S	20	2	16	1	9	1	14	1
female	transgenic	48	E	58	3	60	4	43	2	49	2
female	transgenic	49	W	15	1	15	1	24	2	12	1
female	transgenic	50	N	24	1	17	1	27	2	12	1
female	transgenic	54	S	33	3	48	3	24	1	20	1
female	transgenic	68	E	34	3	53	3	19	1	60	4
				24.7		22.7		23.0		23.3	

Day3				Trials							
Gender	Genotype	ID	Starting position	NW		NE		SW		SE	

male	transgenic	51	N	60	4	45	2	10	1	15	1
male	transgenic	53	S	13	1	6	1	7	1	19	2
male	transgenic	60	E	60	4	60	4	36	2	34	2
male	transgenic	61	W	6	1	9	1	27	2	15	1
male	transgenic	62	N	32	2	45	2	20	1	60	4
male	transgenic	63	S	60	4	11	1	36	2	60	4
male	transgenic	64	E	2	1	20	2	14	1	10	1
female	transgenic	43	N	18	2	42	3	29	2	25	1
female	transgenic	47	S	5	1	25	2	59	3	9	1
female	transgenic	48	E	19	1	16	1	34	2	60	4
female	transgenic	49	W	10	1	57	3	15	1	14	1
female	transgenic	50	N	34	2	6	1	15	2	22	1
female	transgenic	54	S	60	4	22	1	38	2	20	1
female	transgenic	68	E	60	4	60	4	60	4	33	2
				31.4		**30.3**		**28.6**		**28.3**	

G6: PRE-TRAINING GRAPH ANALYSIS

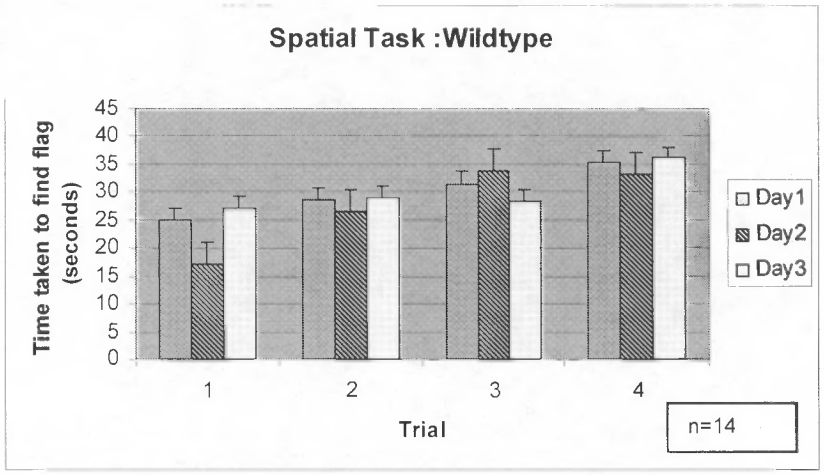


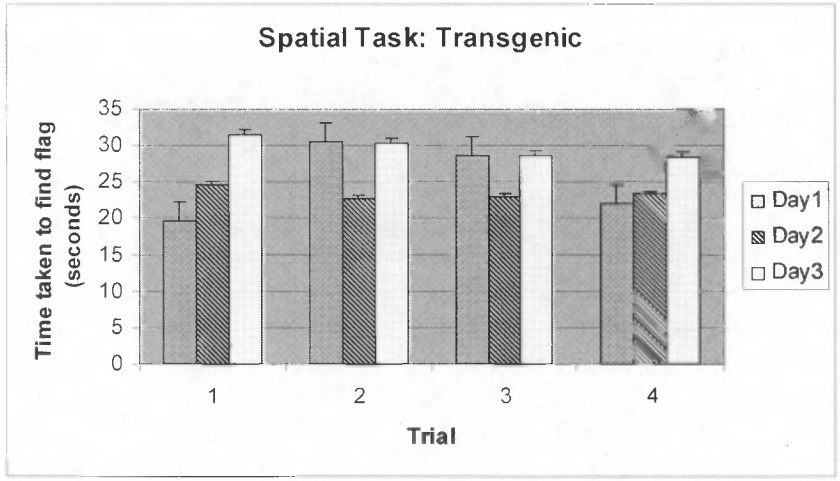


G7: Table to show p-values of significance between wildtype and transgenic mice during the cued task.

