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NEONATAL EXPOSURE TO *STREPTOCOCCUS PNEUMONIAE* IN RAT PUPS AND ITS CONSEQUENCES ON COGNITIVE FUNCTIONNING IN ADOLESCENCE

Tanusha Dukhan



A dissertation submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, South Africa, 2016

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

DECLARATION

I, Tanusha Dukhan declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

…….

Tanusha Dukhan

…….day of ……..[month], 2016

I certify that approval for the use of infectious material in this study was obtained from the Institutional Biosafety Committee (Protocol Number: 20130101) and that all interventions used in this study were approved by the Animal Ethics Screening Committee of the University of Witwatersrand (AESC 2013/11/03, 2013/03/04).

…….

Tanusha Dukhan

…….day of ……..[month], 2016

RESEARCH OUTPUTS

Results arising from this study have been presented in the form of a poster presentation at :

1. The University of the Witwatersrand Faculty of Health Science research day and postgraduate expo hosted by the Faculty of Health Sciences, Johannesburg, South Africa. 17 September 2014.

Tanusha Dukhan, Tanya Swanepoel, Peter V. Adrian, Helen P. Laburn, Duncan Mitchell, Ruth M.Barrientos,Tamzyn L. Baartman, Lois M. Harden. Cognitive functioning of adolescent Sprague-Dawley rats exposed to Streptococcus pneumoniae infection neonatally.

2. The 41st Annual Conference of the Physiology Society of Southern Africa hosted by the University of Limpopo (Medunsa campus), Pretoria, South Africa. 15-18 September 2013.

Dukhan T, Swanepoel T, Adrian PV, Clarke L, Laburn HP, Baartman TL, Barrientos RM, Mitchell D and Harden LM. A neonatal and adolescent model of pneumococcal disease.

3. The 3rd Annual DAAD-NRF In-Country scholarship holders meeting hosted by The German Academic Exchange Service (DAAD), Stellenbosch, South Africa. 8-10 November 2013.

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Results arising from this study have been presented in the form of an oral presentation at:

1. The 43rd Annual Congress of the Physiological Society of Southern Africa jointly hosted by the University of Witwatersrand and the University of Johannesburg, Parys, South Africa. 6-9 September 2015

Tanusha Dukhan, Tanya Swanepoel, Peter V. Adrian , Helen P. Laburn, Duncan Mitchell , Ruth M.Barrientos,Tamzyn L. Bartmaan, Lois M. Harden.Cognitive functioning of Sprague-Dawley rats neonatally exposed to *Streptococcus pneumoniae*.

2. Invited talk at the Institute of Veterinary-Physiology and – Biochemistry at Justus-Liebig University Giessen, Germany. 14 January 2015.

Tanusha Dukhan, Tanya Swanepoel, Peter V. Adrian , Helen P. Laburn, Duncan Mitchell , Ruth M.Barrientos,Tamzyn L. Bartmaan, Lois M. Harden. Neonatal exposure to Streptococcus pneumoniae in rat pups and its consequences on cognitive functioning in adolescence.

3. 5th International Symposium on the Physiology and Pharmacology of Temperature Regulation (PPTR), hosted by the Brain function Research Unit, University of Witwatersrand, Mpumalanga, South Africa. 7-12 September 2014.

Tanusha Dukhan, Tanya Swanepoel, Peter V. Adrian , Helen P. Laburn, Duncan Mitchell , Ruth M.Barrientos,Tamzyn L. Bartmaan, Lois M. Harden. Cognitive functioning of adolescent Sprague-Dawley rats exposed to Streptococcus pneumoniae infection neonatally

4. 6th WITS Cross- faculty graduate symposium hosted by the Graduate Support Division , Johannesburg, South Africa. 28-29 October 2014.

Tanusha Dukhan, Tanya Swanepoel, Peter V. Adrian , Helen P. Laburn, Duncan Mitchell , Ruth M.Barrientos,Tamzyn L. Bartmaan, Lois M. Harden. Cognitive functioning of adolescent Sprague-Dawley rats exposed to Streptococcus pneumoniae infection neonatally

ABSTRACT

Pneumococcal meningitis, an infection caused by the *Streptococcus pneumoniae* (*S. pneumoniae*) bacterium, is a leading cause of serious illness in children and when it is experienced during critical periods of neurodevelopment, particularly in males, it appears to be associated with decreased cognitive functioning later in life. Studies, which have investigated the long-term sequelae of bacterial meningitis, do not separate *S. pneumoniae*-induced sequelae from those sequelae caused by other bacteria. Therefore, using a rat model of neonatal haematogenous meningitis I aimed to determine if neonatal haematogenous meningitis induced by *S. pneumoniae* would affect cognitive functioning in adolescent rats in a gender-specific manner.

Postnatal day (P) 4 rat pups received an intra-peritoneal (i.p.) injection of either *S. pneumoniae* (mean ± SD: 46 ± 35 colony forming units (CFU) in 250 ul) or 0.9% sterile saline. Calibrated microchips were used to obtain skin temperature measurements to evaluate the progression of disease. Cerebral spinal fluid (CSF) and tail blood were collected between 16 and 24 hours after infection once the pups showed signs of illness. A bacterial load of $1x10^7$ CFU/ml was detected in the blood and $1x10^5$ CFU/ml was found in the CSF. Infected pups were treated with ceftriaxone (100 mg/kg, intramuscularly). No gender-specific differences were found in the spread of disease.

Learning and memory of pups was tested on P30 using two behavioural models: the Morris water maze and contextual fear conditioning. In the Morris water maze, both male and female pups that received either saline or *S. pneumoniae* learned to find the location of the platform equally well. In the probe test, both genders of pups that received either saline or *S. pneumoniae* found the location of the platform significantly faster than the cut-off time of 30 seconds. Freezing behaviour during contextual fear testing did not differ between gender or intervention groups.

Results from my study appear to suggest that an acute episode of severe neonatal haematogenous meningitis may not affect hippocampal-dependent spatial, or associative, learning and memory in adolescence. My findings are contradictory to existing literature which suggests that an early life infection leads to an overproduction of pro-inflammatory cytokines which may cause the cognitive impairments seen in later life. To confirm that the *S. pneumoniae* used in my study does indeed activate the immune system and induce pro-inflammatory cytokine release I stimulated micro-cultures of the rat hippocampus with ethanol-treated *S. pneumoniae* ($5x10^3$, $5x10^4$, $5x10^5$ and $5x10^6$ CFU/ml) to determine the effect of *S. pneumoniae* on nuclear factor- interleukin 6 (NF-IL6) activation. NF-IL6 is a transcription factor which regulates the expression of pro-inflammatory cytokines.

NF-IL6 immunoreactivity was increased in hippocampal cells that were stimulated with alcohol-treated *S. pneumoniae* in a dose dependant manner. Neonatal hippocampal cells exposed to high concentrations (5×10^6 CFU/ml) of alcohol-treated *S. pneumoniae* expressed NF-IL6 particularly in astrocytes and microglia. Therefore the presence of a greater number of *S. pneumoniae* CFU in the brain during neonatal life could result in a greater activation of astrocytes and microglia. This increased activation of astrocytes and microglia could then lead to an overproduction of pro-inflammatory cytokines that is capable of inducing hippocampal damage and long-lasting hippocampal-dependant memory impairment.

In conclusion my results suggest that an acute episode of severe neonatal haematogenous meningitis induced by *S. pneumoniae* does not affect hippocampal-dependant memory in adolescent Sprague-Dawley rats. However, my results cannot be directly applied to human cases of pneumococcal meningitis. To fully understand the impact of pneumococcal meningitis on neurocognitive function in humans, a longitudinal case-control based study is needed. There is a high prevalence of bacterial meningitis caused by *S. pneumoniae* in the South African population therefore it is imperative that research continues in the field.

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LIST OF ABBREVIATIONS

CA:	*Cornu Amonis*
CFU:	Colony forming units
CNS:	Central nervous system
CSF:	Cerebral spinal fluid
DAPI:	2-(4-amidinophenyl)-1H-indole-6-carboxyamidine
E-coli:	*Escherichia coli*
GBSS:	Gey's Balanced Salt Solution
GFAP:	Glial fibrillary acidic protein
HBSS:	Hank's Balanced Salt Solution
IPD:	Invasive pneumococcal disease
LPS:	Lipopolysaccharide
NF-IL6:	Nuclear factor interleukin-6
P:	Postnatal day
PBS:	Phosphate buffered saline
S. pneumoniae:	*Streptococcus pneumoniae*

Chapter 1
Introduction

1.1 Long-term consequences of early life infections

Streptococcus pneumoniae (*S. pneumoniae*) or pneumococcus is a Gram-positive bacterium which behaves as an opportunistic pathogen that causes either mild, non-invasive infections in the middle ear or sinuses, or fatal, invasive diseases in which *S. pneumoniae* invades sterile body fluids, such as blood or cerebrospinal fluid (CSF; Driver, 2012). bacterial meningitis caused by *S. pneumoniae* is referred to as pneumococcal meningitis. The long-term cognitive outcomes resulting from exposure to *S. pneumoniae* infections during childhood in a South African context has not been explored, despite the high prevalence of *S. pneumoniae* infections in South Africa.

In 2008 a total of 14.5 million severe infections and 826 000 deaths in children under the age of five were attributed to *S. pneumoniae* (Black *et al*., 2010). Statistics from the Group for Enteric Respiratory and Meningeal disease Surveillance in South Africa (GERM-SA) reported 2724 cases of invasive pneumococcal disease (IPD) in 2013. The highest number of cases was reported amongst children less than a year old (GERM-SA Annual report, 2013). Although statistics for 2015 are unavailable (World Health Statistics, 2015) current epidemiological studies report high carriage rates of *S. pneumoniae* in children (Al-Lahhan and van der Linden, 2014; Mills *et al.*, 2015).

Brain cells acutely exposed to infectious pathogens, like *S. pneumoniae*, during prenatal and postnatal periods of rapid neurodevelopment display altered structure and function, the effects of which persist into adulthood (see review Mwaniki *et al*., 2012). Acute perinatal immune activation has been linked to the development of schizophrenia, autism and cerebral palsy in adulthood (see review Boksa, 2010). In addition exposure to bacterial infections during postnatal life have been associated with impaired behaviour, motor, sensory and executive functions which include impairments in speech, language, numerical ability,

attention and information processing (see review Mwaniki *et al*., 2012). For the purposes of my literature review I will focus on human and animal studies which investigated the long-term cognitive consequences of a bacterial infection during the postnatal period.

1.2 Early life bacterial infections and cognition during childhood

Bacterial meningitis occurs when bacteria or bacterial products invade the central nervous system and cause inflammation of the brain specifically the meninges (Kim, 2010). Intellectual deficits are the most frequently reported consequence of bacterial meningitis (Chandran *et al*., 2011, Antoniuk *et al*., 2011, Singhi *et al*., 2007, Ritchi *et al*., 2008, Clark *et al*., 2013). Studies summarised in Table 1.1 show the cognitive function of children who were exposed to bacterial meningitis in comparison to the cognitive function of children who had no history of bacterial meningitis. The cognitive function of all children (those who had a history of infection and those who did not have a history of infection) was assessed using either questionnaires filled out by parents or teachers, neurological tests conducted by trained individuals or academic results obtained from examinations undertaken using the national education system.

Table 1.1: Summary of studies that investigated the long-term outcomes of childhood bacterial meningitis

Table 1.1: Summary of studies that investigated the long-term outcomes of childhood bacterial meningitis.

Previously infected	No history of infection	illness	up			
107	96	3 months - 14 years	10-18 years	Decreased executive function	*Haemophilus influemzae, Neisseria meningitides, Staphylococcus pneumoniae,*	Anderson *et al*., 2004
97	93	<14 years	3-20 years	Decreased executive function	*Streptococcus pneumoniae*	Christie *et al*., 2011
739	480	<12 months	16 years	Decreased executive function	*Haemophilus influemzae, Neisseria meningitides, Streptococcus pneumoniae, Group B Streptococcus, Escherichia coli or Listeria monocytogenes*	De Louvois *et al.*, 2007
115	115	1 month -15 years	8-25 years	Decreased gross and fine motor skills and executive function	*Neisseria meningitides*	Fellick *et al*., 2001

Table 1.1: Summary of studies that investigated the long-term outcomes of childhood bacterial meningitis continued…

Number of participants that completed the study		Age at onset of illness	Age of follow up	Cognitive function compared to controls	Pathogens identified	Reference
Previously infected	**No history of infection**					
130	130	3 months - 14 years	Mean 8 years	Decreased neurosensory and executive function	*Haemophilius influemzae, Neisseria meningitides, Streptococcus pneumoniae,*	Grimwood *et al., 1*995
31	6494	<18 months	7- 13 years	Decreased executive function	Not identified	Khandaker *et al.*,2015
680	304	<9 years	3- 14 years	Decreased gross and fine motor skills, neurosensory and executive function	*Neisseria meningitides, Streptococcus pneumoniae, Streptococcus agalactiae, Escherichia coli or Listeria monocytogenes*	Koomen *et al.*,2003
70	66	<14 years	5-20 years	Decreased neurosensory function however motor skills and executive function were comparable.	*Streptococcus pneumoniae*	Legood *et al.,* 2009

Table 1.1 shows that children with a history of bacterial meningitis perform poorly in cognitive tests, that tests executive function, when compared to children who did not have bacterial meningitis during their early childhood. Interestingly *S. pneumoniae* was frequently isolated from children diagnosed with bacterial meningitis. However, studies which have investigated the long-term sequelae of bacterial meningitis do not separate *S. pneumoniae*-induced sequelae from those sequelae caused by other bacteria (Table 1.1: Grimwood *et al*., 1995; Fellick *et al*., 2001; Koomen *et al*.,2003; Anderson *et al*., 2004; De Louvois *et al*., 2007).

There were only two studies that focused on pneumococcal meningitis. However, one study found executive function deficits in children with a history of meningitis while the other did not (Legood *et al*., 2009; Christie *et al*., 2011). Due to a lack of strong evidence for an association between childhood pneumococcal meningitis and long-term adverse cognitive outcomes, neurocognitive assessments are not included in the treatment of patients diagnosed with pneumococcal meningitis (Clark *et al.*, 2013). Consequently, children who develop cognitive impairments are not identified and cannot receive the educational support that they need. The need to conduct neurocognitive assessments should be a concern for clinicians given the high prevalence of *S. pneumoniae* infections.

The use of questionnaires and neurological testing in African countries is limited by illiteracy within the population, language barriers and cultural differences (Kihara *et al.*, 2012). Animal models of infection bypass these limitations. Moreover, the rapid growth rate of animals like rats in comparison to human growth rates, allows for the assessment of potential long-term effects of an early life infection on cognition within a short period of time. Therefore, animal models may be a valuable approach to use to strengthen the evidence for an association between childhood pneumococcal meningitis and long-term adverse cognitive outcomes.

In this introductory chapter I will cover the following sections: (1) the colonisation, invasion and disease progression *S. pneumoniae* from the periphery to the brain; (2) animal models of *S. pneumoniae* infections; (3) serotypes of *S. pneumoniae*; (4) cognitive outcomes of neonatal *S. pneumoniae* infections in animals.

1.3 *Streptococcus pneumoniae*

S. pneumoniae appears as green, lancet shaped, alpha haemolytic colonies when grown on blood agar (see Chapter 2: Figure 2.1; Bridy-Pappas *et al*., 2005). The bacterium is surrounded by a thick negatively charged outer capsule which is composed of repeating units of two to five oligosaccharides, acid constituents, sugar alcohols, amino sugars, choline, dideoxyhexoes, acetyl, phosphoryl groups and neutral sugars (Bruyn *et al*., 1992). The presence or absence of these chemicals gives rise to over 90 different capsular types which are referred to as serotypes (Catterall, 1999).

Figure 1.1 depicts the cell composition of *S. pneumoniae* (adapted from Kadioglu *et al*., 2008). The capsule encloses a typical Gram-positive cell wall that is composed of peptidoglycan and teichoic acid (Sørensen *et al.*, 1988). The cell wall contains surface proteins (pneumococcal protein A and pneumococcal protein C) and enzymes (neuraminidase A, neuraminidase B, betagalactosidase A and beta-N- acetylglucosaminidase) which aids survival within the host. The cell wall also protects the cytoplasmic contents which include the pore forming toxin, pneumolysin (Kadioglu *et al*., 2008).

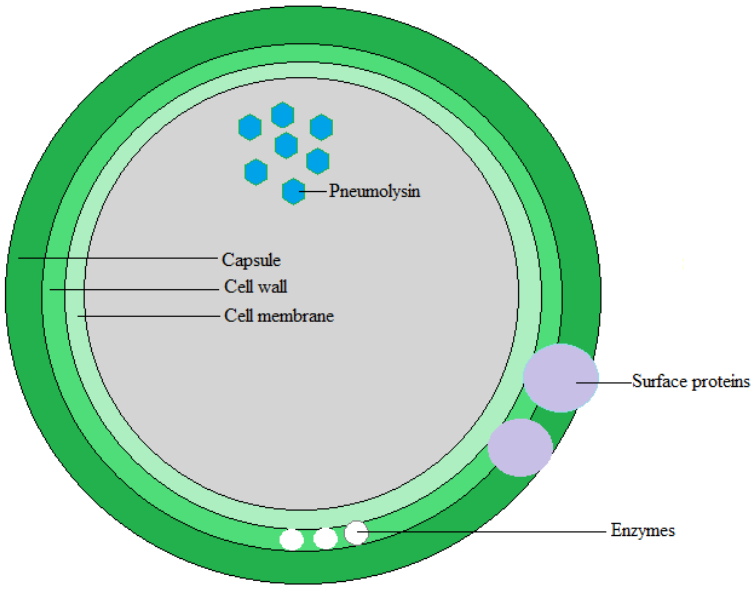


Figure 1.1: Schematic diagram depicting the composition of *S. pneumoniae*, adapted from Kadioglu *et al.*,2008.

1.3.1 Colonisation, invasion and disease progression from the periphery to the brain

The structure and composition of *S. pneumoniae* allows the bacterium to escape innate immune responses, such as mucus entrapment, lysosome destruction, antibody activation and complement activation within the nasopharynx in order to adhere to the epithelial cells and enter the blood (Mook-Kanamori *et al.*, 2011). Figure 1.2 summaries the process involved in colonisation and invasion of *S. pneumoniae*. The thick negatively charged pneumococcal capsule enables the bacterium to escape mucus entrapment by repelling the sialic acid residues in mucus (Nelson *et al.*, 2007). The capsule also prevents interactions with secretory immunoglobulin A (IgA) (Fasching *et al.*, 2007). Secretory IgA is an antibody that prevents the binding of *S. pneumoniae* to the nasopharynx and enables opsonisation (Mook-Kanamori *et al.*, 2011).

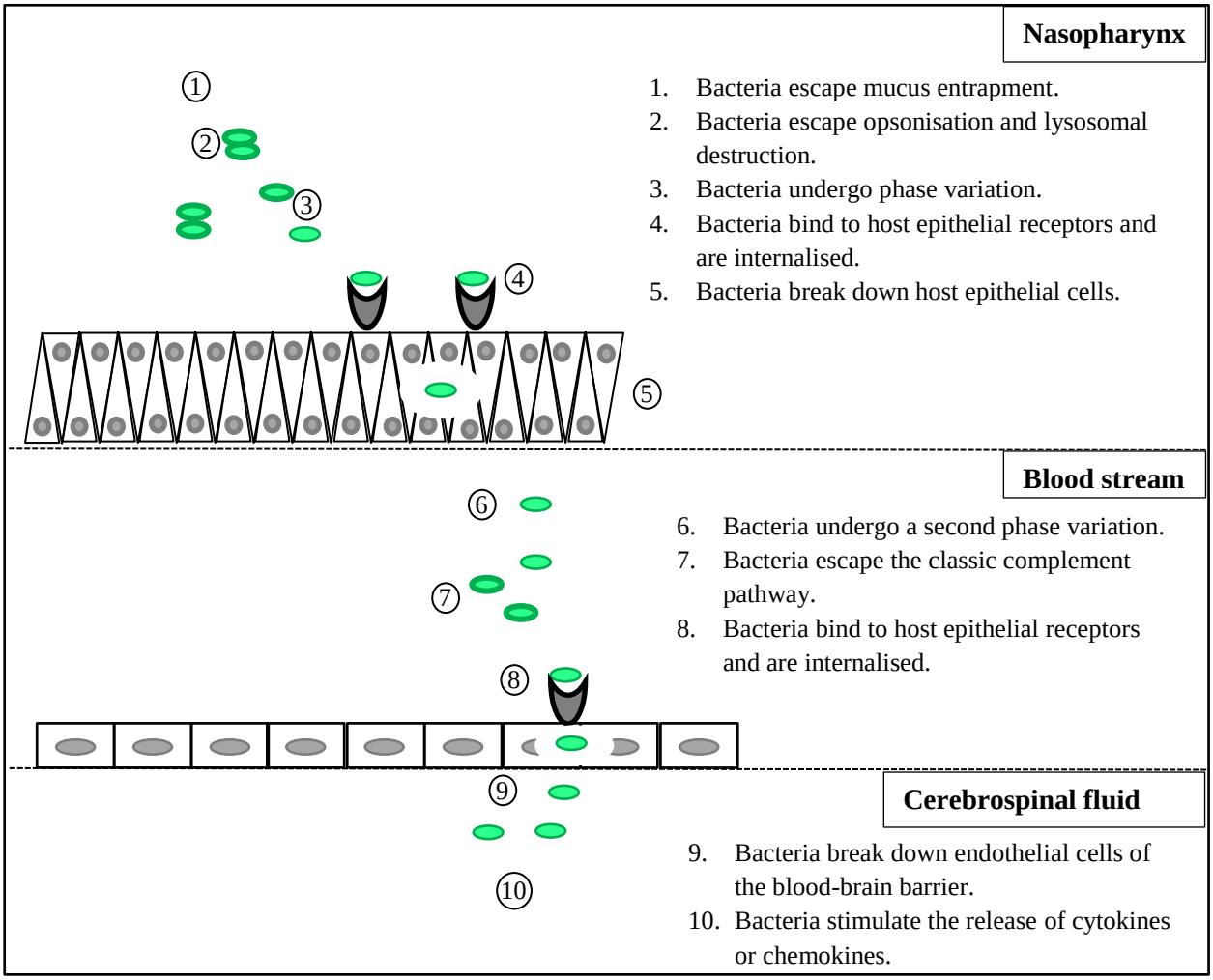


Figure 1.2. Colonisation and invasion of *S. pneumoniae* adapted from Koedel *et al*., 2002.

Opsonisation by IgA is hindered by the pneumococcal enzyme known as pneumococcal IgA1 protease (Weiser *et al*., 2003). By encasing the pneumococcal cell wall the capsule further impedes complement-mediated opsonisation. Additional pneumococcal enzymes within the cell wall of the bacterium (neuraminidase A, neuraminidase B, betagalactosidase A and beta-N- acetylglucosaminidase) reduce mucus viscosity by removing carbohydrates from glycocongates in mucus (Burnaugh, *et al*., 2008). *S. pneumoniae* also contains pneumolysin, an intracellular toxin, which constrains the beating of cilia (Feldman *et* al., 1990) and decreases opsonisation activated via the complement pathway (Quin, *et al*., 2007).

Complement-mediated opsonisation is further inhibited by pneumococcal surface protein C (Psp C) and pneumococcal surface protein A (Psp A). Both surface proteins inhibit opsonisation by hindering the binding of complement component C3, or by binding to inhibiting factors that prevent the activation of the complement pathway (Quin, *et al*., 2007). *S. pneumoniae* is able to escape lysosomal destruction by modifying the molecules on the pneumococcal cell wall that are susceptible to host lysosomal activity. N- acetylglucosamine-deacetylase A and O-acetyltransferase are pneumococcal enzymes that deacetylate pneumococcal cell wall peptidoglycans thus inhibiting lysosomal interaction (Davis *et al.,* 2008).

Once *S. pneumoniae* has overcome the host innate immune responses the bacterium must adhere to the host epithelial cell surface (Mook-Kanamori *et al.*, 2011). Although the thick capsule is advantageous during colonisation, the thickness of the polysaccharide covering discourages binding of the pneumococcus to host epithelial cells. Phase variation allows the bacterium to reduce the amount of polysaccharide in the capsule resulting in the exposure of binding molecules (Weiser *et al.,* 1994). Phase variation describes the ability of bacteria to alter the expression of surface antigen. Pneumococcal phosphorylcholine (ChoP) is a cell wall component which binds to platelet activating factor (PAF) receptor found on the epithelial cell of the hosts nasopharynx (Mook-Kanamori *et al.*, 2011). Once bound to the PAF receptor, the receptor is internalised, subsequently transferring the bacterium from the surface into the epithelial cell of the nasopharynx. Similarly, *S. pneumoniae* can be internalised by the binding of Psp C to polymeric immunoglobulin receptor (Kaekzel, 2001). After internalisation pneumococcus can travel within the host cell to the endothelial lining which the bacterium must then breach to enter the bloodstream. Hyaluronan lyase is a pneumococcal enzyme which breaks down hyaluronan present in the host endothelial cells, thus allowing *S. pneumoniae* to gain access into the bloodstream (Berry *et al*, 1994).

Once in the bloodstream, *S. pneumoniae* must replicate to overwhelming concentrations and avoid immune cell clearance within the blood to cause sepsis. The classic complement pathway is responsible for bacterial clearance within blood (Jarva *et al.*, 2003). Through a second phase variation (alteration of the expression of surface molecules) pneumococcus can use its thick capsule to shield the cell wall from complement components thereby preventing opsonisation and phagocytosis (Mook-Kanamori *et al.*, 2011). Exposed surface proteins (Psp C and Psp A) inhibits complement- mediated opsonisation using the same mechanism as before i.e. by hindering the binding of complement component C3 or by binding to inhibiting factors that prevent the activation of the complement pathway (Mook-Kanamori *et al.*, 2011).

To progress from septicaemia to meningitis, *S. pneumoniae* must breach the blood-brain barrier and overcome immune responses within the central nervous system (CNS). The blood-brain barrier is composed of cerebro-microvascular endothelial cells which contain PAF receptors (Cundell *et al*., 1995). *S. pneumoniae* is said to breach the blood-brain barrier by binding to the receptor and transferring into the cerebrospinal fluid (CSF) when the receptor is internalised (Cundell *et al*., 1995). Recognition of the pathogen-associated molecular patterns (PAMP) of *S. pneumoniae*, such as cell wall components, polysaccharides and surface proteins by antigen presenting cells of the host elicits an immune response within the brain (Paterson and Mitchell, 2006). Pathogen recognition receptors, namely toll-like receptors 2, 4, 9 and Nod-like receptors, which are found on antigen presenting cells of host, recognise specific bacterial components of *S. pneumoniae* (Table 1.2; Mook-Kanamori *et al.*, 2011).

Table 1.2: Pathogen recognition receptors and the pathogen-associated molecular patterns of *S. pneumoniae*.

Pathogen recognition receptor	Bacterial component recognised
Toll-like receptor 2	Lipoteichoic acid on bacterial cell wall
Toll-like receptor 4	Pneumolysin
Toll-like receptor 9	Bacterial DNA (CpG repeat units)
Nod-like receptor 2	Meso-diamiopimelic acid in pneumococcal peptidoglycan

Upon interaction with *S. pneumoniae* or its bacterial components, host pathogen recognition receptors elicit an intracellular cascade resulting in the release of pro-inflammatory cytokines or chemokines in the brain (Mook-Kanamori *et al.*, 2011). Cytokine or chemokine activation results in the activation of effector immune cells. Interleukin (IL)-1, IL-6 and tumour necrosis factor alpha (TNF- α), the first pro-inflammatory cytokines to be produced in response to pneumococcal recognition, stimulates an influx of leucocytes into the CSF. Leukocyte stimulation results in the destruction of pneumococcus through the release of reactive oxygen species (nitric oxide, superoxide anions and perioxynitrite; Freyer *et al.*,1999). However the reactive oxygen species are also toxic to the surrounding brain tissue (Driver, 2012).

It appears that the release of reactive oxygen species during an early-life episode may be one of the mechanisms which result in impaired neurocognitive functioning later on in life (Barichello *et al*., 2012a). A good understanding of the mechanisms which result in cerebral damage will have a great impact on the treatment of bacterial meningitis. Some of the mechanisms discussed above have been elucidated through the use of animal models of

infection (Azeh *et al.*, 1998; Kastenbauer *et al.*, 1999; Braun *et al.*, 2002; Echchannaoui *et al.*, 2002).

1.3.2 Animal models of *Streptococcus pneumoniae* infection

Over the years different animal models have been developed and refined to produce optimal results. Different techniques can be used to mimic specific types of infection. For example, experimental pneumonia can be induced in animals by introducing *S. pneumoniae* through intranasal aspiration or via intratracheal inoculation (Azoulay-Dupuis *et al*., 1991; Canvin *et al*., 1995; Saladino *et al*., 1997).

S. pneumoniae has been introduced into the living systems of rats, mice and rabbits, but infant models of *S. pneumoniae* infection have been developed only in mice and rats (see review Chiavolini *et al*., 2008). Murine models of neonatal meningitis allows for the investigation of neurological consequences of neonatal pneumococcal meningitis. While the recently established infant mouse model allows for the use of knock-out or transgenic models, due to the small body size of mice, the model does not allow for the collection of adequate amounts of blood or CSF samples. Without the collection of biological samples no information can be gathered regarding the concentration of pro-inflammatory cytokines present or the bacterial load within the different organs. Thus one cannot assess the severity of the infection. Nevertheless the infant mouse model has successfully been used to mimic brain damage and determine the protective role of specific host protein molecules against *S. pneumoniae* infections (Tan *et al*., 1995; Grandgirard *et al*., 2007).

Infant rat models of *S. pneumoniae* infections on the other hand have been used to: (1) test drug efficacy, (2) investigate the development of meningitis secondary to ear infections and (3) determine the role of the host immune response in neuronal damage (Tsai *et al*., 1990; Rodriguez *et al*., 1991; Leib *et al*., 2000). The infant rat model consistently produces reliable

robust results and allow for the collection of multiple samples which can be used to quantify inflammation and bacterial concentration within different organs (see review Chiavolini *et al*., 2008). Thus infant rat models can be used to assess the severity of the infection and to assess factors that can determine the severity of the infection. For example, different serotypes (i.e. different chemical variations in the *S. pneumoniae* capsule) of *S. pneumoniae* have been found to be responsible for different types of infection (Butler *et al*., 1995).

1.3.3 Serotypes of *Streptococcus pneumoniae*

Technically introducing any one of the 94 *S. pneumoniae* serotypes into a living system of animals and humans can potentially cause disease however, the majority of infections caused by *S. pneumoniae* are attributed to only 23 serotypes (Bridy-Pappas *et al.*, 2005). Table 1.3 summaries the serotypes used in neonatal animal studies of meningitis caused by *S. pneumoniae*. Serotype 3 is a clinically prevalent serotype that is often isolated from children and has been used to: (1) investigate the neurocognitive outcomes of a *S. pneumoniae* infection, (2) develop animal models of infection, (3) determine the pathophysiology involved in meningitis caused by *S. pneumoniae* and (4) investigate the efficacy of antibiotics. No study has used serotype 2 to develop animal-based *neonatal* models of infection or to determine the neurocognitive outcome, especially the effect on **learning and memory** of an infection with of **serotype 2** (see Table 1.3).

Table 1.3 Summary of serotypes used in animal models of neonatal meningitis.

Serotype	Aim of study	Reference
3	To investigate neurocognitive outcomes.	Loeffler *et al*., 2001
3		Leib *et al*., 2003
3		Barichello *et al*., 2010a
3		Barichello *et al*., 2014
6	To establish a model of infection.	Rodriguez *et al*., 1991
3		Grandgirard *et al*., 2007a
6	To investigate the pathophysiology of an infection	Tan *et al*., 1995
3		Leib *et al*., 2000
3		Bifrare *et al*., 2003
3		Barichello *et al*., 2011
3		Barichello *et al*., 2012b
3	To investigate the therapeutic efficacy of drugs	Tsai *et al*., 1990
3		Auer *et al*., 2000
3		Grandgirard *et al*., 2017b
3		Blaser *et al*., 2011
3		Grandgirard *et al*., 2012

S. pneumoniae serotype 2, strain D39 is a relatively stable and virulent serotype (Lanie *et al.*, 2007). An animal study showed that the i.p. administration of two D39 isolates, which were obtained and stored 21 years apart, produced infections that were comparable in severity after 24 hours of injection (Lanie *et al.*, 2007). Moreover infections in rodents produced using *S. pneumoniae* D39 has been reproducible over the years across various laboratories (Berry *et al.*, 1989; Rosenow *et al.*, 1997; Marra *et al.*, 2002; Hirst *et al.*, 2003; Orihuela *et al.*, 2004; Ogunniyi *et al.*, 2007).

The ease with which *S. pneumoniae* D39 can be genetically manipulated has resulted in the identification of many virulence factors within the bacterium (Benton *et al.*, 1995; Rosenow *et al.*, 1997; Shaper *et al.*, 2004; Ogunniyi *et al.*, 2007). The deletion of virulence factors (such as pneumolysin, pneumococcal surface protein A, pneumococcal surface protein C and choline binding protein A) has resulted in the identification of specific factors that are responsible for the progression of disease from colonisation to invasive disease (Rosenow *et*

al., 1997; Ogunniyi *et al.*, 2007). Thus the stable and reproducible nature of *S. pneumoniae* D39 makes the strain a good standard laboratory strain to use *in vivo*.

1.3.4 Route of infection

S. pneumoniae can be introduced into rodents either through direct injections into CNS (cisterna magna, right forebrain or sub arachnoid or ventricular spaces) or through indirect injections into body cavities (the peritoneum or nasal cavities) (Leib *et al*., 2000; Tan *et al*., 1995; Zwijnenburg *et al*., 2001). Direct introduction of bacteria into the CNS bypasses the natural pathogenic process allowing for the study of physiological events that occur once meningitis is established. Indirect introduction of bacteria via intranasal inoculation permits the study of pathogenesis from the point of colonisation thus mimicking the natural progression of disease. However intranasal inoculations can only cause an infection if synthetic hyaluronidase (an endoglycosidase which allows *S. pneumoniae* to gain access to host cells via degradation of the endothelial lining (Berry *et al.*, 1994)) is added to the inoculum (Zwijnenburg *et al.*, 2001).

Furthermore, the intranasal administration of *S. pneumoniae* produces inconsistent lung infections and bacteraemia in rodents (Steinhoff, 2007). Although the intraperitoneal route of infection does not allow for the study of the natural progression of meningitis it does produce more consistent infections in rodents (Steinhoff, 2007). An intraperitoneal injection of *S. pneumoniae* results in the development of meningitis secondary to bacteraemia, thus producing a model of haematogenous meningitis (Kim *et al*., 1997). Thus far a direct inoculation of *S. pneumoniae* into the cisterna magna has been used in animal studies to assess the cognitive effects (for example, learning and memory) of an early life *S. pneumoniae* infection (Loeffler *et al*., 2001; Leib *et al*., 2003; Barichello *et al*., 2010a;

Barichello *et al*., 2014). No study has assessed the effects of an early life *S. pneumoniae* infection produced by a model of **haematogenous meningitis** on **learning and memory**.

1.3.5 Learning and memory in animals

Learning is the modification of behaviour based on experience whereas memory refers to the ability to store and recall information (Pooters *et al*., 2015). The recollection of memory can occur within a few seconds of requiring the information or after years of first obtaining the information (Morris, 2001). Information that is recalled after a short time (seconds to hours) is referred to as short-term memory whereas long-term memory refers to the recollection of a memory days or years after the memory was formed. Long-term memory is further divided into non-declarative memory and declarative memory based on the manner in which information is processed (see review Morellini, 2013).

The processing of information involved in learning procedures (such as riding a bike) and unconsciously recalling the information required to perform the procedure (the movement involved in pedalling) is referred to as non-declarative memory (see review Morellini, 2013). Declarative memory on the other hand refers to the processing and recall of factual information. Factual information includes general knowledge (sematic memory) and information dependant on personal experience (episodic memory) (see review Morellini, 2013).

Spatial memory is classified as episodic memory. The Morris water maze is one of the tools used to measure spatial learning and memory, a specific type of learning and memory which process spatiotemporal information in rats (Morris, 1984). The maze consists of a circular pool that is filled with opaque water and surrounded by visual cues. A rat's natural instinct when placed in a body of water is to find an escape route. In the Morris water maze a small circular platform placed in a fixed position provides escape from the water. Rats are unable to

see, hear or smell the platform and must therefore use visual cues around the pool to locate the platform (Morris, 1984).

Place, grid and head direction cells in the hippocampal formation are involved in processing spatiotemporal information used to locate the escape platform (Taube *et al*., 1990; Lenck-Santini *et al*., 2002; Hafting *et al*., 2005). Place and grid cell firing continue to develop in postnatal life with experience and exploration (Wills *et al*., 2010). Thus an early life infection can disrupt hippocampal function and affect performance in the Morris water maze in later life (Loeffler *et al*., 2001; Leib *et al*., 2003; Barichello *et al*., 2010a; Barichello *et al*., 2014). Investigations using the Morris water maze have been able to detect learning and memory impairments in rodents that were infected neonatally (see Table 1.4). Subcutaneous administration of *Escherichia coli* to neonatal (Postnatal day (P) 4)) rats resulted in impaired memory retention during adulthood (Bilbo, 2010; Williamson and Bilbo, 2014). In addition intracerebral injections of *S.pneumoniae*, serotype 3 administered to neonatal (P11) rats produced impaired learning in the Morris water maze (Leib *et al*., 2003; Loeffler *et al*., 2001).

Table 1.4: Summary of studies which assessed the neurodevelopmental effects of an early life infection

Infecting organism	Species	Age at time of infection	Learning and memory test administered during adulthood or adolescence	Outcome	Reference
			Live organisms		
***S. pneumoniae*, serotype 3**	Wistar rats	P 3- 4	Step down inhibitory avoidance task	Impaired memory compared to controls	Barichello *et al*., 2010a
	Wistar rats	P11	Step down inhibitory avoidance task	Impaired memory compared to controls	Barichello *et al*., 2014
	Sprague-Dawley rats	P 11	Morris water maze	Impaired learning compared to controls	Loeffler *et al*., 2001
	Sprague-Dawley rats	P 11	Morris water maze	Impaired learning compared to controls	Leib *et al*., 2003
Group B *Streptococcus*	Wistar rats	P 3- 4	Step down inhibitory avoidance task	Impaired memory compared to controls	Barichello *et al*., 2013
Viruses: Influenza Borna	Lewis rats	P 1	Acoustic startle stimulation test	Impaired memory compared to controls	Pletnikov *et al*., 1999
Escherichia coli	Sprague-Dawley rats	P 4	Contextual conditioning	No difference detected between infected rats and controls	Bilbo *et al*., 2005
	Sprague-Dawley rats	P 4	Morris water maze Context pre-exposure test Ambiguous cue test	Impaired memory compared to controls in Morris water maze No difference detected between infected rats and controls in the contextual pre exposure Impaired memory compared to controls in ambiguous cue test	Bilbo, 2010
	Sprague-Dawley rats	P 4	Morris water maze Working memory	No difference detected between infected rats and controls in the Morris water maze Impaired working memory compared to controls	Williamson and Bilbo, 2014

Table 1.4: Summary of studies which assessed the neurodevelopmental effects of an early life infection continued…

Infecting organism	Species	Age at time of infection	Learning and memory test administered during adulthood or adolescence	Outcome	Reference
			Pathogenic moieties		
Endotoxin from *Salmonella enteriditis*	Long Evans rats	P 3 or 5	Acoustic startle response	Impaired acoustic startle response	Walker *et al*., 2008
Polyinosinic: polycytidylic acid	Sprague-Dawley rats	P 14	Morris water maze Contextual conditioning	No difference detected between infected rats and controls in the Morris water maze Impaired memory compared to controls for contextual conditioning	Galic *et al*., 2009
Lipopolysaccharide	Sprague-Dawley rats	P 5, 14, 30 or 77	Morris water maze Contextual conditioning	P5 and P 30 impaired memory compared to controls in both tests	Harrè *et al*., 2008
	Sprague-Dawley rats	P 14	Novel object exploration	Impaired memory compared to controls	Spencer *et al*., 2005

P = Postnatal day

In addition to the Morris water maze, contextual fear conditioning which uses Pavlovian conditioning principals can also be used to test learning and memory, particularly associative learning and memory (Anagnostaras *et al*., 2001). Typically, the contextual apparatus consists of a cage with a metallic grid floor. Rats are placed in the cage and given time to explore the features of the cage (such as light fixtures or odours). Once the exploration time is over, a low voltage foot shock is administered to elicit fear. After this adverse event rats are placed in their home cages and left undisturbed.

During this period of rest, the *Cornu Amonis* (CA) 3 region of the hippocampus encodes all the features of the cage into a single representation which is temporally stored by the CA1 region of the hippocampus (Raineki *et al*., 2010). Output signals from the hippocampus to the amygdala result in an association of the context with the adverse experience of being shocked. As a result, rats develop a fear of the cage. When re-exposed to the cage rats adopt a defensive behaviour referred to as freezing behaviour (Rudy *et al*., 2004). The freezing behaviour is characterised by immobility and is used as a measure of fear and an indirect measure of associative learning and memory.

Contextual fear conditioning has been used in previous studies which assessed the neurodevelopmental effects of an early life infection (Table 1.4). In juvenile (P24) rats inhibition of the hippocampus using a $GABA_A$ receptor agonist results in an inability to associate the cage with the experience of being foot shocked (Raineki *et al*., 2010). In addition decreased freezing behaviour was observed in adult rats that were exposed to Lipopolysaccharides (LPS) during neonatal life (Harrè *et al*., 2008). LPS was shown to down regulate N methyl-D-aspartate receptor mRNA. Nmethyl-D-aspartate receptors are responsible for synaptic plasticity and connectivity in the hippocampus (Harrè *et al*., 2008). Neonatal infections (P1) with viral pathogens also resulted in decreased freezing behaviour

during adulthood (Pletnikov *et al*., 1999). Thus, early life infections that affect hippocampal function may also produce altered freezing behaviour in contextual fear conditioning.

1.3.6 Consequences of *S. pneumoniae* invasion of the brain

In experimental animal models of pneumococcal meningitis hippocampal apoptosis is a common finding (see review Mook-Kanamori *et al.*, 2011). The hippocampus, composed of CA fields, the dentate gyrus and subicular complex, forms part of a system which is responsible for declarative memory (Squire and Zola-Morgan, 1991). Declarative memory includes the recollection of personal events and factual details (Squire and Zola-Morgan, 1991). One of the responsibilities of the hippocampus is the processing, storage and retrieval of spatial details of an event (Morris *et al*., 1982). The processing of spatial details is said to begin with the CA3 region of the hippocampus which receives perceptual information about a scene and the position of objects within a scene from the entorhinal cortex (Eichenbaum, 2004). CA3 cells within the hippocampus connect to each other forming a recurrent collateral network that is capable of associating different objects within a scene. Thus the identification of a specific object can produce rapid firing in the CA3 network resulting in the recollection of the entire scene (Eichenbaum, 2004; Rolls, 2010).

The recollection of events by CA3 neurons is time dependant. The CA3 network can clearly recollect events within a short space of time i.e. within seconds to hours after the event occurred (Kesner and Hunsaker, 2010). Long-term (days to years) recollection is dependent on CA1 neurons of the hippocampus (Kesner and Hunsaker, 2010). Information about the scene is transferred from the CA3 region to the CA1 region via Schaffer collaterals. The CA 1 cells are responsible for decoding the information and delivering the information to the cortex in a user friendly format where it is stored indefinitely (Kesner and Hunsaker, 2010).

Spatial memory is therefore one of the aspects of memory that may be affected by damage to the hippocampus. For example, in patients with hippocampal damage a nine box test was used to test spatial, working and reference memory (Abrahams *et al.*, 1999). Patients were given 9 items to memorise. Once they were familiar with these items, the items were placed in nine identical containers. The containers were fixed on a table that was surrounded by four identical chairs. The room in which the test was conducted had constant features such as a window, cabinet and picture frames. Patients were seated in one of four chairs and taught the location of four specific items. They were then given a booklet with the four items and asked to change their orientation by switching to another seat. After one minute patients were asked to recall the location of the learnt items.

Patients with right temporal lesions affecting the hippocampus found it difficult to re-orientate themselves as they displayed more spatial memory errors than healthy control participants or patients with left temporal lobe resections (Abrahams *et al.*, 1999). Similarly, patients in whom the hippocampus was resected displayed impaired spatial memory in object-location tests (Crane *et al.*, 2005). The removal of the hippocampus from monkeys and rats mimic spatial impairments seen in human patients and have therefore been used to further investigate hippocampal function and development (Morris *et al.*, 1982; Parkinson *et al*., 1988; Bunsey and Eichenbaum, 1996).

Damage to the hippocampus during development can potentially be more severe than damage to the hippocampus during adulthood. Neonatal rats that received an electrolytic lesion in the hippocampus were unable to learn and recall the position of a hidden platform when tested in a Morris water maze eight weeks after the lesion. The same degree of impairment was seen twenty weeks after the lesion (van Praag *et al.*, 1998). Compared to neonatal rats, adult rats that received an electrolytic lesion to the hippocampus were able to learn the location of the hidden platform (van Praag *et al.*, 1998).

In rats the pyramidal cells of the CA1-3 region and the polymorph cells of the dentate gyrus start to develop by gestational day 15 (Arnold and Trojanowski, 1996). The large and medium size pyramidal cells of the CA1-3 regions are completely developed by gestational day 20 while small neurons, glial cells of the CA1 region and granule cells of the dentate gyrus continue to develop during the postnatal life (Rice and Barone Jr, 2000; Bayer and Altman, 1974). Similarly, in humans the CA1-3 region of the hippocampus starts to develop during gestational weeks 15-19 and is fully formed at birth. In the first nine months of human postnatal life the size of the cells increase, the granule cells of dentate gyrus complete development and axons and dentrites in the hippocampus increase in size.

In vitro studies using animal tissue show that *S. pneumoniae*, serotype 2, directly causes hippocampal damage by one of three processes. Firstly, the increased production of hydrogen peroxide by *S. pneumoniae* stimulates the release of calcium. An increased influx of calcium into hippocampal cells weaken the stability of the mitochondrial membrane resulting in hippocampal apoptosis (Braun *et al*., 2002).

Secondly pneumolysin causes mitochondrial damage by forming pores in the mitochondrial membrane thus inducing apoptosis inducing factor (Braun *et al*., 2007). Apoptosis inducing factor causes cell death via chromatin condensation and DNA fragmentation. Thirdly hippocampal damage can result from caspase dependant apoptosis which occurs in response to the binding of pneumococcal cell wall to toll-like receptor 2 on microglial cells (Mitchell *et al*., 2004).

Hippocampal function in rats could also be indirectly impaired by increased production of pro-inflammatory cytokines in response to *S. pneumoniae* invasion (Too *et al*., 2014). Elevated cytokine levels during periods of rapid neurodevelopment may produce cognitive deficits by altering the neural connections responsible for learning and memory (Stephan *et*

al., 2012). During the first two weeks of life weak all inappropriate connections are removed through the process of synaptic pruning. Immature astrocytes stimulate synaptic pruning by activating C1q, an initiating protein of the complement cascade. The binding of C1q to weak or inappropriate neural connections results in the deposition of complement protein C3 (Stevens *et al.*, 2007). Microglia, the resident immunocompetent cells in the brain, has C3 receptors that recognise the C3 protein deposits and initiate phagocytosis thus removing the connection (Paolicelli *et al.*, 2011).

Thus an over production of pro-inflammatory cytokines, in particular interleukin (IL) - 6 and tumour necrosis alpha (TNF-α), have been shown to impair learning and memory. However, studies have also shown that a low concentration of IL-1 (a pro-inflammatory cytokine) produced in a healthy brain helps regulate learning and memory (Yirmiya and Goshen, 2011). Low levels of interleukin (IL)-1 facilitate hippocampal dependant learning and are required for the induction and maintenance of long-term potentiation. Long-term potentiation refers to the physiological process of learning whereby repeated exposure to information produces increased firing of cells and thus an increase in synaptic strength.