Trial Number	p-value
1	[F(1,14)=6.53(p=0.02)]
2	[F(1,14)=24.28(p=0.0002)]
3	[F(1,14)=24.96(p=0.0002)]
4	[F(1,14)=22.96(p=0,0003)]

G8: SPATIAL TASK ANALYSIS





G9: PROBE PHASE DATA AND ANALYSIS

Probe Task

Trial 1					Anova: Single Factor	**Trial 1**					
wt	wt+saline	Tg	Tg+saline	Tg+VCP							
10	24	60	31	8	SUMMARY						
15	60	51	57	12	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
23	8	60	14	60	wt	12	278	23.16667	322.8788		
38	19	23	45	60	wt+saline	12	335	27.91667	257.3561		
60	19	60	60	59	Tg	12	553	46.08333	194.6288		
40	46	54	60	26	Tg+saline	12	538	44.83333	340.3333		
5	4	39	60	10	Tg+VCP	12	381	31.75	502.3864		
4	40	29	60	60							
1	23	32	16	14							
16	40	60	60	32	ANOVA						
30	28	33	24	33	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
36	24	52	51	7	Between Groups	5039.833	4	1259.958	3.89457	**0.007429**	2.539686
23.2	**27.9**	**46.1**	**44.8**	**31.8**	Within Groups	17793.42	55	323.5167			
					Total	22833.25	59				

Trial 2					Anova: Single Factor	**Trial 2**					
wt	wt+saline	Tg	Tg+saline	Tg+VCP							
15	12	60	60	46	SUMMARY						
60	55	60	60	26	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
27	41	60	60	60	wt	12	433	36.08333	474.8106		
29	21	60	60	60	wt+saline	12	419	34.91667	414.6288		
19	29	40	60	34	Tg	12	632	52.66667	194.2424		
60	37	17	60	60	Tg+saline	12	720	60	0		
9	3	60	60	54	Tg+VCP	12	603	50.25	208.3864		
60	60	60	60	60							
13	60	60	60	60							
21	60	58	60	60	ANOVA						
60	16	60	60	23	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
60	25	37	60	60	Between Groups	5719.433	4	1429.858	5.533215	**0.000813**	2.539686
36.1	**34.9**	**52.7**	**60.0**	**50.3**	Within Groups	14212.75	55	258.4136			
					Total	19932.18	59				

Trial 3					Anova: Single Factor	**Trial 3**					
wt	wt+saline	Tg	Tg+saline	Tg+VCP							
60	8	60	60	26	SUMMARY						
17	56	49	49	57	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
13	60	60	58	60	wt	12	428	35.66667	437.697		
60	46	60	30	60	wt+saline	12	434	36.16667	343.4242		
24	26	45	60	60	Tg	12	613	51.08333	271.3561		
60	32	32	60	19	Tg+saline	12	617	51.41667	188.0833		
26	4	60	60	60	Tg+VCP	12	602	50.16667	244.1515		
16	35	60	38	30							
28	47	60	60	60							
53	28	60	22	60	ANOVA						
11	32	60	60	50	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
60	60	7	60	60	Between Groups	3239.567	4	809.8917	2.727437	**0.038309**	2.539686
35.7	**36.2**	**51.1**	**51.4**	**50.2**	Within Groups	16331.83	55	296.9424			
					Total	19571.4	59				

Trial 4					Anova: Single	**Trial 4**				

					Factor						
wt	wt+saline	Tg	Tg+saline	Tg+VCP							
60	12	14	13	15	SUMMARY						
55	36	60	60	13	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
6	41	20	15	10	wt	12	368	30.66667	502.7879		
60	27	22	60	14	wt+saline	12	436	36.33333	338.9697		
8	22	60	15	60	Tg	12	395	32.91667	486.6288		
19	60	4	16	60	Tg+saline	12	469	39.08333	440.6288		
23	5	12	60	60	Tg+VCP	12	455	37.91667	555.1742		
4	60	60	60	60							
20	43	35	50	60							
36	49	37	60	60	ANOVA						
17	56	60	29	13	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
60	25	11	31	30	Between Groups	592.1	4	148.025	0.318444	**0.864439**	2.539686
30.7	**36.3**	**32.9**	**39.1**	**37.9**	Within Groups	25566.08	55	464.8379			
					Total	26158.18	59				

Probe task analysis : Trial 2 VCP vs Saline

Trial 2	

Tg+saline	Tg+VCP	Anova: Single Factor						
60	46							
60	26	SUMMARY						
60	60	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
60	60	Tg+saline	12	720	60	0		
60	34	Tg+VCP	12	603	50.25	208.3864		
60	60							
60	54							
60	60	ANOVA						
60	60	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
60	60	Between Groups	570.375	1	570.375	5.474207	**0.02878**	4.300944
60	23	Within Groups	2292.25	22	104.1932			
60	60							
60.0	50.3	Total	2862.625	23				