An increase in synaptic strength allows one to easily recall the information (Yirmiya and Goshen, 2011). The role of IL-1 in learning and memory has been established through a series of independent studies which demonstrated that IL-1 gene expression is induced 24 hours after learning (Goshen *et al*., 2007) and that administration of low levels of IL-1 improves memory (Song *et al*., 2003). Additional studies found memory impairments when the IL-1 receptor was blocked (Yirmiya *et al*., 2002) or genetically knocked out (Avital *et al*., 2003; Goshen *et al*., 2007).

Lipopolysaccharides (LPS), a cell wall component of *Escherichia coli*, administrated via intracerebral injections (1mg/kg) to five day old rats resulted in increased levels of IL-1 in the

brain and increased activation of microglia that could be detected 66 days (at the age of 71 days) after infection (Wang *et al*., 2013). Since the over expression of the IL-1 gene results in long-term hippocampal dependant memory impairment (Hein *et al*., 2009) it is possible that an infection with *S. pneumoniae* during the neonatal period, a period of rapid neurodevelopment, could induce an overexpression of pro-inflammatory cytokine genes that lead to cognitive deficits.

The expression of pro-inflammatory cytokines is regulated by transcription factors such as nuclear factor kappa B (NF-κB). NF-κB is found in the cytoplasm of resting cells and bound to the inhibitory protein IκB. The binding of the *S. pneumoniae* cell wall to toll-like receptor 2 on microglia activates NF-κB through the phosphorylation and degradation of IκB (Traenckner *et al*., 1995). In addition reactive oxygen species produced during infections acts as a stimulus for the activation of NFκB (Schmidt *et al*., 1995). Active NFκB translocate to the nucleus where it increases the expression of pro-inflammatory genes including IL-1, IL-6 and TNF-α. Inhibition of the NFκB activation reduces IL-6 concentrations in the cerebral spinal fluid of infected rats and limits intracerebral complications associated with bacterial meningitis (Koedel *et al*., 2000).

Nuclear factor- interleukin 6 (NF-IL6) is another transcription factor which is highly expressed in the CA1 region of the hippocampus (Taubenfeld *et al*., 2001). Increased expression of NF-IL6 is associated with the formation of new memories in the hippocampus (Taubenfeld *et al*., 2001). NF-IL6 is also capable of increasing the expression of pro-inflammatory genes (Akira *et al*., 1990). Activation of NF-IL6 occurs a few hours after NFκB suggesting that during an infection NFκB induces the productions of pro-inflammatory cytokines while NF-IL6 maintains the production of cytokines (Poli, 1998; Damm *et al*., 2011). An infection with *S. pneumoniae* during a period of rapid neurodevelopment could induce an overexpression of pro-inflammatory cytokine genes via NFκB dysregulation or

through dysregulation of NF-IL 6. The activation of hippocampal NF-IL6 in response to *S. pneumoniae* has not been explored.

1.3.7 Gender as a risk factor for cognitive outcomes of early life infection

Microglia are cells that are responsible for the release of pro-inflammatory cytokines. The hippocampi of male (P4) rats have more microglia as compared to female rats of the same age (Schwarz *et al*., 2012). Thus an early life infection with *S. pneumoniae* may have more severe consequences for males as compared to females. An infection with *Escherichia coli* on P4 with a subsequent LPS infection in adulthood resulted in memory deficits in male rats that were not detected with female rats given the same intervention (Bilbo *et al*., 2012).

Male mice were also found to be more susceptible to *S. pneumoniae* (serotype 2, strain D39) compared to female rats of the same age. Intravenous administration of 5×10^4 colony forming units (CFU) of *S. pneumoniae* (serotype 2, strain D39) resulted in 100 % mortality within 48 hours of infection in male mice whereas 55 % mortality within 72 hours was reported in female mice (Kadioglu *et al*., 2011). Male mice displayed more severe sickness behaviours (significant weight loss and hypothermia) compared to female mice (Kadioglu *et al*., 2011). Moreover cytokine concentrations taken at 24 and 48 hours after infection were significantly higher in male mice as compared to female mice (Kadioglu *et al*., 2011). The observation that males are more susceptible to *S. pneumoniae* infection and thus have a higher risk of developing neurological sequelue is further supported by human based studies investigating the outcomes of bacterial meningitis (Antoniul *et al*., 2011; Koomen *et al*., 2003).

1.4 Dissertation aims

The findings presented in the sections above have highlighted the possible association between early life infection and the development of cognitive impairment in later life. Although *S. pneumoniae* infections are prevalent in the paediatric population particularly in children under five, no study to date has examined the long-term cognitive effects of haematogenous meningitis induced by *S. pneumoniae* in early life. Therefore the aims of my dissertation are as follows:

1. To establish an animal model of haematogenous meningitis induced by *S. pneumoniae* (serotype 2, strain D39).
2. To determine if an early life episode of haematogenous meningitis induced by *S. pneumoniae* results in learning and memory deficits during adolescence in a gender specific manner.
3. To determine the expression of NF-IL6 in the hippocampus in response to *S. pneumoniae.*

Chapter 2
Methods

2.1 Animals and housing.

Thirty adult Sprague-Dawley rats (males n = 15, females n = 15) were obtained from the National Health Laboratory Services (NHLS, Johannesburg, South Africa) and housed in same sex pairs with food (Pelleted rat chow, Epol, Centurion, South Africa) and water provided *ad libitum*. Rats were given a two week period to acclimatize to rooms maintained at a constant temperature of 22 ± 2 °C with a 12h :12h light:dark cycle (lights on at 07:00). Piperazine [(2 g Piperazine / 500 ml drinking water), Kyron Laboratory (PTY) LTD, Benrose, South Africa] was administered as part of the standard care provided to laboratory animals. Piperazine prevents common roundworm (ascariasis) and pinworm (enterobiasis, oxyuriasis) infections. Once treatment was complete rats were divided into breeding pairs and allowed to mate.

Upon visual evidence of conception (i.e. the release of a mucus plug from the female rat) breeding pairs were separated and pregnant females were kept under observation until the day of delivery. If I observed a dam deliver before 09:00 on a given day then that day was defined as the date of birth i.e. postnatal day (P) 0. If litters were born after 09:00 then the next day was defined as P0 (Schmidt *et al.*, 2002). To prevent developmental stunting that is seen in large litters (i.e. more than 13 pups in a litter, Rödel *et al.*, 2008) we reduced the number of pups per litter to a maximum of 12. Pups were housed with their dams in standard Perspex cages layered with saw dust. Cages were cleaned three times a week and routine health checks were conducted twice daily by myself and animal technicians. Pups were handled during cage changes and on procedural days.

All procedures adhered to the animal care regulations set by the Animal Ethics and Control Committee of the University of Witwatersrand and all interventions used in this investigation

were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (Ethics clearance number 2012/32/04; 2013/11/03; 2013/03/04).

2.2 Infecting organism

A clinical isolate of *S. pneumoniae* (serotype 2, strain D39) obtained from the Respiratory and Meningeal Pathogens Research Unit (Chris Hani Baragwanath Hospital, Johannesburg, South Africa) was used to create passaged bacterial colonies. The use of passaged bacterial colonies improves the likelihood of infection within a litter by increasing the virulence of the bacteria (Saladino *et al.*, 1997). Passaged colonies were created by injecting the clinical isolate of *S. pneumoniae* intraperitoneally into an adolescent rat (P30). Once the rat displayed signs of illness (pale skin, erect fur and hunched position), blood (50 ul) was collected via a cardiac puncture using a 25 G needle attached to a 1 ml syringe. The blood was streaked onto a blood agar plate [(5 % sheep blood supplemented with gentamycin), Media Mage, Johannesburg, South Africa] to obtain single colonies of the passaged strain. The passaged strain was cultured overnight in a carbon dioxide incubator maintained at 37 °C with 5 % carbon dioxide. The passaged strain was confirmed as *S. pneumoniae* by colony morphology, optochin sensitivity and bile solubility.

Single colonises (Figure 2.1) were isolated from the passaged culture and grown in Todd-Hewitt broth (Sigma, Johannesburg, South Africa) to an early log-phase (i.e. an optical density of 0.1). The Todd-Hewitt broth containing the passaged strain, yeast extract [(5 %), Sigma, Johannesburg, South Africa] and glycerol [(15 %), Sigma, Johannesburg, South Africa] was dispensed in 1 ml aliquots, snap frozen and stored at -80 °C. The aliquots were thawed 5 minutes before infection procedures and re-suspended in sterile saline to a concentration of $1x10^2$ colony forming units (CFU)/ml. The amount of bacteria present in a solution containing $1x10^2$ CFU/ml has been shown to induce severe illness with low

mortality rates (data not shown). In our experience doses higher than $1x10^2$ CFU/ml resulted in high mortality rates (data not shown). The exact number of live bacterial colonies varied between vials thus confirmation of the exact concentration used to infect each litter was conducted after each infection procedure using quantitative cultures.

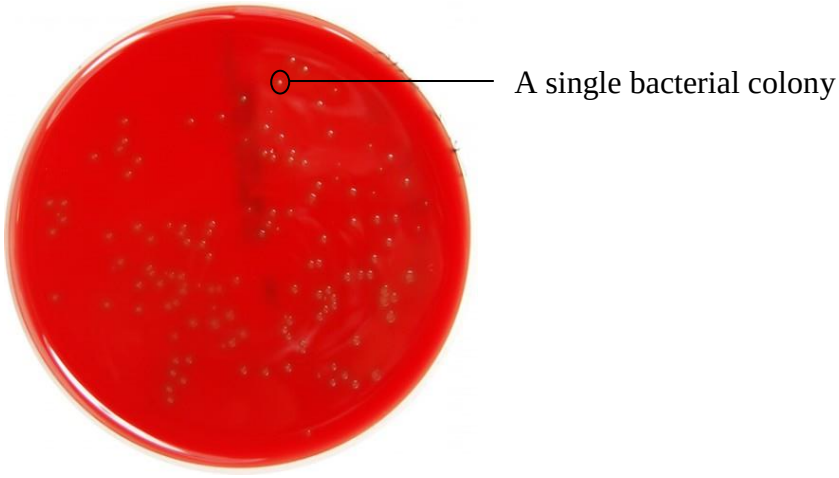


Figure 2.1 Culture of *S. pneumoniae* on a 5 % sheep blood agar plate supplemented with gentamycin.

2.3 Infection procedures

Four day old pups were anaesthetised using isoflourane induced at 5% (v/v) and maintained at 2% (v/v). Once the pain reflex of each rat was diminished, the skin between the shoulder blades was disinfected using chlorhexidine gluconate (Health and Hygiene, Johannesburg, South Africa). Sterile microchip transponders (Figure 2.2A, FDX-B 15 Digit Biotherm Identipet, Pretoria, South Africa) were then inserted subcutaneously using a sterile 12 G needle fixed to a syringe (Figure 2.2B). Once the microchip transponders were secured with a single suture, rats were injected intraperitoneally with 250 ul of *S. pneumoniae* (mean ± SD: 46 ± 35 CFU) or 0.9 % sterile saline. All intra-peritoneal injections were conducted in a biosafety class II cabinet as per bio-safety recommendations.

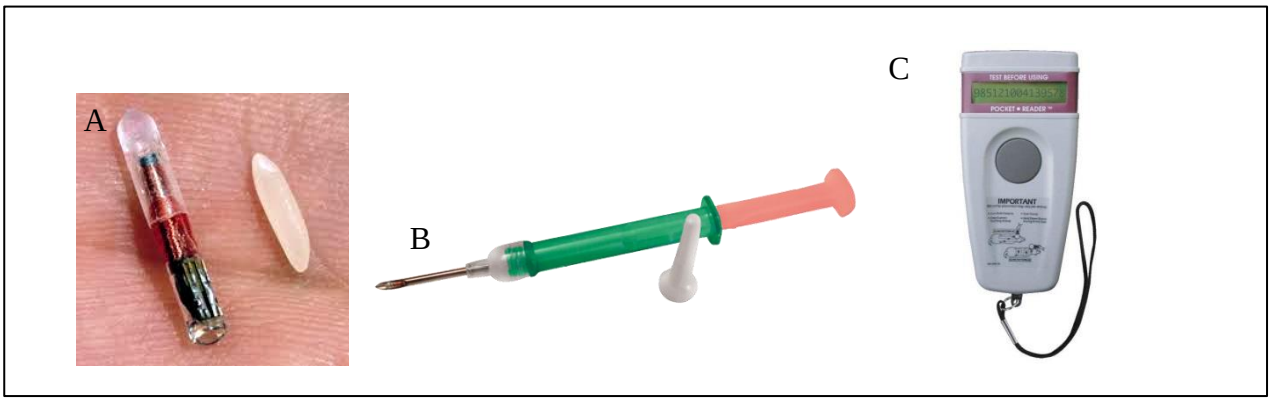


Figure 2.2 The Bio Therm microchip (A), insertion syringe (B) and BioThermo reader (C) used to measure skin temperature in rats.

2.4 Skin temperature

In animal models, haematogenous meningitis often results in death due to septic shock (Rodriguez *et al*., 1991). A decrease in body temperature is often observed in rats before death (Kort *et al*., 1998). Therefore, in this study skin temperature was taken to monitor the progression of illness and thereby ensure that the infection did not progress to the point of death. Previous studies have used rectal probes to assess body temperature in neonatal rat pups (Conklin and Heggeness, 1971; Palmer *et al.*, 1993; Arnal *et al.*, 2013). However, the procedure used to attain rectal temperature measurements can induce stress (Warn *et al.*, 2003), which is known to influence neurodevelopment (Oitzl *et al.*, 2000). Thus, rectal temperature measurement was not a suitable tool to use in a study seeking to determine the neurodevelopmental effects of a neonatal infection. Kort and colleagues (1998) proposed the use of microchip transponders as a non-invasive alternative to rectal probes after their study found that temperature measurements obtained subcutaneously were not significantly different from temperature measurements made using a rectal probe in rats and mice (Kort *et al.*, 1998). Based on these results I chose to measure the skin temperature of infected rats to assess changes in body temperature produced in response to a *S. pneumoniae* infection.

Skin temperature was monitored using rod-shaped microchip transponders (12 x 2.1 mm, 0.06 g) encapsulated in a BioBond® anit-migration cap (Figure 2.2A). Each transponder was pre-programmed with a unique identification number by the suppliers (Identipet, Pretoria, South Africa) thus allowing us to record the temperature of individual pups within a litter. Transponders were calibrated against a high accuracy thermometer (Quat 100, Heraeus, Germany) revealed that the transponders had an accuracy of 0.1 °C with a resolution of 0.1 °C. Skin temperature was measured using a handheld BioThermo Pocket reader (Figure 2.2C, Identipet Pretoria, South Africa). When held 20-50 mm away from the transponder the handheld reader emits low frequency radio signals that activate the biosensitive transponder. The activation of the transponder resulted in instantaneous, non-invasive temperature readings.

2.5 Learning and memory

2.5.1 Morris water maze

The Morris water maze, developed by Richard G.M. Morris (Morris, 1981), is a well-established research tool that has been used since its development to assess the spatial learning and memory of rodents (Loeffler *et al*., 2001; Leib *et al*., 2003; Williamson and Bilbo, 2014). The Morris water maze generally consists of a circular pool that is filled with opaque water and surrounded by four distinct visual cues. The pool is divided into four virtual quadrants with the target quadrant containing a fixed platform. Rats are natural swimmers however when placed in a pool of water rats look for an escape route (Morris, 1981). Thus the fixed platform provides rats with an escape from the water.

The platform is submerged 1 cm below the surface of the water, hence rats cannot see the platform. Odour cues to the platform location are eliminated through the use of a disinfectant while audio cues to the platform location are eliminated through the use of a white noise

generator. Thus rats are forced to use the distal visual cues to learn the platform location. Generally healthy rats are able to learn the location of the platform relative to the distal visual cues and hence swim in a direct path to the escape platform (Morris, 1981). The time taken (latency) to swim to the platform should decrease with each swim trial thus the latency to the platform is used as a measure of learning. Morris showed that rats are able to learn the location of the platform within 20 swim trails (Morris, 1981). In my protocol 16 swim trails were shown to produce sufficient learning of the platform location (see Experiment 1: validation of Morris water maze and contextual fear conditioning). Spatial memory is then tested in a probe trial by removing the platform from the target quadrant. A rat that remembers the location of the platform should spend more time exploring the quadrant which housed the platform during the learning trials (Morris, 1981).

The Morris water maze has been successfully used to test the effects on spatial learning and memory after neonatal Gram-negative bacterial infections (Bilbo 2010, Williamson and Bilbo 2014), neonatal injury (Henderson *et al.*, 2014) and maternal deprivation (Oitzl *et al.*, 2000) during adulthood. Over the years results from the Morris water maze have proven to be reliable and robust (Sharma, 2009) therefore, the Morris water maze was chosen to test spatial learning and memory in adolescent (P29 – P34) rats, neonatally exposed to *S. pneumoniae*.

2.5.1.1 Apparatus and testing conditions

The Morris water maze apparatus consisted of a black circular pool measuring 0.5 m in height and 1.75 m in diameter. The pool was divided into four virtual quadrants (see Figure 2.3) with the target quadrant containing an adjustable escape platform (diameter = 100 mm). Four visual cues pinned onto poster boards surrounded the pool. The visual cues consisted of black geometric shapes (◯, △, ▢, +, =) placed against a white background (see Figure 2.4). A

camera (Canon Digital Video Camcorder, Model DM-MV550iE, Canon, Johannesburg, South Africa) and video tracking system (ANY-maze; Stoelting, Illinios, USA) were used to record the latency, distance and speed of rats swimming in the pool.

On the test days the pool was filled with water to a depth of 0.45 m. The temperature of the water was maintained between 25 and 26 °C for the duration of the testing period. Potassium permanganate was added to the water turning the water opaque and also acted as a disinfectant. The testing room was maintained at 22 °C and had dim lighting illuminating the pool to reduce reflections on the water surface. White noise (generated by ANY-maze software; Stoelting, Illinios, USA) played in the background to reduce any auditory cues that could distract rats during their performance in the pool. On test days rats were removed from their home cages and transported in individual cages to the testing room. Rats were given an hour to acclimatize to the testing conditions before testing begun. During this time vaginal smears were collected from female rats to determine the stage of the oestrous cycle (see section 2.6).

2.5.1.2 Testing procedure

A modified four day Morris water maze protocol was used to assess learning and memory in this study. Figure 2.4 summarises the procedures conducted over the four day testing period. The protocol consisted of a habituation trial, two cued tests, two learning sessions (composed of 8 learning trials each) and one probe trial.

Habituation trial

The habituation process allows rats to habituate to the pool and its surroundings (Morris, 1981; Vorhees and Williams, 2006). The habituation trial was conducted on day 1 (between 08:00 and 09:00) to reduce stress and anxiety associated with swimming. For the habituation

trial rats were released into the water tail first, with their head facing the wall, from release point 1 (see Figure 2.3). Rats were allowed to swim freely for 120 seconds before they were removed from the water, dried and returned to their cages. No platform was used during the habituation trial.

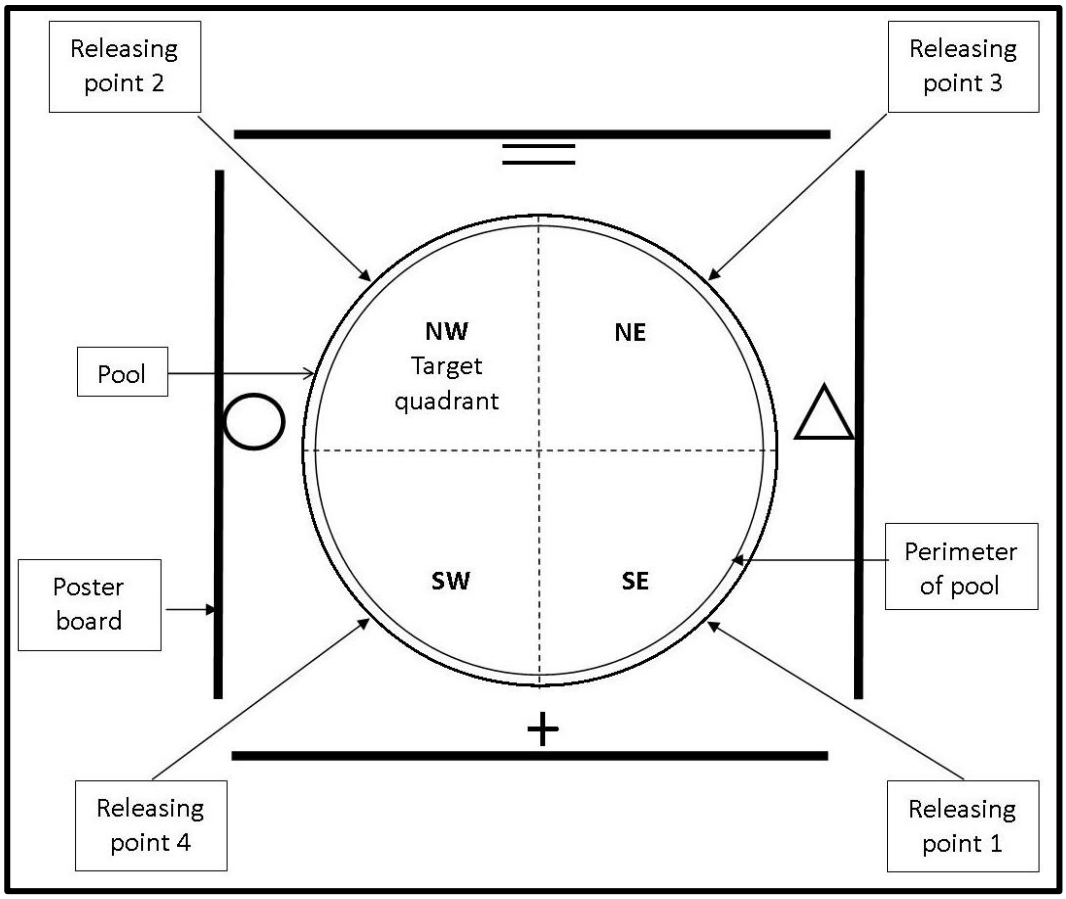


Figure 2.3 Schematic representation of the Morris water maze experimental set up, indicating the four release points from which rats were released into the water and the four virtual quadrants (NW, NE, SE, SW).

Cued test 1

A cued test was conducted 30 minutes after rats went through the habituation process. Performance in the Morris water maze requires visual acuity, the ability to swim and motivation to escape the water (Morris, 1984). I used the cued test as a control procedure to ensure that a rat does not have physical or visual disabilities or an inherent disincentive to escape water (Vorhees and Williams, 2006). During the cued test the platform was placed in

the centre of the pool, 1 cm above the water level with a 12 cm flag attached to the platform. Thus the platform was clearly visible to rats when they were swimming. Rats were given 60 seconds to swim (from release point 1) to the visible platform and thus escape from the water. Generally a healthy rat should see the platform and swim directly towards it as fast as possible. Thus swim speed is used as an indication of swimming ability and motivation to escape the water (Morris, 1984; Vorhees and Williams, 2006). Performance in the cued test was also measured using the latency and distance to the visible platform. The successful completion of the cued test indicated that rats were physically and visually able to participate in the learning trials conducted during acquisition training.

Acquisition training

Rats were given 16 chances i.e. 16 learning trials to learn the location of a hidden platform. The platform was submerged (10 mm below the water level) in the northwest (NW) quadrant. Rats were released from four release points. Learning trials were split over 2 days with 2 sessions (8 learning trials) conducted on each day. To spatially orientate themselves using the distal cues, rats were placed on the submerged platform for 10 seconds at the start of each day. After 10 seconds of orientation the rats were placed into the pool tail first, with the head facing the wall, from one of four starting points (see Figure 2.3). The order in which rats were released into the water (Table 2.1) was kept consistent for all rats. Rats were allowed a maximum of 60 seconds (per trial) to find the submerged platform. If rats failed to reach the platform within 60 seconds, they were guided to the platform by the experimenter and given another 3 seconds of orientation. Learning during the acquisition training was measured using the latency, distance and speed to the submerged platform. The inter-trial interval was limited to 15 seconds.

Table 2.1 Order of release of rats during acquisition training in the Morris water maze.

	Release point 1	Release point 2	Release point 3	Release point 4
Trials	1, 5, 9, 13	2, 6, 10, 14	3, 7, 11, 15	4, 8, 12, 16

Probe trial

Rats were given a 30 second probe trial 24 hours after the last (16th) learning trial. The probe trial is used to assess reference memory. For this trial the platform was removed and rats were released into the water from release point 1, tail first with the head facing the wall. After 30 seconds rats were removed from the pool, dried and returned to the cages. Typically a rat that remembers the location of the platform should swim directly to the target quadrant (the quadrant which housed the platform during the learning trials) and spend more time exploring that quadrant in search of the escape platform (Morris, 1981). Performance during the probe trial was therefore measured using the time spent in each quadrant as well as the latency, distance and speed to the former platform position.

Cued test 2

A second cued test (similar to the first cued test) was performed 30 minutes after the probe trial to ensure that rats did not acquire impairments during the experimental process. Cued test 2 was conducted as described for cued test 1.

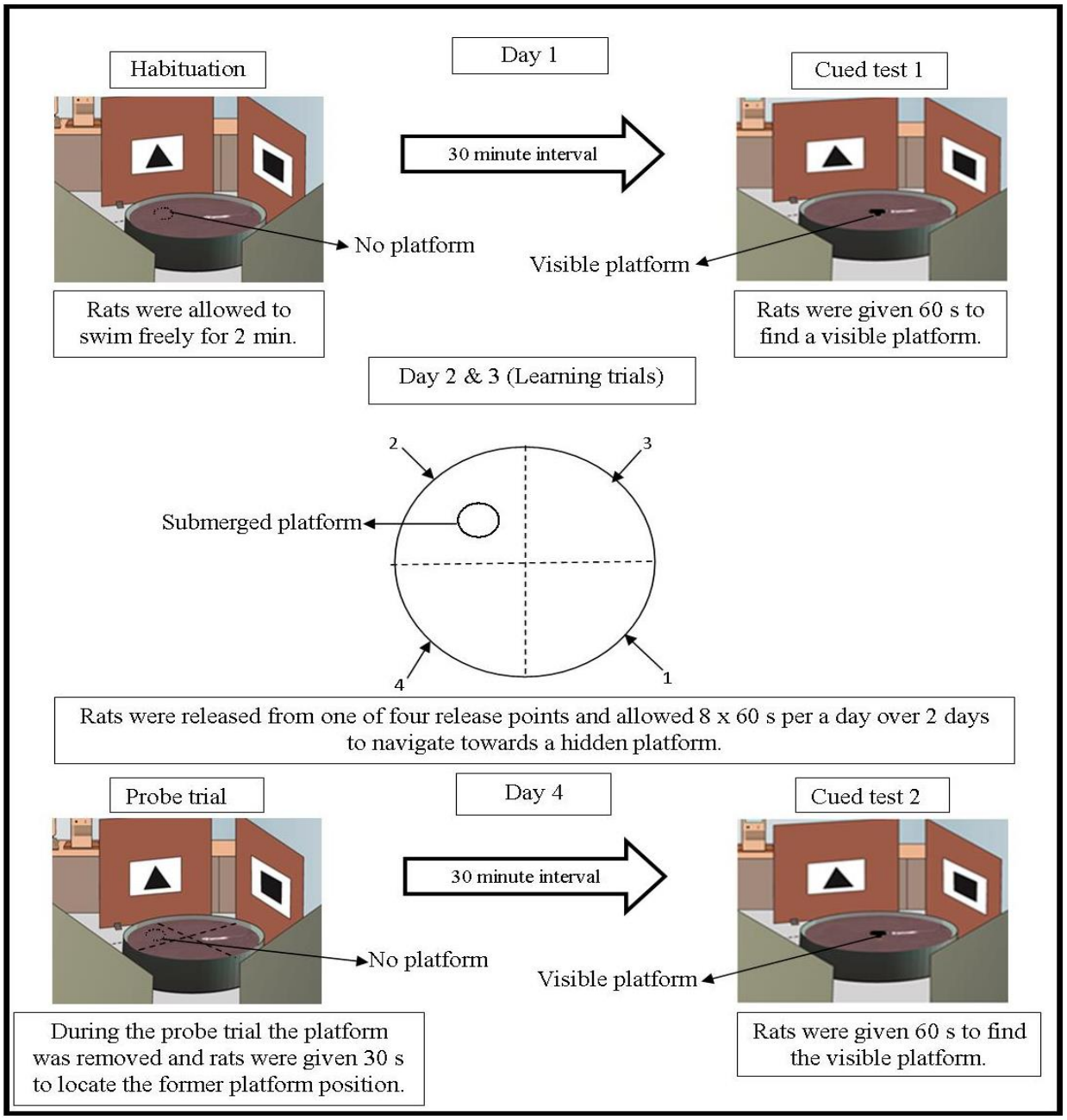


Figure 2.4 A summary of the four day Morris water maze protocol used in this study.

2.5.2 Contextual fear conditioning.

Contextual fear conditioning in rats, is based on Pavlovian fear conditioning. The model pairs a harmless conditioned stimulus such as a chamber made of metal walls with an adverse unconditioned stimulus such as a 1.5 mA foot shock (Le Doux, 2003). Rats exposed to the pairing, of a conditioned stimulus with an unconditioned stimulus, develop a fear of the chamber (the context) in which the shock was administered. As a result rats exhibit freezing behaviour. The act of freezing is an innate defensive reaction produced by rats in response to

fear and can be measured (Rudy *et al*., 2004). Contextual fear conditioning has been used to assess memory in adult rats that were infected neonatally with bacterial (E-*coli* or LPS) or viral pathogens (Pletnikov *et al*., 1999; Bilbo *et al.*, 2005; Harrè *et al.*, 2008). Thus for my study, contextual fear conditioning was chosen as a suitable and reliable tool to assess associative learning and memory in adolescent (P37) rats, neonatally exposed to *S. pneumoniae*.

2.5.2.1 Apparatus and behavioural procedures

Conditioning and testing of rats were conducted using a startle response system (Figure 2.5A) which comprised of two identical sound proof isolation chambers (0.79 x 0.52 x 0.45 m; Coulbourn Instruments, Whitehall, PA). The isolation chambers had white interiors and each housed a context chamber (0.23 x 0.24 x 0.20 m; Coulbourn Instruments, Whitehall, PA). The back and front walls of the context chambers were composed of clear Plexiglas while the two lateral walls and the ceiling were composed of stainless steel. A light fixture was situated on the right wall of the chamber. The removable metallic grid floor (16 stainless steel rods that are 1.5 mm in diameter, set 1.2 cm apart) was attached to a current generator (Coulbourn Instruments, Whitehall, PA) which produced a 1.5 mA foot shock using a Habitest self-powered line (Coulbourn Instruments, Whitehall, PA). Protocols used for conditioning and testing were created using Graphic state Notation 4 software (Coulbourn Instruments, Whitehall, PA). Conditioning and testing was conducted in a quiet, well-lit room maintained at 22 ± 2 °C.

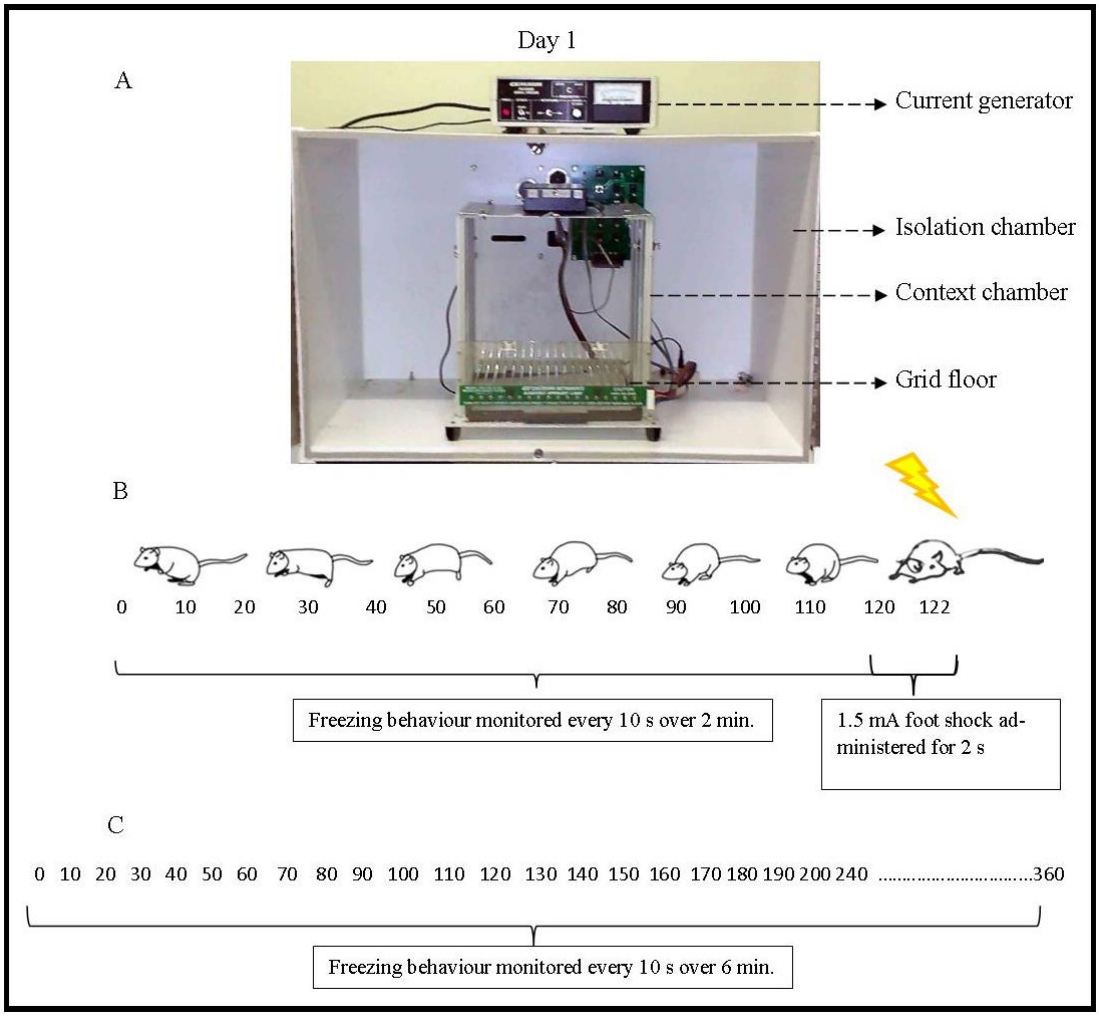


Figure 2.5 Schematic representation of the fear conditioning apparatus (A) that was used for the conditioning of rats (B) and in the assessment of memory for the fear of the context (C).

The conditioning process required rats to be exposed to a novel environment (context chamber) in which they encountered an adverse event (foot shock). Rats were conditioned between 08:00 and 10:00. On the day of conditioning rats were removed from their home cages and transported two at a time to the testing room. Each rat was transported in a black bucket with a lid. Rats were then placed individually into a novel environment i.e. the context chamber and allowed to explore the chamber for 120 seconds. Thereafter a 1.5 mA foot shock was administered for 2 seconds. Each rat's behaviour (movement or no movement i.e. freezing) was noted every 10 seconds for the duration of the conditioning process (Figure

2.5B). Rats were then transported back to their home cages in individual black buckets with lids. Both the chambers and the transportation buckets were cleaned and disinfected between test subjects (F10 Veterinary disinfectants, Health and Hygiene, Sunninghill, South Africa). After the conditioning process rats were returned to their home cages and left undisturbed for 48 hours.

Normally during the 48hour period that follows the hippocampus is thought to process all the features of the cage (odour, floor texture and lighting) and combine them to form a complete representation of the context. The memory of the context is then associated with the adverse experience of being foot shocked (Rudy *et al*., 2004). Thus, if hippocampal-dependant memory is intact a second exposure of the rat to the context chamber without the 1.5 mA foot shock, should evoke a fear response in rats. Freezing behaviour, defined as 'the absence of visible movement except for respiration', is a noticeable defensive response to fear which can be measured during testing (Rudy *et al*., 2004).

Testing for the memory of fear for the context was assessed between 08:00 and 10:00. Rats were transported back to the testing room using the same black transportation buckets with lids. Rats were once again placed in the context chamber and were observed for freezing behaviour by two trained observers. The observers who had no previous knowledge of the project were instructed to note the presence or absence of freezing behaviour every 10 seconds for a total of 6 minutes (Figure 2.5C). No foot shock was administered during the testing session. Vaginal smears were collection one hour before conditioning and testing to determine the stage of the oestrous cycle (see section 2.6).

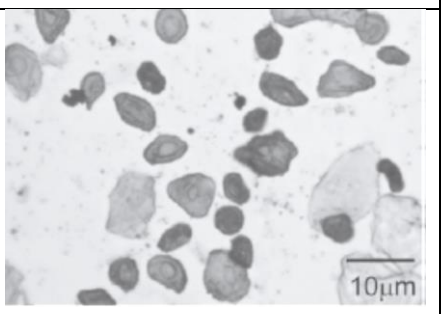
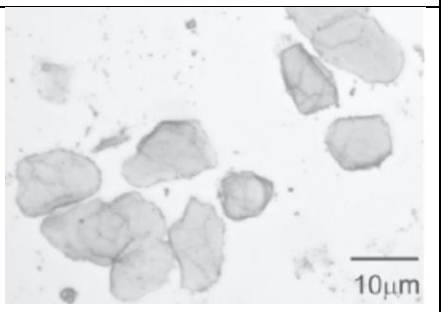
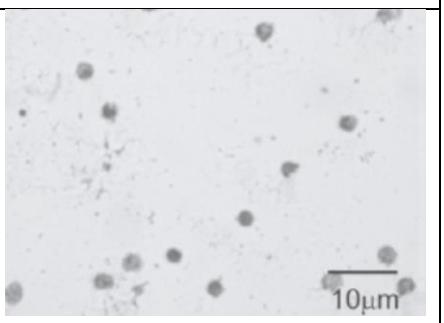
2.6 Determination of oestrus cycle stage.

In associative memory tasks adult female rats freeze less when conditioned and tested during the proestrus stage of the cycle than when conditioned and tested during the oestrus stage of

the cycle (Markus and Zecevic, 1997). Similarly, in spatial tasks adult female rats perform optimally during the oestrus phase of the cycle and worse during the proestrus phase of the cycle (Warren and Juraska, 1997; D' Hooge and De Deyn, 2001). Adolescent rats typically start cycling from P32 and are more likely to experience irregular cycle patterns than adult rats (Goldman *et al.,* 2007). Therefore vaginal cytology was conducted on female rats used in the study to determine any effect that the stage of the oestrus cycle might have on cognitive performance.

Vaginal lavages were performed an hour before behavioural testing began. For the lavage the tail of the rat was raised and a sterile plastic tip containing 10 ul of sterile saline was inserted 1 mm into the vagina. Cells from the vaginal epithelium were collected and transferred onto a glass microscope slide (1-1.2 mm; Lasec, Centurion, South Africa). Slides were then stained with 0.1 % toluidine blue and left to dry before analysis occurred. Cell populations were determined under a light microscope at 40 x magnification. Table 2.2 shows the criteria used to determine the point of the oestrous cycle phase.

Table 2.2: Cell populations used to determine the phase of the oestrus cycle.

Phase of cycle	Dominant cell population	Photomicrohraph*
Proestrus	Nucleated epithelial cells	
Oestrous	Anucleated cornified cells	
Diestrus	Leucocytes	

*Photomicrographs adapted from Garcina *et al*, 2011

2.7 Experimental procedures

Differences in behavioural apparatus used, the conditions under which tests are conducted or differences in protocols used may influence performance and outcomes of behavioural tests (Vorhees and Williams, 2006). Thus in experiment one a validation study was conducted to verify that the conditions and protocol used in both the Morris water maze and contextual fear conditioning are able to detect impairments in learning and memory. The validation study also provided an opportunity for me to familiarise myself with the apparatus and

protocols. In experiment two, four day old pups were anaesthetised and injected with 250 ul of *S. pneumoniae* to characterise the spread of bacteria throughout the periphery and the brain. The results from experiment two were then used to design and investigate the effects of an early-life infection on learning and memory in rats (experiment three). An additional experiment was conducted to investigate the ability of *S. pneumoniae* to induce nuclear factor interleukin-6 (NF-IL6) expression in the hippocampus.

2.7.1 Experiment 1 – Validation of the Morris water maze and contextual fear conditioning protocols.

A pharmacological challenge with scopolamine hydrobromide was used to validate the Morris water maze and contextual fear conditioning protocols in adolescent rats. Scopolamine hydrobromide is a muscarinic, cholinergic antagonist which acts on the basal forebrain cholinergic system to impair memory acquisition, consolidation and retrieval (for review see Deiana *et al.*, 2011).

2.7.1.1 Animals and housing

Adolescent Sprague-Dawley rats (P21: n = 30 males, n = 24 females) were purchased from the NHLS and housed in gender specific rooms. Both rooms were maintained at 22 ± 2 °C with a 12h:12h light:dark cycle (lights on at 07:00). After a week of acclimatization the rats were randomly assigned to one of two experimental groups.

Experimental procedure: Morris water maze

Initially both adolescent male and female were used for the validation of the Morris water maze. However, preliminary results showed no differences in spatial learning and memory between male (n = 15) and female (n = 12) rats that were injected intraperitoneally with

saline (0.9 %, 1 ml/kg, see section 3.1.1). To reduce the number of animals used in this experiment we decided to complete the validation study using only male rats (n = 15).

Male rats were intraperitoneally injected with scopolamine hydrobromide (0.8 mg/kg; Sigma, Aldrich, St Louis, MO, USA). The dose and time of administration of scopolamine hydrobromide was based on a previous validation study conducted in our laboratory (Swanepoel *et al.*, 2011). Learning and memory of both groups of rats that received either saline or scopolamine hydrobromide were tested within 30 minutes of administration of scopolamine hydrobromide (Figure 2.6). Scopolamine hydrobromide appears to have a half-life of only 30 minutes (Ebert *et al.*, 1998).

Experimental procedure: Contextual fear conditioning

Studies have shown gender differences in results obtained from contextual fear conditioning (Markus and Zecevic, 1997; Jasnow *et al.*, 2006). We therefore intraperitoneally injected adolescent male (n = 12) and female (n = 12) Sprague-Dawley rats with saline (1 ml/kg) and compared freezing behaviour between the genders. The pilot results showed significant differences between the freezing behaviour of male and female rats ($t_{(19)} = 2.15$, $P = 0.04$) therefore we carried out the validation study using both male and female rats. An additional 12 males and 12 females were intraperitoneally injected with scopolamine hydrobromide (0.8 mg/kg; Sigma, Aldrich, St Louis, MO, USA).

Rats were injected with scopolamine hydrobromide or saline 30 minutes before being exposed to the context chamber during conditioning and testing (Figure 2.7). The conditioning and testing procedure was conducted as described in section 2.5.2.1. Two independent observers were present during testing to record the freezing behaviour of rats.

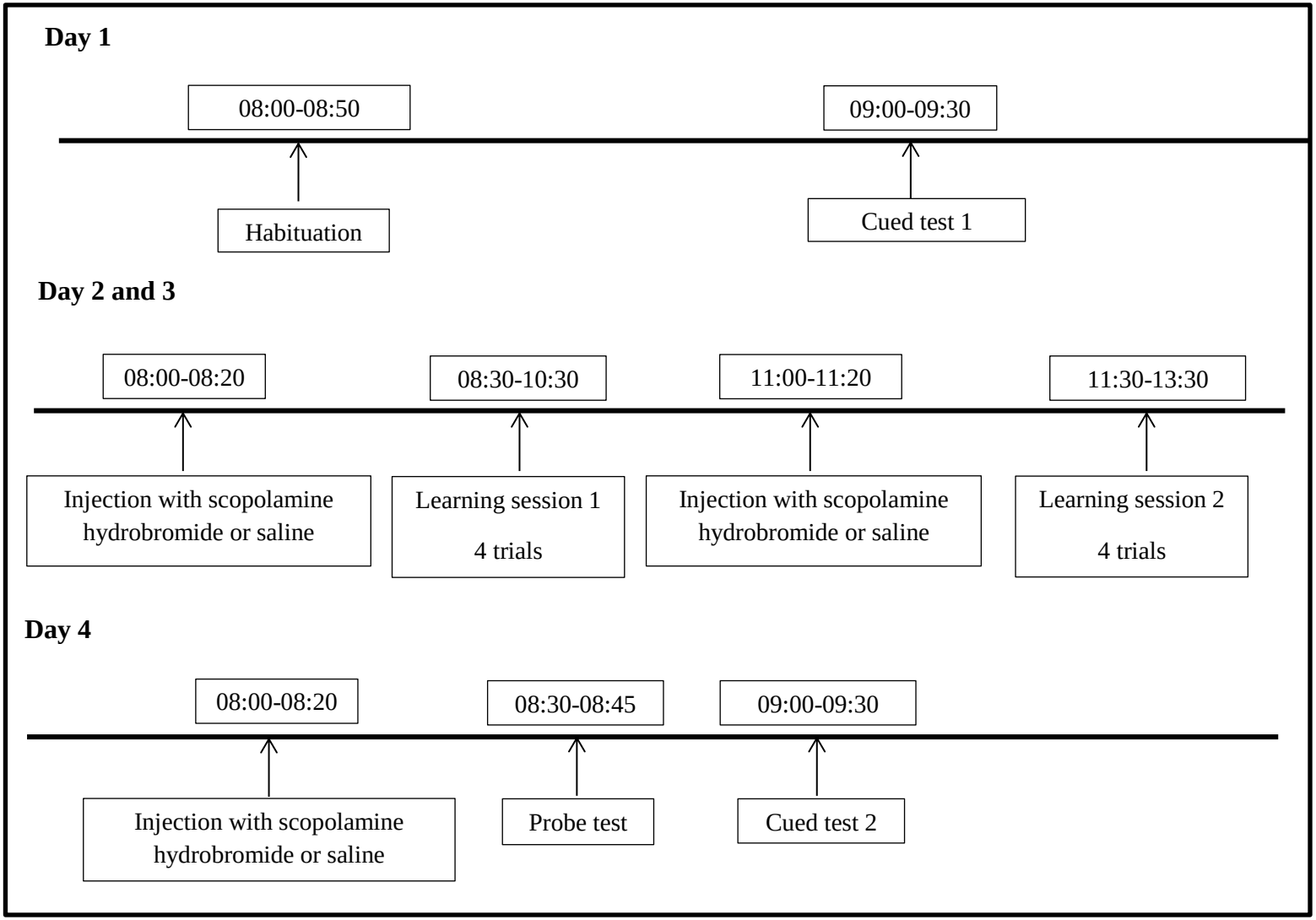


Figure 2.6 The study design used to validate the modified Morris water maze protocol.