Probe Phase: Reverse Task Analysis

Trial 1					Anova: Single Factor	**Trial 1**					
wt	wt+saline	Tg	Tg+saline	Tg+VCP							
10	24	14	4	25	SUMMARY						
15	60	60	23	24	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
23	8	12	60	48	wt	12	278	23.16667	322.8788		
38	19	26	48	18	wt+saline	12	335	27.91667	257.3561		
60	19	33	60	60	Tg	12	445	37.08333	459.7197		
40	46	13	27	41	Tg+saline	12	515	42.91667	410.9924		
5	4	60	31	24	Tg+VCP	12	437	36.41667	216.2652		
4	40	48	60	25							
1	23	60	60	27							
16	40	60	22	60	ANOVA						
30	28	50	60	47	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
36	24	9	60	38	Between Groups	2975.667	4	743.9167	2.23102	**0.07744**	2.539686
23.2	**27.9**	**37.1**	**42.9**	**36.4**	Within Groups	18339.33	55	333.4424			
					Total	21315	59				

Trial 2					Anova: Single Factor	**Trial 2**				

wt	wt+saline	Tg	Tg+saline	Tg+VCP							
15	12	60	14	34	SUMMARY						
60	55	42	20	11	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
27	41	5	43	22	wt	12	433	36.08333	474.8106		
29	21	14	23	14	wt+saline	12	419	34.91667	414.6288		
19	29	45	60	25	Tg	12	438	36.5	340.8182		
60	37	40	39	7	Tg+saline	12	412	34.33333	329.3333		
9	3	36	8	6	Tg+VCP	12	265	22.08333	210.9924		
60	60	60	52	15							
13	60	54	16	24							
21	60	17	45	24	ANOVA						
60	16	45	32	14	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
60	25	20	60	23	Between Groups	1753.767	4	438.4417	1.238128	0.305523	2.539686
36.1	**34.9**	**36.5**	**34.3**	**18.3**	Within Groups	19476.42	55	354.1167			
					Total	21230.18	59				

Trial 3					Anova: Single Factor	**Trial 3**					
wt	wt+saline	Tg	Tg+saline	Tg+VCP							

60	8	17	10	8	SUMMARY						
17	56	17	60	4	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
13	60	5	60	13	wt	12	428	35.66667	437.697		
60	46	60	60	18	wt+saline	12	434	36.16667	343.4242		
24	26	14	38	12	Tg	12	296	24.66667	328.4242		
60	32	6	20	39	Tg+saline	12	493	41.08333	431.5379		
26	4	22	16	8	Tg+VCP	12	285	23.75	396.3864		
16	35	17	24	8							
28	47	28	60	17							
53	28	60	60	55	ANOVA						
11	32	31	60	43	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
60	60	19	25	60	Between Groups	2817.567	4	704.3917	1.817813	0.138503	2.539686
35.7	**36.2**	**24.7**	**41.1**	**23.8**	Within Groups	21312.17	55	387.4939			
					Total	24129.73	59				

Trial 4					Anova: Single Factor	**Trial 4**					
wt	wt+saline	Tg	Tg+saline	Tg+VCP							

60	12	40	43	18	SUMMARY						
55	36	22	36	49	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
6	41	39	60	14	wt	12	368	30.66667	502.7879		
60	27	60	24	60	wt+saline	12	436	36.33333	338.9697		
8	22	42	60	60	Tg	12	581	48.41667	172.6288		
19	60	42	49	34	Tg+saline	12	602	50.16667	131.6061		
23	5	60	60	12	Tg+VCP	12	504	42	361.2727		
4	60	60	51	57							
20	43	60	58	60							
36	49	60	56	34	ANOVA						
17	56	60	45	46	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
60	25	36	60	60	Between Groups	3207.067	4	801.7667	2.659674	0.042174	2.539686
30.7	**36.3**	**48.4**	**50.2**	**42.0**	Within Groups	16579.92	55	301.453			
					Total	19786.98	59				

Reverse Task Analysis: Trial 2 VCP vs Saline

Trial 2		**Anova: Single**	**Trial 2**				

		Factor						
Tg+saline	**Tg+VCP**							
14	34	SUMMARY						
20	11	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
43	22	Tg+saline	12	412	34.33333	329.3333		
23	14	Tg+VCP	12	219	18.25	70.20455		
60	25							
39	7							
8	6	ANOVA						
52	15	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
16	24	Between Groups	1552.042	1	1552.042	7.769184	**0.01074**	4.300944
45	24	Within Groups	4394.917	22	199.7689			
32	14							
60	23	Total	5946.958	23				

Reverse Task Analysis: Trial 3 VCP vs Saline

Trial 3		Anova: Single Factor	**Trial 3**					
Tg+saline	Tg+VCP							
10	8	SUMMARY						
60	4	*Groups*	*Count*	*Sum*	*Average*	*Variance*		
60	13	Tg+saline	12	493	41.08333	431.5379		
60	18	Tg+VCP	12	285	23.75	396.3864		
38	12							
20	39							
16	8	ANOVA						
24	8	*Source of Variation*	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
60	17	Between Groups	1802.667	1	1802.667	4.354666	**0.048709**	4.300944
60	55	Within Groups	9107.167	22	413.9621			
60	43							
25	60	Total	10909.83	23				

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