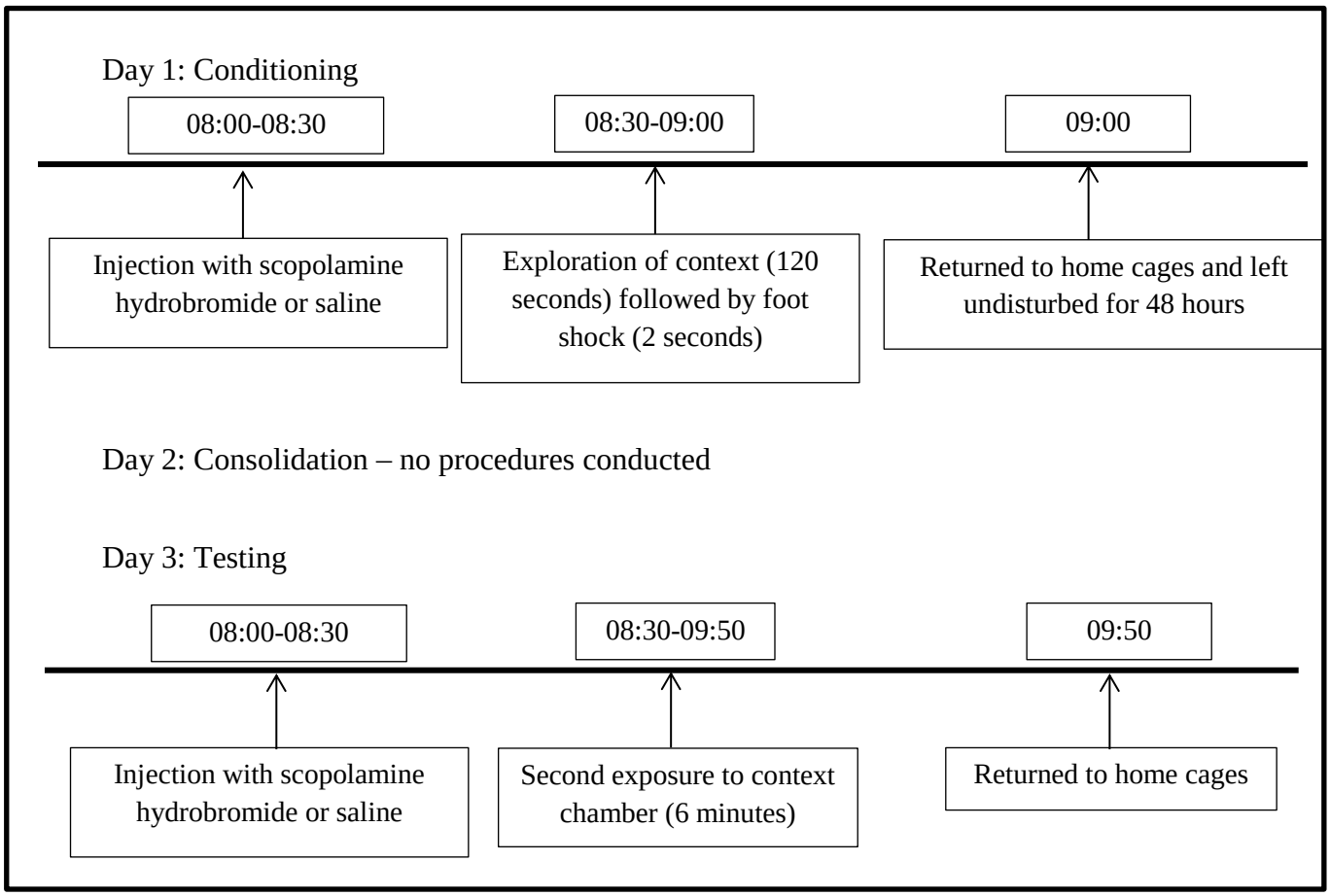


Figure 2.7 The study design used to validate the contextual fear conditioning model.

2.7.2 Experiment 2- Establishing a model of haematogenous meningitis.

Sprague-Dawley rat pups (P4: n = 8 males, n = 8 females) were anaesthetised and implanted with microchip transponders between the shoulder blades (see section 2.3). Rats then received an intraperitoneal injection of *S. pneumoniae* (mean ± SD: 46 ± 35 CFU in 250 ul), between 16:00 and 17:00. Following infection with *S. pneumoniae*, general health (skin colour, activity and feeding) and the skin temperature of rats were monitored hourly over a 24 hour period. Once rats showed signs of illness (pale skin colour, decreased activity and decreased feeding) a final temperature measurement was made and rats were given a lethal dose (1ml) of sodium pentobarbitone (Eutha-naze, Bayer, Johannesburg, South Africa). As soon as rats were unresponsive, an incision was made along the midline of the body to expose the heart, lungs, liver and spleen. Approximately 20 ul of blood was drawn from the heart

using a 25 G needle flushed with an anticoagulant, heparin (Bodene, Port Elizabeth, South Africa). Thereafter the lungs, liver, spleen and brain were harvested and weighed. The organs were homogenized separately using sterile homogenizers (50 ml glass homogenizers, Lasec, Centurion, South Africa) filled with 1 ml of sterile saline. Cardiac blood and organ homogenates were plated in sterile saline using a 10-fold serial dilution. The blood agar plates (5 % sheep blood supplemented with gentamicin; Media Mage, Johannesburg, South Africa) were placed in an incubator and allowed to grow overnight in 5 % carbon dioxide at 37 °C. Single bacterial colonies were counted after 18 hours of incubation to estimate the amount of bacteria present in each organ and in the blood of each rat.

2.7.3 Experiment 3- Investigating the effects of a neonatal infection with *S. pneumoniae* on learning and memory in adolescent rats

Postnatal day 4 rats were intraperitionealy injected with *S. pneumoniae* (mean ± SD: 46 ± 35 CFU in 250 ul) or 0.9% sterile saline and treated with an intramuscular injection of the antibiotic, ceftriaxone (100 mg/kg 2-3 times a day as recommended by manufacturer, Aspen ceftriaxone, Sandton, South Africa) or its vehicle, injectable water. Ceftriaxone is a broad spectrum cephalosporin that targets a wide variety of Gram-positive bacteria including *S. pneumoniae*. Ceftriaxone is a common antibiotic used in animal developmental studies as it does not lead to developmental stunting (Rothstein *et al.,* 2005). On the day of infection rat pups were randomly assigned to receive injections of one of the following combinations: *S. pneumoniae* + ceftriaxone (n = 10 males, n = 9 females); Saline + ceftriaxone (n = 10 males, n = 8 females); Saline + water (n = 9 males, n = 9 females).

It has been shown that a 5minute period of maternal separation does not alter maternal behaviour (Harre *et al.,* 2008). Therefore all procedures (infection, treatment administration and temperature readings) were limited to a maximum of 5 minutes per pup to ensure our

handling of pups did not induce maternal stress. To avoid cross contamination between pups, pups from the same litter received the same intervention (*S. pneumoniae* or saline). Litter characteristics such as litter size, maternal care and growth rates of pups are considered as confounding factors in developmental studies (Abbey and Howard, 1973) and have a significant effect on each pup's behaviour. Therefore each experimental group contained a maximum of 2 male and 2 female pups per litter.

All litters were infected between 16:00 and 18:00. Hourly skin temperature measurements were used to estimate the progression of the infection in individual pups. Skin temperatures below 34 °C indicated the onset of the illness (based on results from experiment 1, see section 3.2). At this point tail blood and CSF were collected to estimate the bacterial load within each pup (P5). For ethical reasons pups were euthanized when the skin temperature dropped below 32 °C. Tail blood was collected by amputating the tip of the tail of anaesthetized rats using sterile surgical scissors. The blood (20 ul) then was collected using a pipette fitted with a sterile filter tip flushed with an anticoagulant, heparin (Bodene, Port Elizabeth, South Africa). Within five minutes of collection, the blood was plated (on blood agar plates supplemented with gentamicin) using 10-fold dilutions and bacteria present in the blood were allowed to grow overnight.

Single bacterial colonies were counted after 18 hours to provide an estimation of the amount of bacteria present in the blood of rats before antibiotic treatment was administered. Additionally, CSF was collected using a 26 G needle attached to a 1ml syringe via polyethylene tubing. The needle was inserted at the occipital crest through the skin and 5 ul of CSF was drawn from the cisterna magnum. The bacterial load in the CSF of rats was estimated by quantitative cultures as described for the tail blood samples.

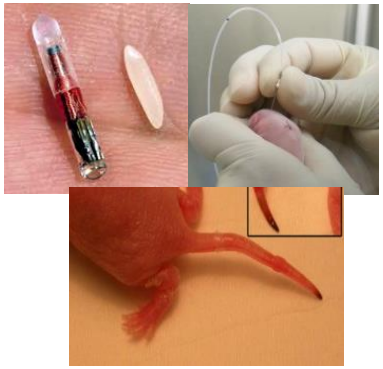
Rats were returned to their home cages after receiving an intramuscular injection of ceftriaxone. To ensure the survival of pups two more doses of ceftriaxone was administered four hours apart. Thereafter ceftriaxone was administered twice a day (08:00 and 16:00) for four consecutive days. The same treatment regime was used for rats injected with saline and for rats that received injectable water.

All pups were weaned on P21, removed from the infectious unit and housed in gender specific rooms in same sex pairs within the same experimental groups. Rats were handled during cage changes and on experimental days. Memory testing in the Morris water maze (refer to section 2.5.1) was conducted from P29 to P34 between 08:00 and 13:00. The same rats were then exposed to contextual fear conditioning (refer to section 2.5.2) on P37 and P39 between 08:00 and 10:00. Figure 2.8 shows a summary of the experimental procedure used for experiment 3.

Neonatal manipulations



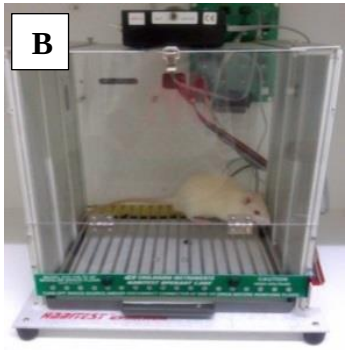
Postnatal day (P4) rats had microchip transponders implanted between their shoulder blades and were injected intraperitoneally with *S.pneumoniae* (mean ± SD: 46 ± 35 CFU in 250 ul) or saline.



Skin temperature was monitored hourly. Once the skin temperature decreased below 34 °C tail blood and CSF were collected. Antibiotic treatment with ceftriaxone (100 mg/kg) or injectable water was administered from P5 to P9.

Adolescent behavioural assessment





Memory was assessed in the Morris water maze (MWM; A) and Contextual fear conditioning (B). Rats had to learn the location of a submerged platform within two learning sessions (composed of 16 learning trials). Reference memory was then assessed in a probe trial the following day. Five days after the MWM experiment rats were exposed to contextual fear conditioning. On the day of conditioning rats were allowed to explore the novel environment (context cage) for two minutes after which they received a 1.5 mA foot shock. Forty-eight hours later the rats were placed back into the context cage and their freezing response was recorded as a measure of memory.

P4 P5 P29 –P39

Figure 2.8 Summary of the procedures used to investigate the effects of an early life infection on learning and memory in adolescent rats.

2.7.4 Experiment 4- Nuclear factor interleukin-6 expression in the rat hippocampus.

Nuclear factor interleukin-6 (NF-IL6) is a transcription factor that plays an important role in memory consolidation (Taubenfeld *et al.*, 2001). Disruption of NF-IL6 function within the hippocampus may result in memory impairments in rodents (Taubenfeld *et al.*, 2001). Therefore we stimulated micro-cultures of the rat hippocampus with ethanol-treated *S. pneumoniae* ($5x10^3$, $5x10^4$, $5x10^5$ and $5x10^6$ CFU/ml) to determine the effect of *S. pneumoniae* on NF-IL6 activation. The experiment was performed at the Institute of Veterinary-Physiology and – Biochemistry at Justus-Liebig University Giessen, Germany. Due to biosafety concerns and shipping regulations the *S. pneumoniae* bacteria used in this experiment was pre-treated with and stored in 70% ethanol. Thus, only dead bacterial cells were used to stimulate hippocampal micro-cultures of rats.

2.7.4.1 Stimulants

A second batch of clinical isolates of *S. pneumoniae* (serotype 2, D39) were obtained from the Respiratory and Meningeal Pathogens Research Unit (Chris Hani Baragwanath Hospital, Johannesburg, South Africa) was grown in Todd-Hewitt broth (Sigma, Johannesburg, South Africa) to an optical density of 0.3. Quantitative cultures were used to calculate the concentration of bacteria present in each solution. The Todd-Hewitt broth containing bacteria and yeast extract [(5 %), Sigma, Johannesburg, South Africa] was then centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the bacterial pellet was re-suspended in 50 ml of 70 % ethanol. The ethanol treated bacterial solution was once again centrifuged at 4000 rpm for 10 minutes. The bacterial pellet formed was re-suspended in 1ml of 70 % ethanol and stored for shipping. Before use the ethanol-treated *S. pneumoniae* solution was re-suspended in 1ml of sterile phosphate buffered saline (PBS).

Lipopolysaccharide (LPS; Sigma-Aldrich, Munich, Germany) was used as a positive control in this experiment. The *E.coli* derived endotoxin was dissolved in 0.1 M/l PBS before being added to hippocampal derived cells.

2.7.4.2 Preparation of hippocampal micro-cultures

Hippocampal micro-cultures were isolated from the brain tissue of P4-6 Wistar rats (gender unknown) obtained from the Justus-Liebig University breeding unit. For the isolation procedure rats were decapitated using sharp surgical scissors and the head was sterilized by immersion in cold 70 % ethanol. The brain was carefully removed from the skull under sterile conditions and mounted onto a Telflon® block using Histoacryl® tissue glue (Braun, Melsungen, Germany). The Teflon block supporting the brain was placed into a chamber containing cold oxygenated Gey's Balanced Salt Solution (GBSS, Biotrend, Cologne, Germany) supplemented with 5 % D- glucose (Sigma-Aldrich, Munich, Germany). Serial coronal slices, 500 um thick, were cut 5.8 mm away from Bregma (Figure 2.9) using a vibratome (752M, Vibroslice,WPI, Berlin, Germany) equipped with fibre optics (1500KL, Schott, Mainz, Germany).

Cells in the CA1 region of the hippocampus have been shown to be activated during spatial and contextual fear memory retrieval (Tsien *et al*., 1996; Hall *et al*., 2001). Therefore the CA1 regions (Figure 2.9) of the rat hippocampus were dissected with fine eye scissors and a stereomicroscope (SMZ-U, Nikon, Düsseldorf, Germany). CA1 fragments were placed into a pertri dish containing Hanks Balanced Salt Solution (HBSS, Biochrom, Berlin, Germany) that lacked calcium and magnesium but was supplemented with 20mM/L HEPES (Sigma-Aldrich, Munich, Germany), pH 7.4.

The fragments were then enzymatically treated with 2 ml dispase-1 (1.0 U/ml; Roche Diagnostics, Mannheim, Germany) in oxygenated HBSS with 20 mM/l HEPES at 37 °C. After 60 minutes of enzymatic treatment, the enzyme was deactivated by washing the fragments with HBSS containing 1.0 mM/l EDTA (Sigma-Aldrich, Munich, Germany). The fragments were then washed with 3 ml of medium (Neurobasal medium A supplemented with 2 % B 27 [Invitrogen,Karlsruhe, Germany], penicillin [100 U/ml], streptomycin [0.1 mg/ml] and 2 mM/l L-glutamine [Biochrom, Berlin, Germany]).

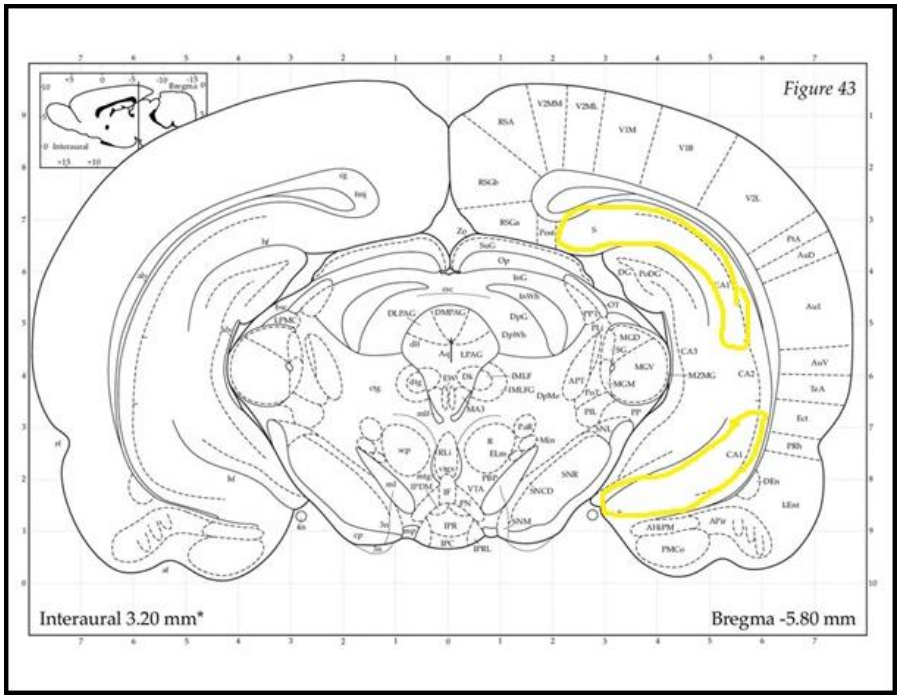


Figure 2.9 The position at which coronal slices were taken (Extract from Paxinos and Watson, 2005).

Thereafter the fragments were dissociated in 1ml of medium by repeated trituration with the tip of a 1ml pipette. The dissociated cells were plated in reusable Flexiperm-micro-12 well (6 mm diameter; Greiner Bio-One GmbH, Solingen, Germany) that were pre-warmed and coated with poly-L-lysine (1.0 mg/ml H2O;Sigma Aldrich) which fixed the flexiperm onto CELLocate® glass coverslips (Eppendorf,Hamburg, Germany). Cells were grown over 3

days with 5 % CO_2 at 37 °C. Media was changed the day after preparation and cells remained undisturbed for the remainder of the time.

Seventy-two hours after preparation cells were stimulated with one of the solutions in Table 2.3. Stimulation was conducted for 24 hours after which the supernatant was removed and the cells were fixed with 4 % paraformaldehyde (Merck, Darmstadt, Germany) for 15 minutes. The paraformaldehyde was washed off by rinsing cells three times with PBS. Fixed cells were then incubated for 2 hours in blocking buffer containing 10 % Fetal Calf Serum (PAA, Pasching, Austria) diluted in PBS containing 0.05 % TritonX-100 (PBS-Triton; Sigma-Aldrich). Once the blocking solution was removed the cells were incubated with NF-IL6 (1:5000) and one of three primary antibodies diluted in blocking buffer (anti-GFAP 1:1000, anti-MAP2a+b 1:800 and anti-ED-1 1:1000). Incubation with the primary antibody was conducted overnight.

Table 2.3 The stimulant used in experiment 4.

	Stimulant
1	200 ul of 5x10e^6 CFU/ml *S. pneumoniae* D39 + 1800ul of Media
2	200 ul of 5x10e^5 CFU/ml *S. pneumoniae* D39 + 1800ul of Media
3	200 ul of 5x10e^4 CFU/ml *S. pneumoniae* D39 + 1800ul of Media
4	200 ul of 5x10e^3 CFU/ml *S. pneumoniae* D39 + 1800ul of Media
5	20 ul of 1 mg/ml LPS + 1980ul of Media
6	200 ul of PBS + 1800ul of Media

Thereafter the unbound antibodies were removed using PBS-Triton and cells were incubated for 2 hours with fluorophore-coupled secondary antisera (Alexa-488 donkey anti-rabbit IgG [H+L], diluted at 1:500 in blocking buffer and Cy^3 goat anti-mouse IgG [H+L] diluted at

1:1000 in blocking buffer [Dianova, Hamburg, Germany]). After incubation cells were washed (three times) with PBS-Triton and the cellular nuclei were labelled with 2-(4-amidinophenyl)-1H-indole-6-carboxyamidine (DAPI; Mobitec, Göttingen, Germany). The coverslips containing the fixed cells stained for NF-IL6 and three different cell types were embedded onto microscope slides using a glycerol/PBS solution (Citifluor Ltd., London, UK).The slides were studied and photographed with an Olympus BX50 epifluorescence microscope (Olympus Optical) equipped with the appropriate filter sets. A five point scale (Table 2.4) was used to rate NF-IL6 immunoreactivity.

Table 2.4 Five point rating scale used to assess NF-IL6 immunoreactivity.

Score	Interpretation
+++	High density of nuclear signal
++(+)	Moderate to high density of nuclear signal
++	Moderate density of nuclear signal
+	Low density of nuclear signal
-	No nuclear signal

2.8 Data analysis

Data was analysed using Graphpad Prism version 5 (Graph-pad Software Inc., San Diego, USA) and Statistica version 12 software (StatSoft Inc., Oklahoma, USA). All data are expressed as means and standard deviations (±SD). Statistical significance for all comparisons were set at $\alpha < 0.05$ and corrected to < 0.01 if multiple comparisons were conducted. The bacterial load present in different sites of the body in male and female pups was expressed as log values per gram of tissue or ml of blood and were compared using a two-way (gender x body site) analysis of variance (ANOVA).

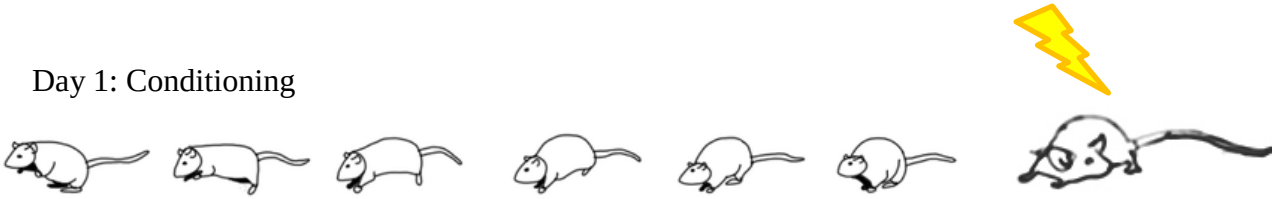
Performance in cued test 1 and 2 (measured using the mean latency, distance and speed of a group) was analysed using an unpaired t-test (scopolamine x saline , Experiment 1) and a two-way (gender x treatment) ANOVA (Experiment 3). Spatial learning (measured using the mean latency, speed and distance of a group for each session) was analysed using a two-way (treatment x session) ANOVA (Experiment 1) and a three-way (gender x treatment x session) ANOVA (Experiment 3). Performance during the probe trial (measured the mean latency, distance and speed to the former platform position) was analysed using an unpaired t-test (scopolamine x saline, Experiment 1) and a two-way (gender x treatment) ANOVA (Experiment 3). The time spent in each quadrant was analysed with a three-way (gender x treatment x quadrant) ANOVA. In addition a one-sample t-test was used to determine if all animals reached the platform under the cut off time of 30 seconds.

Performance in contextual fear conditioning was measured by two trained observers. Each observer was provided with a score card (Figure 2.10) designed to numerically record the freezing behaviour of each animal. Each block on the score grid represented a 10 second observation point in which the observer recorded whether the rat was in motion (indicated by '0') or stationary i.e. freezing (indicated by '1'). The number of positive freezing observations ('1') that were recorded was added for each observer. The freezing percentage of rats was then calculated by taking the sum of all positive freezing observations and dividing it by the total number of observation points.

The result was then converted to a percentage. The average percentage of freezing between the observers for each rat was then analysed using a two-way (gender x treatment) ANOVA. The inter-rater reliability exceeded 94% for all groups tested using contextual fear conditioning. The inter-rater reliability was calculated by finding the correlation co-efficient

between all positive freezing observations noted by both observers. Bonferroni *post hoc* tests were performed when statistical significance was detected.

Day 1: Conditioning



0 10 20 30 40 50 60 70 80 90 100 110 120 122

Rat 1	0	0	0	0	0	0	0	0	0	0	0	Reacted to shock

Day 3: Testing



0 10 20 30 40 50 60 70 80 90 100 110 360

Rat 1	0	0	1	1	1	1	1	1	1	1	1	1

Figure 2.10 Example of the scoring procedure used to assess freezing behaviour in rats during conditioning and testing using fear conditioning.

Chapter 3

Results

3.1 Experiment 1– Validation of the Morris water maze and contextual fear conditioning protocols.

3.1.1 Morris water maze validation

Spatial learning and memory of male and female rats were analysed to determine the necessity of testing both gender groups in the validation protocols for the Morris water maze. Figure 3.1A shows that the mean time taken (latency) to reaching the submerged platform (measured during the learning trials) decreased for male and female rats that received an injection of saline (intraperitoneal, 1 ml/kg) (Main effect of time: $F_{(3,75)} = 30.01$, $P < 0.0001$). Importantly, there was a significant difference between the mean latency during learning session 1 and learning session 4 for all rats tested, indicating that rats had learnt (Bonferroni's multiple comparisons test, $P < 0.01$). No gender differences were observed in the latency to reaching the submerged platform (Main effect of gender: $F_{(1,25)} = 0.04$, $P > 0.05$). In addition, no interaction effects between gender and time (learning sessions) were observed (Main effect of interaction: $F_{(3, 75)} = 1.27$, $P > 0.05$).

Figure 3.1B shows that the mean latencies to the former platform position (tested during the probe test in which the platform was removed) for both gender groups were significantly shorter than the 30 second cut off, indicating that rats had remembered the platform location (one sample t-test for male and female rats: $t_{(14)} = 9.79$ and $t_{(11)} = 5.01$, $P < 0.001$). Moreover, there were no significant gender differences in the time taken to find the former location of the platform (unpaired t-test: $t_{(25)} = 0.94$, $P > 0.05$). The results show that both gender groups display similar patterns of spatial learning and memory in our version of the Morris water maze. Therefore, the results that follow for the validation of the Morris water maze protocol using scopolamine are for male rats only.

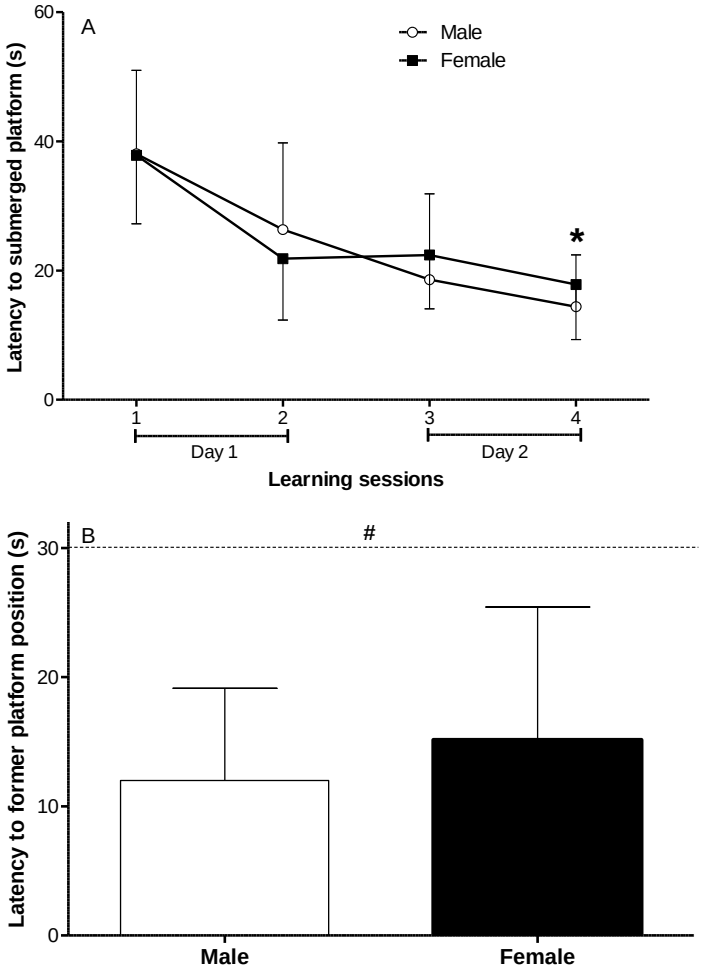


Figure 3.1 Latencies to reaching the submerged platform during the learning sessions (A) or the former platform position during the probe trial (B) for male (n =15) and female (n =12) rats that received an intraperitoneal injection of saline (1 ml/kg). Rats were trained over 16 trials (2 learning sessions a day comprising of 4 trials per a session) to find a submerged platform. The mean value for the four trials per session is presented (A). * Indicates a significant difference between learning session 1 and learning session 4 (Bonferroni's multiple comparisons test, $P < 0.01$). No gender differences were noted for the latency to reaching the submerged platform (Main effect of gender: $F_{(1, 25)} = 0.04$, $P > 0.05$). No interaction effects were detected ($F_{(3, 75)} = 1.27$, $P > 0.05$). To assess memory, a probe trial was performed 24 hours after the last learning session. # Indicates that both male and female rats were able to find the location of the former platform position in under 30 seconds (one sample t-test for male and female rats: $t_{(14)} = 9.79$ and $t_{(11)} = 5.01$, $P < 0.001$) and no gender differences in the latency to the former platform position was detected (unpaired t-test: $t_{(25)} = 0.94$,$P > 0.05$). Results are expressed as mean and standard deviation.

Cued tests

Results from cued test 1, which was conducted before the administration of any substance, showed no difference in performance in the Morris water maze between adolescent male rats (P29) that would later receive an intraperitoneal injection of either saline or scopolamine hydrobromide. The latency, distance and speed to the visible platform were similar for all rats indicating that rats were not physically impaired (Data not shown. Cued test 1 for latency, distance and speed, Unpaired t-test: $t_{(28)} = 1.66$, 1.39 and 1.18; $P > 0.05$).

Cued test 2 was conducted after the learning sessions and probe trial and therefore equal 5 doses of scopolamine hydrobromide (0.8 mg/kg) or saline (1 ml/kg). The latency, distance and speed to the visible platform were not different between rats that received scopolamine hydrobromide and those that received saline despite multiple administration of scopolamine hydrobromide over 3 consecutive days (Data not shown. Cued test 2 treatment effect for latency, distance and speed, Unpaired t-test: $t_{(28)} = 0.42$, 0.33 and 0.22; $P > 0.05$).

Acquisition training

Figure 3.2A shows that over the four learning sessions the mean latency to reach the submerged platform decreased significantly for male adolescent rats (P30) that received scopolamine hydrobromide and male adolescent rats that received saline (Main effect of time: $F_{(3, 84)} = 18.30$, $P < 0.0001$). A significant difference between the mean latency to reaching the submerged platform for learning session 1 and learning session 4 was found indicating that all rats had learnt to locate the platform (Bonferroni's multiple comparisons test, $P < 0.001$). The mean latency to reaching the submerged platform was significantly different between adolescent rats that received scopolamine hydrobromide and adolescent rats that received saline (Main effects of treatment: $F_{(1,28)} = 18.36$, $P < 0.001$). On the second day of acquisition training (during sessions 3 and 4) rats that received scopolamine hydrobromide

took a longer time to reach the submerged platform compared to rats that received saline (Bonferroni's multiple comparisons test, $P < 0.01$). No interaction between treatment and learning sessions were detected for latencies measured during acquisition training (Main effect of interaction effect latency: $F_{(3,84)} = 0.86$, $P > 0.05$).

Figure 3.2B the distance swum to reach the submerged platform for adolescent male rats (P30). Over the four learning sessions the distance swum to reach the submerged platform decreased significantly for male adolescent rats that received scopolamine hydrobromide and male adolescent rats that received saline (Main effect of time: $F_{(3, 84)} = 13.23$, $P < 0.0001$). The distance swum to reach the submerged platform during learning session 4 was significantly less than the distance to reaching the platform during learning session 1 for both groups of rats (Bonferroni's multiple comparisons test, $P < 0.001$). The distance swum to reach the submerged platform was significantly different between adolescent rats that received scopolamine hydrobromide and adolescent rats that received saline (Main effects of treatment: $F_{(1,28)} = 26.03$, $P < 0.0001$). Rats that received scopolamine hydrobromide swam longer distances to reach the submerged platform on the second day of acquisition training (learning sessions 3 and 4) as compared to rats that received saline (Bonferroni's multiple comparisons test, $P < 0.01$). No interaction between treatment and time (learning sessions) was detected for distance measured during acquisition training (Main effect of interaction effect distance: $F_{(3,84)} = 0.36$, $P > 0.05$).

Swim speed (Figure 3.2C) was comparable over the four learning sessions between male adolescent rats (P30) that received scopolamine hydrobromide and male adolescent rats that received saline (Main effect of time: $F_{(3, 84)} = 2.94$, $P > 0.01$). Although the speed swum to reach the submerged platform was significantly different between adolescent rats that received scopolamine hydrobromide and adolescent rats that received saline (Main effects of

treatment: $F_{(1,28)} = 10.11$, $P < 0.01$), post hoc tests could not detect significant differences between the two treatment groups over the four learning sessions (Bonferroni's multiple comparisons test, $P > 0.05$). No interaction between treatment and learning sessions were detected for speed measured during acquisition training (Main effect of interaction effect speed: $F_{(3,84)} = 0.66$, $P > 0.05$).

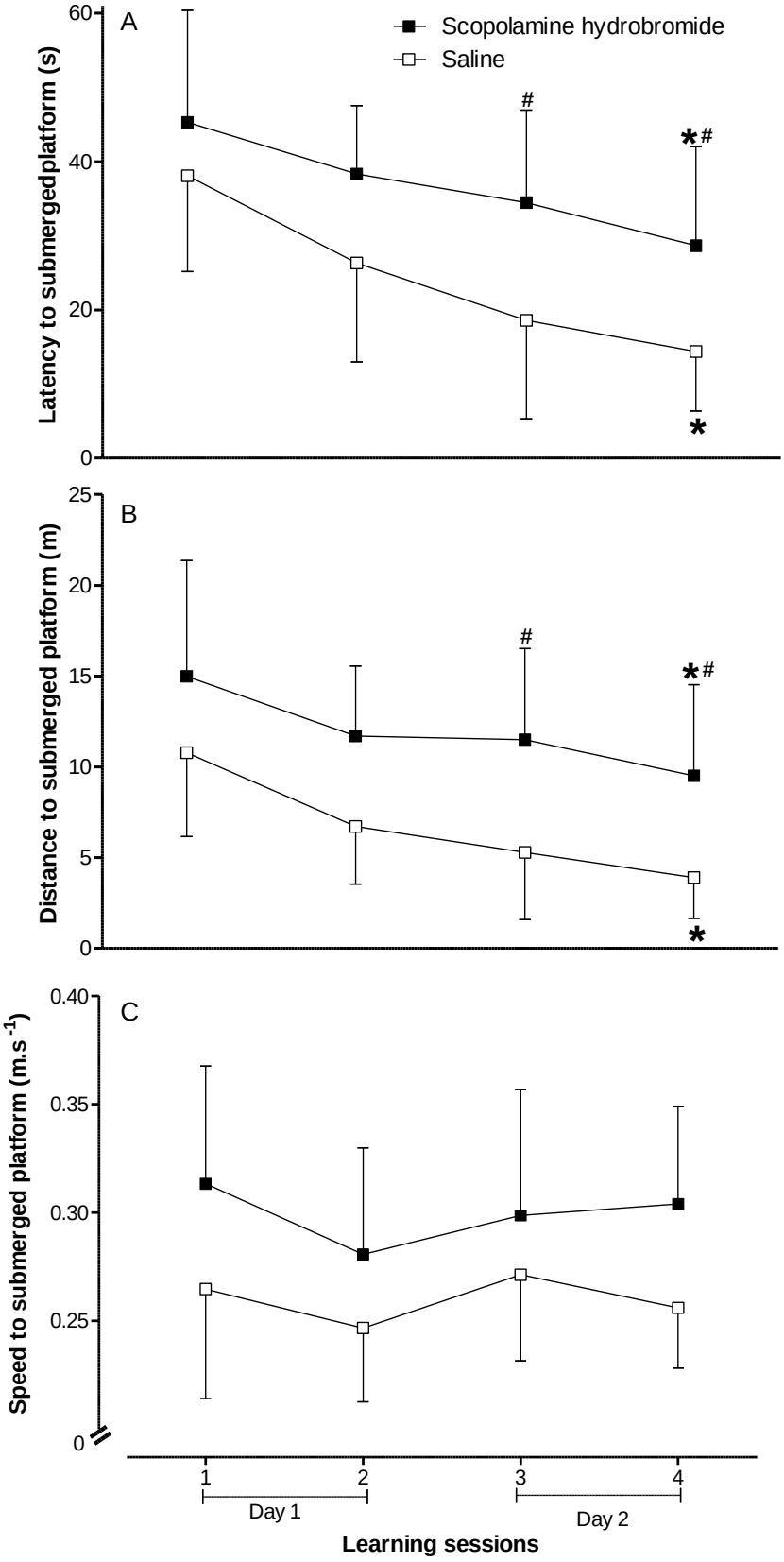


Figure 3.2 Spatial learning, measured using latency (A), distance (B) and speed (C) to the submerged platform, in adolescent (P30 and P31) rats that received an intraperitoneal

injection of either scopolamine hydrobromide (0.8 mg/kg, n =15) or saline (1 ml/kg, n =15). Rats were trained over 2 days (2 learning sessions a day) to find a platform submerged 10 mm below the water. * Indicates a significant difference between learning session 1 and learning session 4 for rats that received scopolamine hydrobromide or saline (Main effect of time for latency and distance: $F_{(3, 84)} = 18.30$ and 13.23; Bonferroni's multiple comparisons test $P < 0.01$). # Indicates a significant difference between rats that received scopolamine hydrobromide and rats that received saline at learning sessions 3 and 4 (Main effects of treatment for latency and distance: $F_{(1, 28)} = 18.36$ and 26.03; Bonferroni's multiple comparisons test, $P < 0.01$). No interaction between treatment and learning sessions were detected for any of the parameters measured (Main effect of interaction for latency, distance and speed: $F_{(3,150)} = 0.86$, 0.36 and 0.66; $P > 0.05$). Results are expressed as mean and standard deviation.

Probe test

Figure 3.3A shows that the mean latency to the former platform position (measured during the probe test, when the platform was removed) for both adolescent (P32) male rats injected with scopolamine hydrobromide or saline was significantly shorter than the 30 second cut off time (one sample t-test for scopolamine hydrobromide and saline rats: $t_{(14)} = 3.72$ and 9.79, $P < 0.01$). However adolescent male rats that received scopolamine hydrobromide took longer to reach the former platform position compared to adolescent male rats that received saline (Unpaired t-test: $t_{(28)} = 2.5$, $P < 0.05$). Similarly figure 3.3B shows that adolescent rats injected with scopolamine hydrobromide swam longer distances to reach the former platform position compared to adolescent male rats injected with saline (Unpaired t-test: $t_{(28)} = 2.67$, $P < 0.05$). Figure 3.3C shows that there was no significant differences between the swim speed of adolescent rats injected with scopolamine hydrobromide and saline during the probe trial (Unpaired t-test: $t_{(28)} = 1.81$, $P > 0.05$).

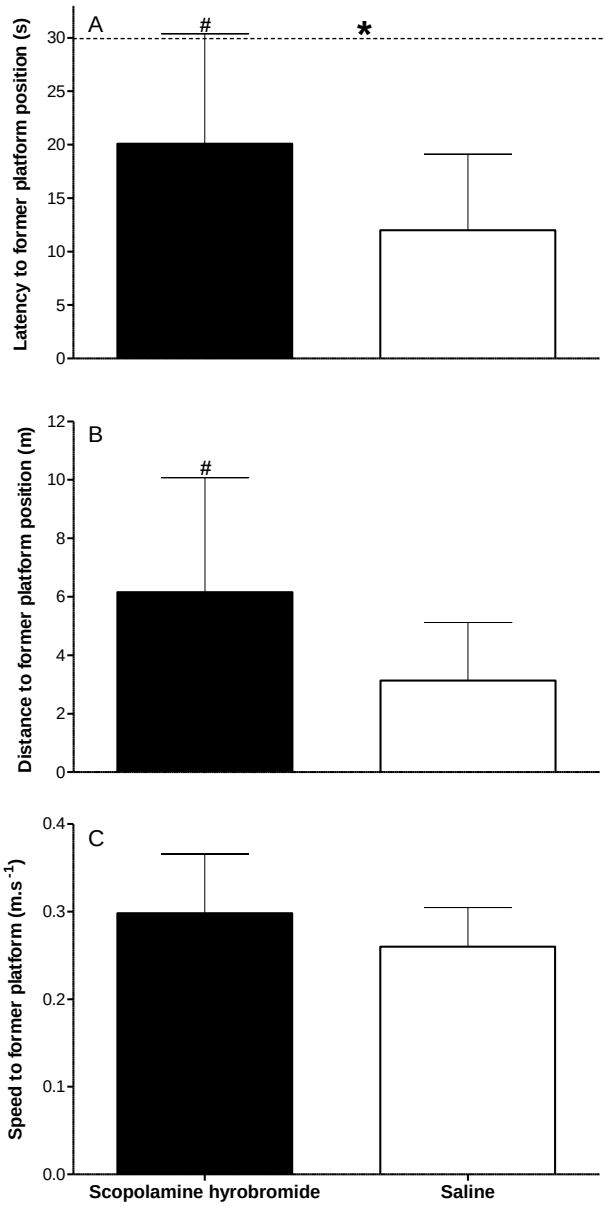


Figure 3.3 Spatial memory, measured using latency (A), distance (B) and speed (C) to the former platform position, in rats that received an intraperitoneal injection of scopolamine hydrobromide (0.8 mg/kg, n =15) or saline (1 ml/kg, n =15). Rats were given 30 seconds to locate the former platform position. * Indicates that all rats were able to find the location of the former platform within 30 seconds (one sample t-test for scopolamine hydrobromide and saline rats: $t_{(14)}$ =3.72 and 9.79, $P < 0.01$). # indicates a significant difference between rats that received scopolamine hydrobromide and rats that received saline (Unpaired t-test for latency and distance: $t_{(28)} = 2.5$ and 2.67, $P \leq 0.02$). Results are expressed as mean and standard deviation.

3.1.2 Contextual fear conditioning

Associative learning and memory of adolescent (P39) male and female rats was analysed to determine the necessity of testing both gender groups in the validation of the contextual fear conditioning protocol used. Results confirmed significant differences between the freezing behaviour of male and female rats (data not shown, Unpaired t test: $t_{(19)} = 2.15$, $P < 0.05$), thus the validation study was completed using both gender groups.

During conditioning adolescent (P37) male and female rats that were injected with scopolamine hydrobromide or saline did not display any fear responses (freezing behaviour) in the context chamber and all rats were responsive to the 1.5 mA foot shock (data not shown). However, when tested for fear of the context (Figure 3.4) male and female adolescent rats which received an intraperitoneal injection of scopolamine hydrobromide displayed significantly less freezing than male and female rats that received an intraperitoneal injection of saline (Main effect of treatment: $F_{(1,37)}= 128.9$, $P < 0.0001$).

In addition a significant gender effect was detected between the freezing behaviour of male and female rats (Main effect of gender: $F_{(1, 37)} = 4.43$, $P < 0.05$). Male rats that received an intraperitoneal injection of saline froze significantly more than female rats that received an intraperitoneal injection of scopolamine hydrobromide (Bonferroni's multiple comparisons test, $P < 0.01$). No significant interaction effect was detected for gender and treatment (Main interaction effect gender x treatment: $F_{(1, 37)} = 3.70$, $P > 0.05$). Four male rats and three female rats were excluded from statistical analysis based on the results of a Grubbs' outlier test.

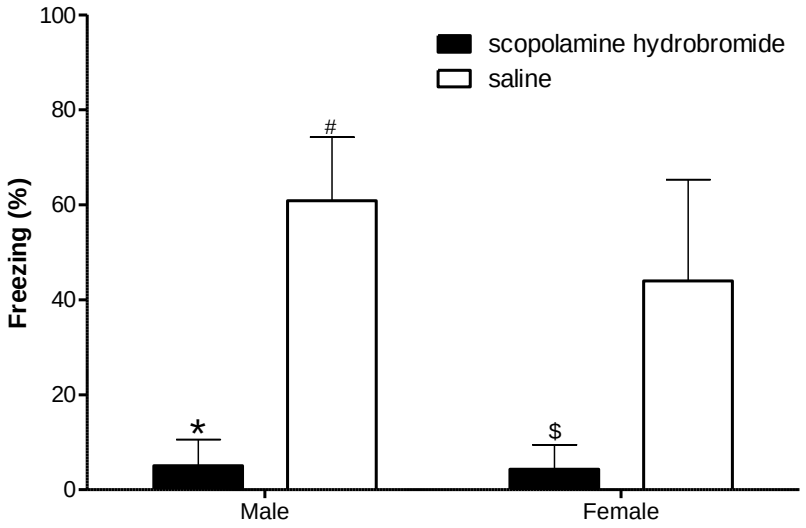


Figure 3.4 Freezing behaviour (fear responses) of adolescent (P39) rats injected intraperitoneally with scopolamine hydrobromide (0.8 mg/kg, n = 10 males and n =10 females) or saline (1 ml/kg, n = 10 males and n =11 females). The freezing response of rats to a context in which they previously received a 1.5 mA foot shock was measured over 6 minutes. * Indicates a significant difference between male rats that received scopolamine hydrobromide and male rats that received saline and **$** indicates a significant difference between female rats that received scopolamine hydrobromide and female rats that received saline (Main effect of treatment: $F_{(1, 37)} = 128.9$, Bonferroni's multiple comparisons test, $P < 0.01$). # Indicates a significant difference between male rats that received saline and female rats that received scopolamine hydrobromide (Main effect of gender: $F_{(1, 37)} = 4.43$; Bonferroni's multiple comparisons test, $P < 0.01$). No significant interaction effect was detected for gender and treatment (Main interaction effect: $F_{(1, 37)} = 3.70$, $P > 0.05$). Results are expressed as mean and standard deviation.

3.1.3 The effects of the different phases of the oestrus cycle on learning and memory in female rats

Morris water maze

A previous study investigating the gender differences in hippocampal-dependant learning found that adult male rats performed better than adult female rats in hippocampal-dependant memory tasks (Warren and Juraska, 1997). Adult female rats in the proestrus phase of the cycle performed poorer than both adult male rats and adult female rats in the oestrus phase of the cycle (Warren and Juraska, 1997). Figure 3.1 shows that the overall performance of adolescent male rats was comparable to the overall performance of adolescent female rats in the Morris water maze validation study. However on the day of memory testing (in the probe trial) the latency to the former platform position for adolescent female rats was more variable than the latency to the former platform position for adolescent male rats. To determine if the variability in the performance during the probe trial was related to female rats being in different phases of the cycle during the learning sessions, I analysed vaginal smears which were taken during both training days (before the learning sessions began) in the Morris water maze.

Table 3.1 shows the different phases of the oestrous cycle for saline treated adolescent (P29 - P32) female rats on the days of behavioural testing in the Morris water maze. Nine out of 11 rats (rats 1-9) displayed a regular four day cycle with two days of diestrus followed by a day of proestrus and then a day of oestrus. On the second day of training (learning sessions 3 and 4), 10 out of 11 rats were determined to be in the proestrus phase of the cycle. Previous results have reported that adult female rats which learnt during the proestrus phase of the cycle displayed impaired hippocampal-dependant memory during the probe trial in the Morris water maze (Warren and Juraska, 1997). However in our validation study the latency

to the submerged platform was comparable between adolescent female rats which learnt during the proestrus phase of the cycle and male rats (Figure 3.5A; $t_{(23)} = 1.56$, $P > 0.05$). Furthermore adolescent female rats which learnt during the proestrus phase of the cycle did not display impairments in memory as the latency to the former platform position for female rats were similar to the latency to the former platform position for male rats (Figure 3.5B; $t_{(23)} = 0.76$, $P > 0.05$).

Table 3.1 Phase of the oestrous cycle of adolescent (P 29- P 32) rats tested in the Morris water maze.

Rat	Habituation and cued test 1	Learning sessions 1 and 2	Learning sessions 3 and 4	Probe
1	Diestrus	Diestrus	Proestrus	Oestrus
2	Diestrus	Diestrus	Proestrus	Oestrus
3	Diestrus	Diestrus	Proestrus	Oestrus
4	Diestrus	Diestrus	Proestrus	Oestrus
5	Diestrus	Diestrus	Proestrus	Oestrus
6	Diestrus	Diestrus	Proestrus	Oestrus
7	Diestrus	Diestrus	Proestrus	Oestrus
8	Diestrus	Diestrus	Proestrus	Oestrus
9	Diestrus	Diestrus	Proestrus	Oestrus
10	Diestrus	**Proestrus**	**Diestrus**	Oestrus
11	Diestrus	**Oestrus**	**Proestrus**	Oestrus

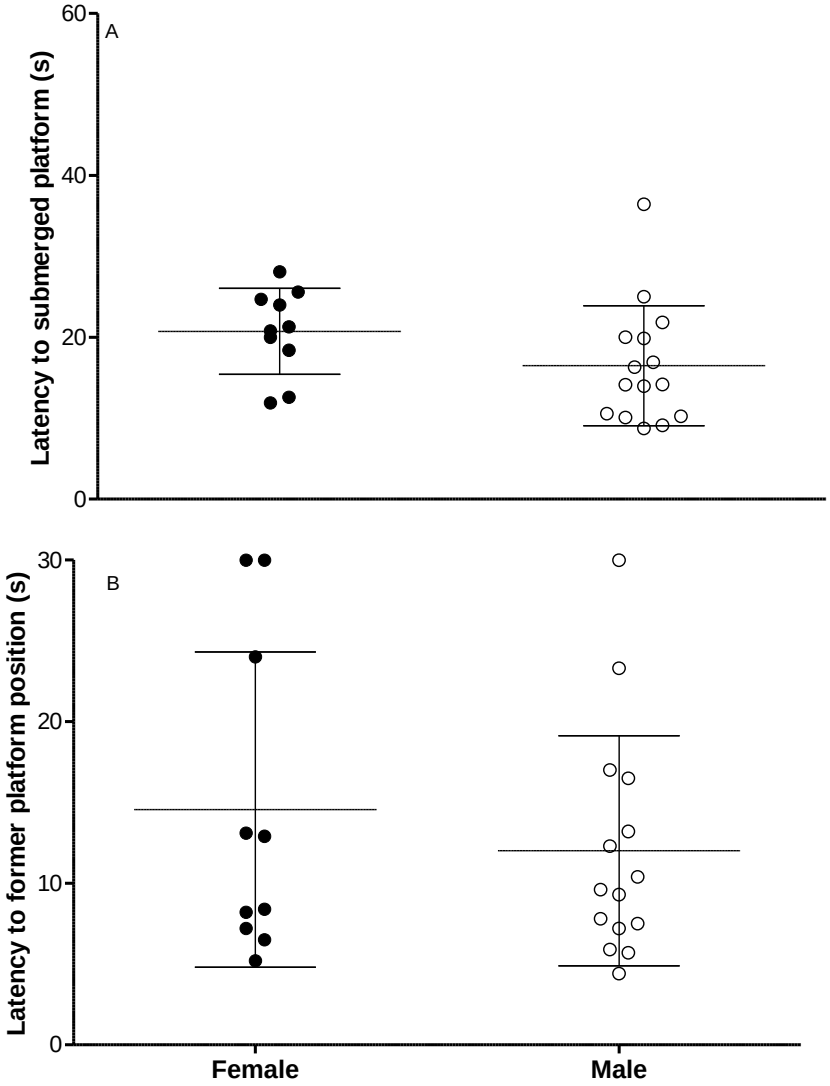


Figure 3.5 Spatial learning (A) measured using latency to the submerged platform and memory (B) measured using latency to the former platform position (probe trial) in adolescent (P32) female and male rats. Vaginal cytology was used to determine the phase of the oestrous cycle in adolescent female rats. No significant differences were detected in spatial learning and memory between female rats (n =10) that learnt during the proestrus phase of the cycle (during learning sessions 3 and 4) and male rats (n =15, Unpaired t-test for learning day 2 (mean of learning sessions 3 and 4) and probe trial: $t_{(24)} = 1.56$ and 0.76, $P > 0.05$). Results are expressed as individual data, mean and standard deviation.

Contextual fear conditioning

Figure 3.4 (section 3.1.2) shows that in our validation study significant overall gender differences in freezing behaviour were exhibited by adolescent (P39) rats during contextual fear conditioning. Previous studies have shown that adult female rats freeze less when conditioned and tested during the proestrus phase of the cycle than when conditioned and tested during the oestrus phase of the cycle (Markus and Zecevic 1997). To explain the differences seen between adolescent male and female rats in our study, we analysed vaginal smears taken during the days of learning (conditioning) and memory (testing).

On the day of conditioning 9 out of 11 rats were in the diestrus phase of the cycle and the remaining two rats were in the oestrus phase of the cycle whereas on the day of testing 10 out of 11 rats were in the diestrus phase of the cycle and the remaining rat was in the oestrus phase of the cycle (data not shown). None of the female rats that were tested using contextual fear conditioning were in the proestrus phase of the cycle during learning (conditioning) or recall (testing) thus the phase of the cycle did not appear to account for the differences noted between male and female rats.

3.2 Experiment 2 - Establishing a model of haematogenous meningitis

Skin temperature

A sudden, distinct decrease in skin temperature was detected in rats that were injected with *S. pneumoniae* between 16 to 29 hours after injection. Although the decrease in skin temperature from 36 °C to below 34 °C was a consistent observation in all rats injected with *S. pneumoniae,* the time at which the decrease in skin temperature occurred varied between rats. Early responders (i.e. rats in which a decrease in temperature was observed from 16 to 20 hours after an injection of *S. pneumoniae*, n = 5) and late responders (i.e. rats in which a decrease in temperature was observed from 20 to 29 hours after an injection of *S.*

pneumoniae, n = 12) recovered their baseline skin temperatures (skin temperature values seen before an injection of *S. pneumoniae*) after receiving the first antibiotic dose. However rats which exhibited a decrease in temperature below 30°C did not survive (n = 24.8 % of infected rats, data not shown).

Figure 3.6 shows the skin temperature of **two** individual P5 rats injected with *S. pneumoniae* plotted against the average skin temperature of a litter of 12 rats (P5) injected with saline. In one rat the skin temperature decreased 16 hours after receiving an injection while in the other the skin temperature decreased 27 hours after receiving an injection.

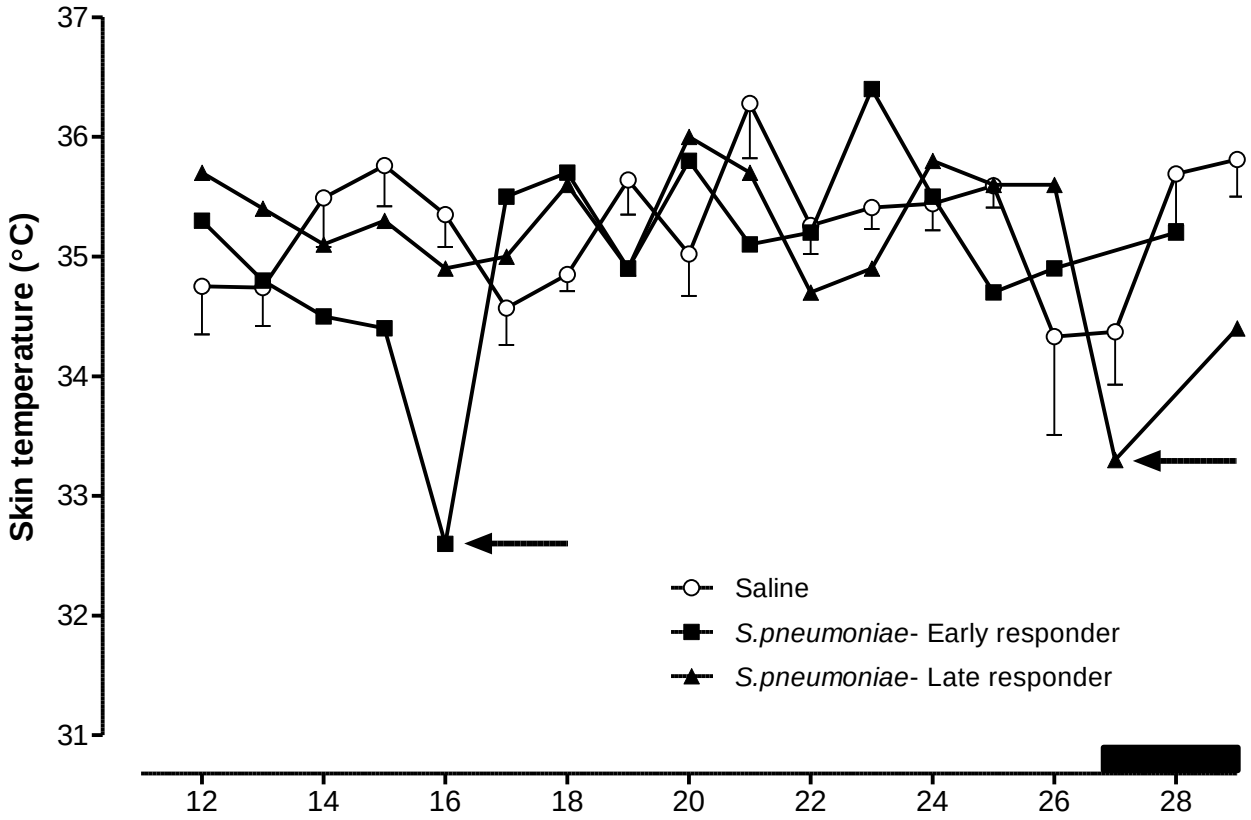


Figure 3.6 Skin temperature measurements of P5 rats that received an intraperitoneal injection of *S. pneumoniae* (250 ul of $1x10^2$ CFU/ml) or saline (250ul) on P4. Data shown are from two individual rats injected with *S. pneumoniae* and the average temperature (and standard deviation) of a litter of 12 rats injected with saline. The arrows indicate a decrease in skin temperature and the first antibiotic treatment. The black bar indicates lights out (19:00 clock time).

Bacterial load in different body sites

Figure 3.7 shows the bacterial load within the blood (cardiac), brain, spleen, liver and lungs of P5 rats injected with *S. pneumoniae* (250 ul of $1x10^2$ CFU/ml). Samples were collected once the skin temperature of each rat decreased below 34 °C. Bacterial colonies were found in all samples taken from all rats. Following administration of *S. pneumoniae* bacterial colonies increased five-fold within the brain, liver, spleen and lungs while a seven-fold increase of bacterial colonies was detected within the blood.

Significant differences were seen in the bacterial load at different body sites of male and female rats (Main effect of body site: $F_{(4,60)} = 12.73$, $P < 0.0001$). A greater amount of bacteria were found in male liver samples as compared to male brain samples, whereas female brain samples had significantly lower bacterial counts compared to female liver, lungs, spleen and blood samples (Bonferroni's multiple comparisons test, $P < 0.01$). The exponential increase in bacterial colonies obtained from different body sites were similar between samples taken from male and female rats (Main effect of gender: $F_{(1, 15)} = 2$, $P > 0.05$). No interaction between body site and gender was detected (Main effect of interaction: $F_{(4,60)} = 0.95$, $P > 0.05$).

Furthermore, no significant differences were found in the bacterial load within the different body sites between early responders (rats which displayed a decrease in temperature from 16 to 20 hours after infection, n = 5) and late responders (rats which displayed a decrease in temperature from 20 to 29 hours after infection, n = 12; Data not shown, Main effect of time of collection: $F_{(1, 60)} = 0.29$, $P > 0.05$). No interaction effects were noted between the time of collection and the body site (Main interaction effect: $F_{(4, 60)} = 1.51$, $P > 0.05$). A significant difference was detected between the bacterial load at different body sites (Main effect of body site: $F_{(4,60)} = 35$, $P < 0.0001$). A greater amount of bacteria was found in the brain compared to the liver, lungs, spleen and blood (Bonferroni's multiple comparisons test, P <

0.01). The pattern of bacterial dispersion confirms that our model of *S. pneumoniae* infection is one of haematogenous meningitis.

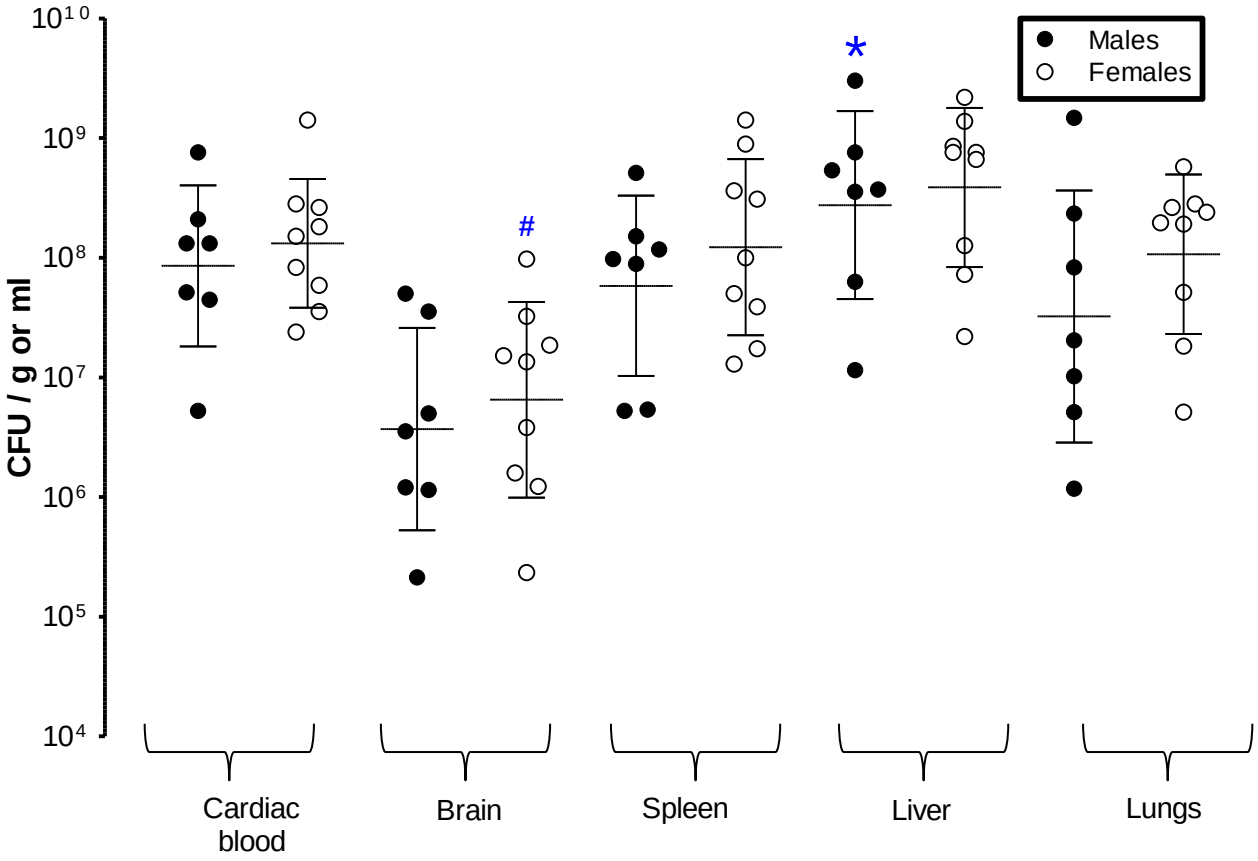


Figure 3.7 Characterisation of the spread of *S. pneumoniae* from the peritoneum to the brain, spleen, liver and lungs following an intraperitoneal injection of *S. pneumoniae* (250 ul of $1x10^2$ CFU/ ml). * Indicates a significant difference between male brain samples and male liver samples (Bonferroni's multiple comparisons test, $P < 0.01$). # Indicates a significant difference between female brain samples and female blood, liver, lungs and spleen samples (Bonferroni's multiple comparisons test, $P < 0.01$). No significant gender differences were detected between bacterial counts within the liver, lungs, spleen, brain and blood (Main effect of gender: $F_{(1, 15)} = 2$, $P > 0.05$) No significant interaction effects were detected (Main effect of interaction: $F_{(4,60)} = 0.95$, $P > 0.05$). Results are expressed as log transformed values and are shown as mean and standard deviation.

3.3 Experiment 3 - The effects of a neonatal infection with *S. pneumoniae* on learning and memory in adolescent male and female rats.

Bacterial load within tail blood and cerebrospinal fluid (CSF)

Figure 3.8 shows results from quantitative cultures of tail blood and CSF samples collected from neonatal (P5) rats, 16 - 29 hours after receiving an intra-peritoneal injection of *S. pneumoniae*. Samples were collected once the skin temperature decreased below 34°C. The bacterial load within the blood was significantly greater than the bacterial load within the CSF for both male and female rats (Main effect of body site: $F_{(1,17)} = 43.17$, $P < 0.0001$). Similar bacterial loads were found between blood and CSF samples for neonatal male and female rats (Main effect of gender: $F_{(1,\ 17)} = 0.10$, $P > 0.05$). No interaction effect was detected (Main effect of interaction: $F_{(1,\ 17)} = 0.32$, $P > 0.05$).

Furthermore no significant differences were found in the bacterial load within the blood and CSF samples between early responders (rats which displayed a decrease in temperature from 16 to 20 hours after infection, n = 7) and late responders (rats which displayed a decrease in temperature from 20 to 29 hours after infection, n = 12; Data not shown, Main effect of time of collection: $F_{(1,\ 17)} = 3.55$, $P > 0.05$). However a significant difference was detected between the bacterial load at different body sites (Main effect of body site: $F_{(1,17)} = 77.15$ $P < 0.0001$). A greater amount of bacteria was found in the blood samples compared to the CSF samples (Bonferroni's multiple comparisons test, $P < 0.01$). A significant interaction effect was detected (Main effect of interaction: $F_{(1,17)} = 9$, $P < 0.01$).

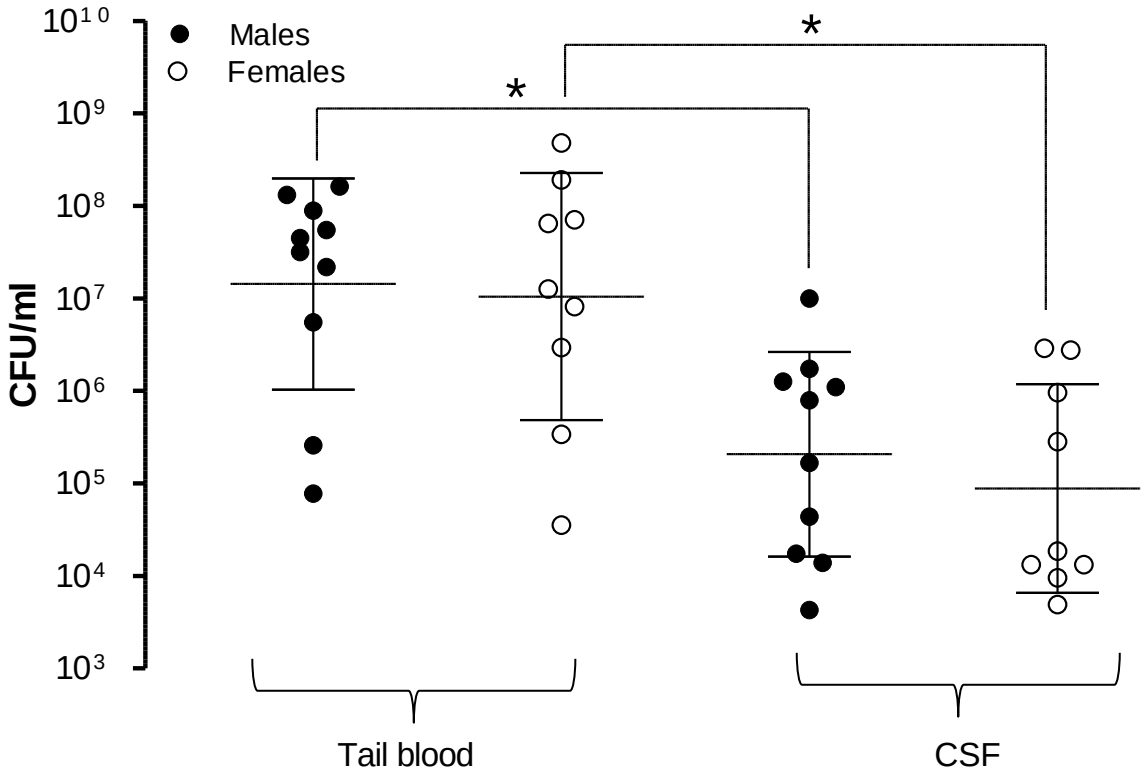


Figure 3.8 Estimation of bacterial load within the blood (tail) and CSF taken from neonatal (P5) rats 16 – 29 hours after receiving an intraperitoneal injection of *S. pneumoniae*. * Indicates a significant difference between bacterial counts within the CSF and bacterial counts within the blood (Main effect of collection site: $F_{(1, 17)} = 43.17$, $P < 0.0001$). No significant gender and interaction effects were detected (Main effect of gender and interaction: $F_{(1, 17)} = 0.10$ and 0.32, $P > 0.05$) Results are expressed as log transformed values and are shown as mean and standard deviation.

Learning and memory tested in the Morris water maze

Cued test

Figure 3.9 shows the performance of male and female rats during both cued tests. In cued test 1(performed before acquisition training) adolescent (P 29) male (A) and female (B) rats that received *S. pneumoniae* or saline as neonates (P4) took longer to find the visible platform as compared to cued test 2 (performed after acquisition training and probe testing; Main effect of time for males and females: $F_{(1, 27)} = 24.02$, $F_{(1, 23)} = 99.86$, $P < 0.0001$). No significant differences were seen in the latency to the visible platform for adolescent male and female rats that received either *S. pneumoniae* or saline (Main effect of treatment for males and females: $F_{(1, 27)} = 0.09$, $F_{(1, 23)} = 0.42$, $P > 0.05$). In addition no significant interaction was detected between the two cued tests and treatment (Main effect of interaction for males and females: $F_{(1, 27)} = 0.003$, $F_{(1, 23)} = 0.008$, $P > 0.05$).

Correspondingly the distance swum to the visible platform (data not shown) was longer in cued test 1 as compared to cued test 2 (Main effect of time for males and females: $F_{(1, 27)} = 25.33$, $F_{(1, 23)} = 76.15$, $P < 0.001$). The distance swum to the visible platform was comparable between adolescent male rats that received *S. pneumoniae* and adolescent male rats that received saline (Main effect of treatment for males and females: $F_{(1, 27)} = 1.06$, $F_{(1, 23)} = 0.08$, $P > 0.05$). In addition no significant interaction effect was detected between the two cued tests and treatment (Main effect of interaction for males and females: $F_{(1, 27)} = 0.48$, $F_{(1, 23)} = 0.002$, $P > 0.05$).

The swim speed to the visible platform (data not shown) was comparable between cued test 1 and cued test 2 for male rats (Main effect of time: $F_{(1, 27)} = 0.10$, $P > 0.05$) while female rats swam slower in cued test 1 as compared to cued test 2 (Main effect of time: $F_{(1, 23)} = 17.83$, $P < 0.001$). No significant differences were seen for the swim speed swum to the visible

platform between adolescent male and female rats that received *S. pneumoniae* and adolescent male rats that received saline (Main effect of treatment for males and females: $F_{(1, 27)} = 1.12$, $F_{(1, 23)} = 0.27$, $P > 0.05$). In addition no significant interaction effect was detected between the two cued tests and treatment (Main effect of interaction for males and females: $F_{(1, 27)} = 0.001$, $F_{(1, 27)} = 0.61$, $P > 0.05$).

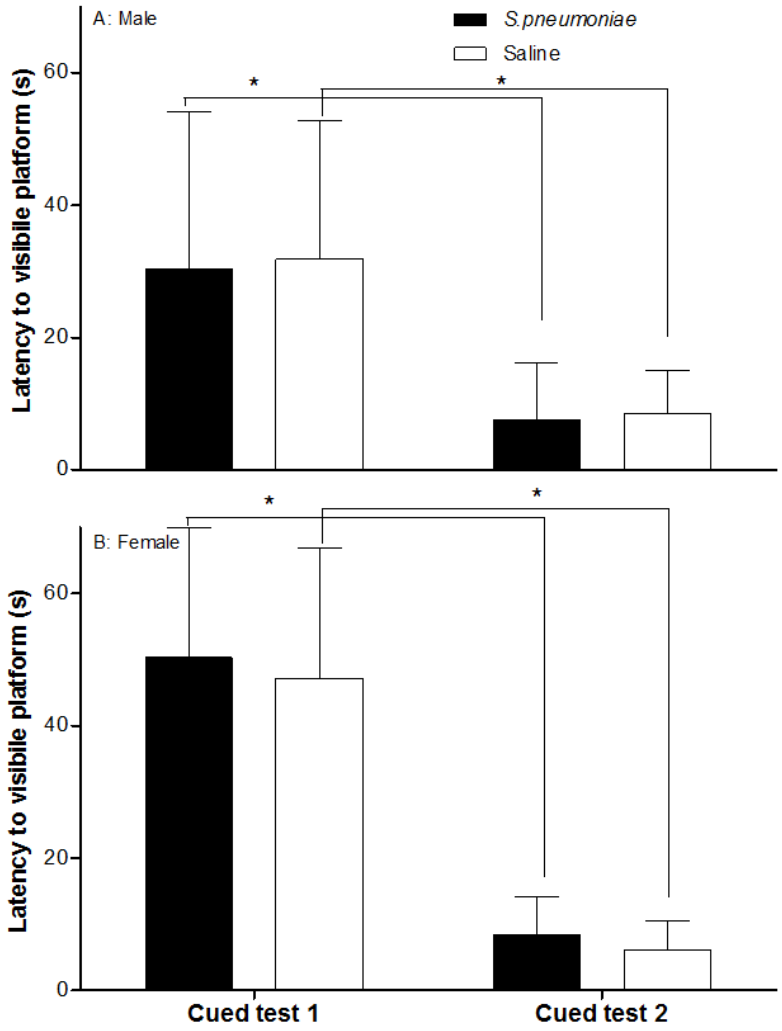


Figure 3.9 Performance in the cued test of adolescent (P29) male (A) and female (B) rats that received an intraperitoneal injection of *S. pneumoniae* (250 ul of $1x10^2$ CFU/ml, n = 10 males and n = 9 females) or saline (250 ul, n = 19 males and n =16 females) as neonates (P4). Rats were given 60 seconds to locate a visible platform placed in the middle of the pool 10 mm above the water level. * Indicates a significant difference between cued test 1 (performed before acquisition training) and cued test 2 (performed after acquisition training and probe testing; Main effect of time for adolescent male and female rats: $F_{(1, 27)} = 24.02$ and $F_{(1, 23)} = 99.86$). No treatment or interaction effects were detected (Main effect of treatment and interaction for adolescent male rats: $F_{(1, 27)} = 0.09$ and 0.003, $P > 0.05$; Main effect of treatment and interaction for adolescent female rats: $F_{(1, 23)} = 0.42$ and 0.008, $P > 0.05$). Results are expressed as mean and standard deviation

Acquisition training

Figure 3.10A shows that over the four learning sessions the latency to reaching the submerged platform decreased significantly for male and female adolescent (P30) rats that received *S. pneumoniae* as neonates (P4) and male and female adolescent (P30) rats that received saline as neonates (P4) (Main effect of time: $F_{(3, 150)} = 72.7$, $P < 0.0001$). A significant difference was detected in the latency to reaching the submerged platform between learning session 1 and learning session 4 (Bonferroni's multiple comparisons test, $P < 0.01$). The latency to reaching the submerged platform was comparable between rats in both treatment and gender groups (Main effects of treatment and gender: $F_{(1, 50)} = 1.2$ and 0.2, $P > 0.05$). No interaction between gender, treatment and learning sessions were detected for latencies measured during acquisition training (Main effect of interaction: $F_{(3,150)} = 0.90$, $P > 0.05$).

Figure 3.10B shows that over the four learning sessions the distance swum to reach the submerged platform decreased similarly for male and female adolescent (P30) rats that received *S. pneumoniae* as neonates (P4) and male and female adolescent (P30) rats that received saline as neonates (P4) (Main effect of time: $F_{(3, 150)} = 59.77$, $P < 0.0001$). A significant difference was detected in the distance swum to reach the submerged platform between learning session 1 and learning session 4 (Bonferroni's multiple comparisons test, $P < 0.01$). No statistical differences between the distances swum to reach the submerged platform was found for rats in either treatment or gender groups (Main effects of treatment and gender: $F_{(1, 50)} = 2.62$ and 0.46, $P > 0.05$). No interaction between gender, treatment and learning sessions were detected for distances measured during acquisition training (Main effect of interaction: $F_{(3,150)} = 0.42$, $P > 0.05$).

Figure 3.10C shows that the swim speed over the four learning sessions decreased significantly for male and female adolescent rats that received *S. pneumoniae* as neonates (P4) and male and female adolescent (P30) rats that received saline as neonates (P4) (Main effect of time: $F_{(3, 150)} = 5.64$, $P = 0.001$). A statistical difference was detected in the speed swum to reach the submerged platform between learning session 1 and learning session 4 (Bonferroni's multiple comparisons test, $P < 0.01$). The swim speed was comparable between rats in both treatment and gender groups (Main effects of treatment and gender: $F_{(1, 50)} = 2.21$ and 0.92, $P > 0.05$). No interaction between gender, treatment and learning sessions were detected for the swim speed measured during acquisition training (Main effect of interaction: $F_{(3,150)} = 0.78$, $P > 0.05$).

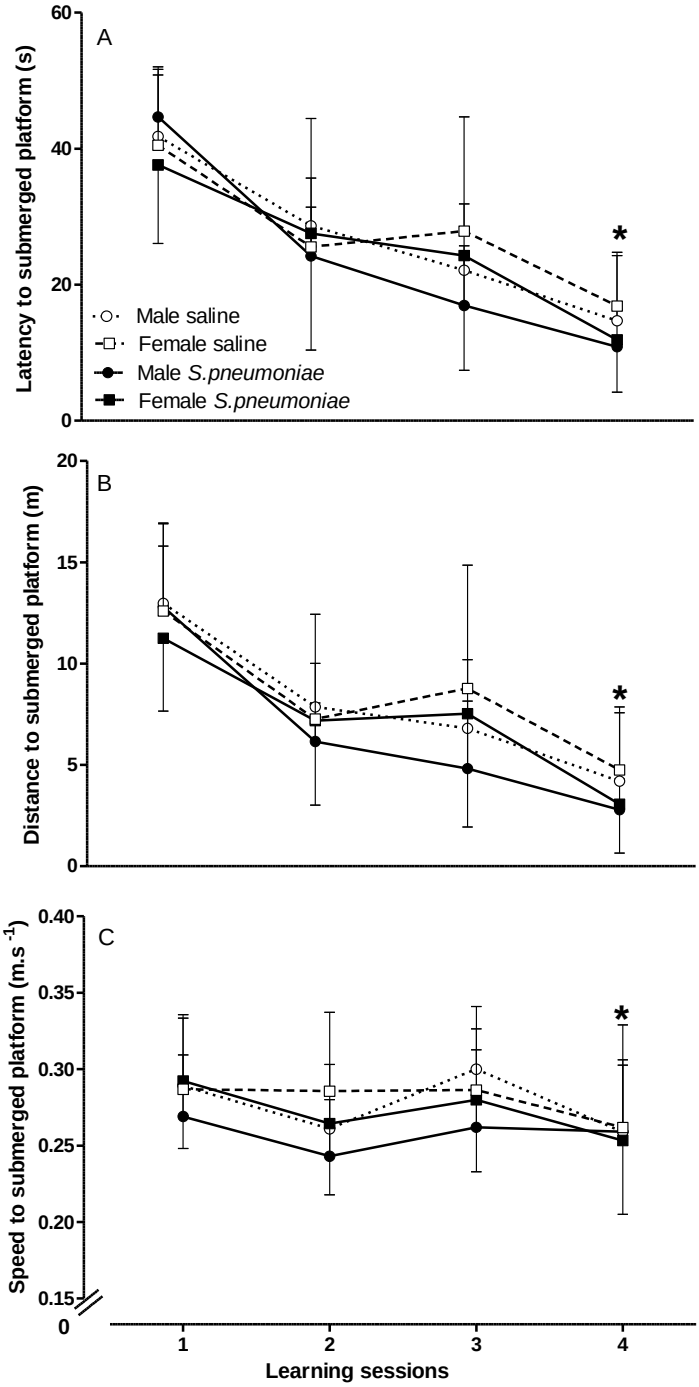


Figure 3.10 Spatial learning, measured using latency (A), distance (B) and speed (C) to the submerged platform, in adolescent (P30) rats that received an intraperitoneal injection of *S. pneumoniae* (250 ul of $1x10^2$ CFU/ml, n = 10 males and n =9 females) or saline (250 ul, n = 19 males and n =16 females) as neonates (P4). Rats were trained over 2 days (2 sessions a day) to find a submerged platform. * Indicates a statistical difference between learning session 1and learning session 4 (Main effect of time for latency, distance and speed: $F_{(3, 150)}$ = 72.7, 59.77 and 5.64, Bonferroni's multiple comparisons test, $P < 0.01$). However, treatment and gender did not influence any of the parameters measured (Main effects of treatment for latency, distance and speed: $F_{(1, 50)}$ = 1.2, 2.62 and 2.21; Main effects of gender for latency, distance and speed: $F_{(1, 50)}$ = 0.2, 0.46 and 0.92, $P > 0.05$). In addition no interaction between gender, treatment and learning sessions were detected for any of the parameters measured (Interaction effect for latency, distance and speed: $F_{(3,150)}$ = 0.90, 0.42 and 0.78, $P > 0.05$). Results are expressed as mean and standard deviation.

Probe test

Figure 3.11A shows that the mean latency to the former platform position for adolescent (P 32) male and female rats injected with *S. pneumoniae* as neonates (P4) and adolescent (P32) male and female rats injected with saline as neonates (P4) was significantly shorter than the 30 second cut off time (one sample t-test for *S. pneumoniae* neonatally treated males; $t_{(9)}$ = 4.17, saline neonatally treated males; $t_{(18)}$ = 6.30, *S. pneumoniae* neonatally treated females; $t_{(8)}$ = 5.91 and saline neonatally treated females; $t_{(15)}$ = 5.92, $P < 0.01$). The latency, distance and speed to the former platform position was similar for male and female rats that received *S. pneumoniae* as neonates and male and female rats that received saline as neonates (Main effect of treatment for latency, distance and speed: $F_{(1, 50)}$ = 0.06, 0.57 and 3.70; $P > 0.05$). Data for the distance and speed to the former platform position are not shown.

No significant differences were detected between the genders for the latency, distance and speed to the former platform position (Main effect of gender for latency, distance and speed $F_{(1, 50)}$ = 0.03, 0.12 and 0.60; $P > 0.05$). Moreover, no interaction effects were detected for the latency, distance and speed swum to the former platform position (Main effect of interaction for latency, distance and speed: $F_{(1, 50)}$ = 0.05, 0.07 and 0.95; $P > 0.05$).

Figure 3.11B shows that adolescent rats that received an intra-peritoneal injection of *S. pneumoniae* as neonates (P4) and adolescent rats received an intra-peritoneal injection of saline as neonates (P4) spent similar amounts of time searching the target quadrant regardless of gender and neonatal treatment received (Main effect of gender and treatment: $F_{(1,50)}$ = 0.31and 0.41, $P > 0.05$). There was no interaction between gender and treatment for the percentage time spent in the target quadrant ($F_{(1,50)}$ = 1.76, $P > 0.05$).

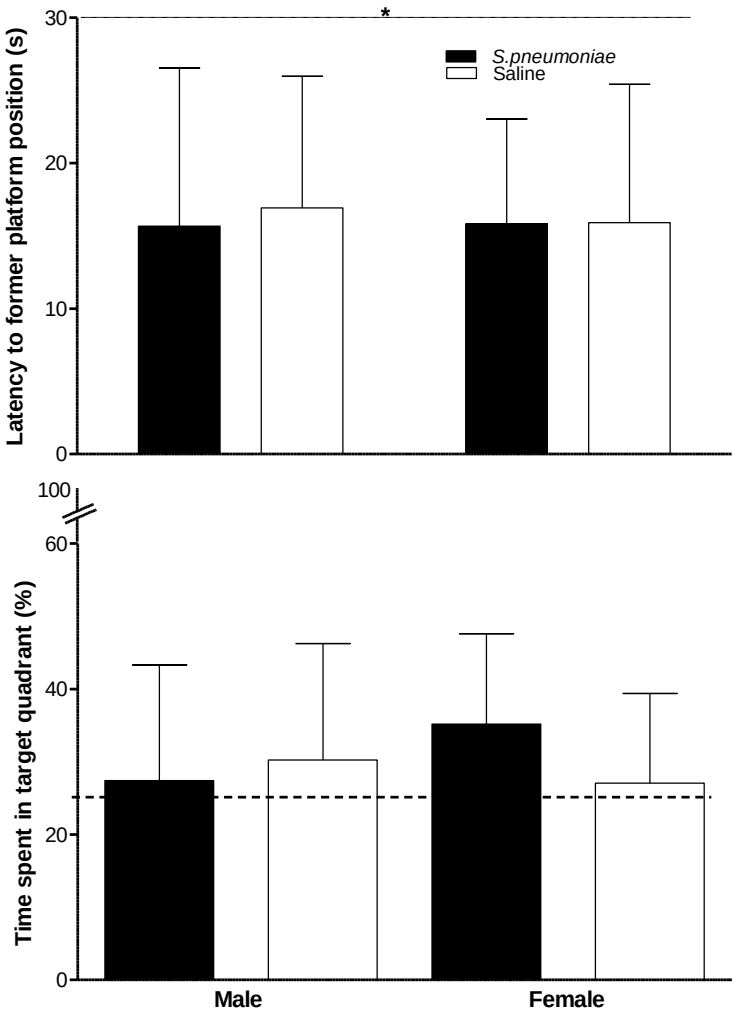


Figure 3.11 Spatial memory measured using the latency to the former platform position (A) and percentage time spent in the target quadrant (B) for adolescent (P32) rats that were injected with *S. pneumoniae* (250 ul of $1x10^2$ CFU/ml, n = 10 males and n = 9 females) or saline (250 ul, n = 19 males and n =16 females) as neonates (P4). Rats were given 30 seconds to locate the former platform position 24 hours after the fourth learning session. * Indicates that all rats were able to find the platform in under 30 seconds (one sample t-test for *S. pneumoniae* neonatally treated males; $t_{(9)} = 4.00$, saline neonatally treated males; $t_{(18)} = 6.30$, *S. pneumoniae* neonatally treated females; $t_{(8)} = 5.91$ and saline neonatally treated females; $t_{(15)} = 5.92$, $P < 0.01$). No gender, treatment or interaction effects were detected for the latency to the former platform (Main effect of gender, treatment and interaction: $F_{(1, 50)} = 0.03$, 0.57 and 0.07; $P > 0.05$). All rats spent more than 25 % of their allocated time in the target quadrant (indicated by 25 % dashed line). There was no gender, treatment or interaction effect detected for the percentage time spent in the target quadrant (Main effect for gender, treatment and interaction: $F_{(1,50)} = 0.31$, 0.41 and 1.76; $P > 0.05$). Results are expressed as mean and standard deviation

Determination of the effects of the different phases of the oestrus cycle on spatial learning and memory.

In rats a regular oestrus cycle pattern consists of one to two days of diestrus followed by a day of proestrus and then a day of oestrus (Marcondes *et al*., 2002). Vaginal smears taken from adolescent rats used in this experiment showed that on the days of testing in the Morris water maze rats did not follow a regular cycle pattern (data not shown). Since no gender differences were detected between adolescent female and male rats during acquisition training and probe testing, the data was not analysed further.

Contextual fear conditioning test

During conditioning adolescent (P37) male and female rats showed interest in the novel environment without displaying any fear responses (freezing behaviour) or signs of lethargy and all rats were responsive to the 1.5 mA foot shock (data not shown). Figure 3.12 shows that there were no significant differences between contextual freezing behaviour of adolescent (P39) rats that were injected with *S. pneumoniae* as neonates (P4) and adolescent (P 39) rats injected with saline as neonates (P4) (Main effect of treatment: $F_{(1, 50)} = 0.35$, $P > 0.05$). A significant difference was detected between the contextual freezing behaviour of adolescent male and female rats (Main effect of gender: $F_{(1, 50)} = 24.60$, $P < 0.0001$).

Adolescent female rats injected with *S. pneumoniae* froze significantly less than adolescent male rats injected with *S. pneumoniae* (Bonferroni's multiple comparisons test, $P < 0.01$) and adolescent female rats injected with *S. pneumoniae* displayed significantly less freezing than adolescent male rats injected with saline (Bonferroni's multiple comparisons test, $P < 0.01$). However, no significant differences were noted between adolescent female rats injected with saline and adolescent male rats injected with saline (Bonferroni's multiple comparisons test,

P > 0.05). A significant interaction was detected between gender and treatment for freezing exhibited in the context (Main effect of interaction: $F_{(1, 50)} = 8.4$, $P < 0.01$).

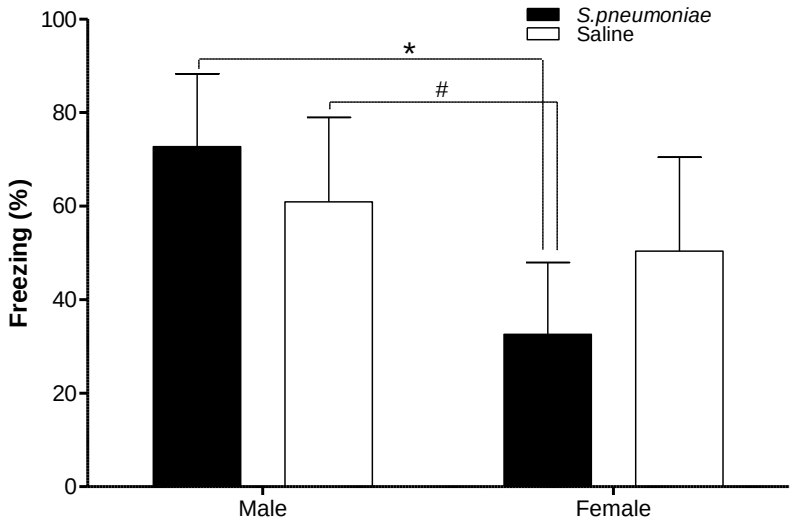


Figure 3.12 Freezing behaviour (fear responses) of adolescent (P39) rats that received an intraperitoneal injection of *S. pneumoniae* (250 ul of $1x10^2$ CFU/ml, n = 10 males and n = 9 females) or saline (250 ul, n = 19 males and n =16 females) as neonates (P4). The freezing response of rats to a context in which they previously received a 1.5 mA foot shock was measured over 6 minutes. * Indicates a significant difference between the contextual freezing responses of female rats that received either *S. pneumoniae* and the contextual freezing responses of male rats that received *S. pneumoniae* (Main effect of gender: $F_{(1, 50)} = 24.60$, Bonferroni's multiple comparisons test $P < 0.01$). # Indicates a significant difference between the contextual freezing responses of female rats injected with *S. pneumoniae* and the contextual freezing responses of male rats injected with saline (Bonferroni's multiple comparisons test, $P < 0.01$). No significant differences were noted between adolescent female rats injected with saline and adolescent male rats injected with saline (Bonferroni's multiple comparisons test, $P > 0.05$). A significant interaction effect was detected (Main effect of interaction: $F_{(1, 50)} = 8.4$, $P < 0.01$). However, no significant treatment effects were detected (Main effect of treatment: $F_{(1, 50)} = 0.35$, $P > 0.05$). Results are expressed as mean and standard deviation.

Determination of the effects of the different phases of the oestrus cycle in contextual fear conditioning

Figure 3.12 shows a significant difference between the contextual freezing behaviour of adolescent male and female rats. To identify if the differences noted in the contextual freezing behaviour between adolescent male and female rats was related to the oestrous cycle, we analysed vaginal smears taken from adolescent female rats during the days of learning (conditioning) and memory (testing). Treatment groups were collapsed for this analysis since both adolescent male and female rats injected with *S. pneumoniae* as neonates exhibited similar freezing responses as adolescent male and female rats injected with saline as neonates.

Adolescent rats were in different phase of the oestrous cycle on the day of conditioning. Seven out of 19 rats were in the proestrus phase of the cycle and 4 out 19 rats were in the oestrus phase of the cycle on the day of conditioning (Data not shown). Figure 3.13 shows a statistical difference between the contextual freezing response of adolescent female rats that were conditioned (learnt) either during the proestrus phase of the cycle or during the oestrus phase of the cycle and adolescent male rats (One-way ANOVA: $F_{(2,37)} = 13.28$, $P < 0.0001$).

Previous results have shown that adult female rats freeze less when conditioned and tested during the proestrus phase of the oestrus cycle than when conditioned and tested during the oestrus phase of the cycle (Markus and Zecevic, 1997). In this study however, no significant difference were noted between the contextual freezing response of adolescent female rats that were conditioned (learnt) during the proestrus phase of the cycle and the contextual freezing response of adolescent female rats that were conditioned during the oestrus phase of the cycle (Bonferroni's multiple comparisons test, $P > 0.05$). Although, a statistical difference was noted between the contextual freezing response of adolescent female rats that were

conditioned either during the proestrus or oestrus phase of the cycle and the contextual freezing response of adolescent male rats (Bonferroni's multiple comparisons test, $P < 0.01$).

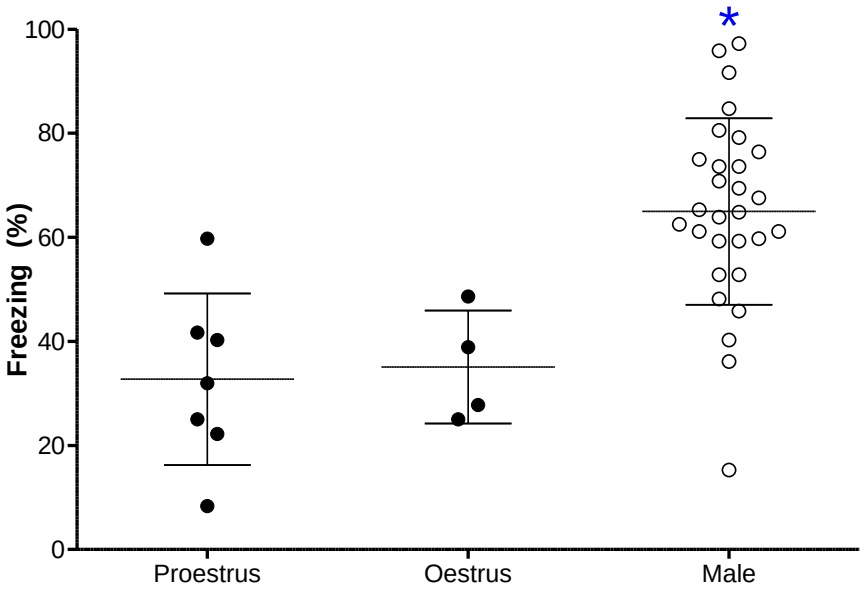


Figure 3.13 Freezing behaviour (fear responses) of adolescent (P39) female rats that were conditioned either during the proestrus or oestrus phases of the cycle and adolescent male rats. Treatment groups for female and male rats were collapsed for this analysis. The freezing response of rats to a context in which they previously received a 1.5 mA foot shock was measured over 6 minutes. * Indicates a statistical significance between the contextual freezing response of adolescent female rats that were conditioned during the proestrus or oestrus phase of the cycle and the contextual freezing response of adolescent male rats (One-way ANOVA: $F_{(2,37)} = 13.2$, Bonferroni's multiple comparisons test, $P < 0.01$). Results are expressed as mean and standard deviation.

3.4 Experiment 4- Nuclear factor interleukin-6 (NF-IL6) expression in the rat hippocampus

Figure 3.14 shows the NF-IL6 immuno-reactivity of hippocampal cells stimulated for 24 hours with alcohol-treated *S. pneumoniae* at doses of $5x10^3$, $5x10^{4,}$ $5x10^5$ and $5x10^6$ CFU/ml. Cells from the CA1 regions of the hippocampus were double stained with antibodies against NF-IL 6 and nuclear DAPI. The co-localization of DAPI (blue) staining and NF-IL 6 (red) signals indicate the nuclear origin of the NF-IL6 signal (pink).

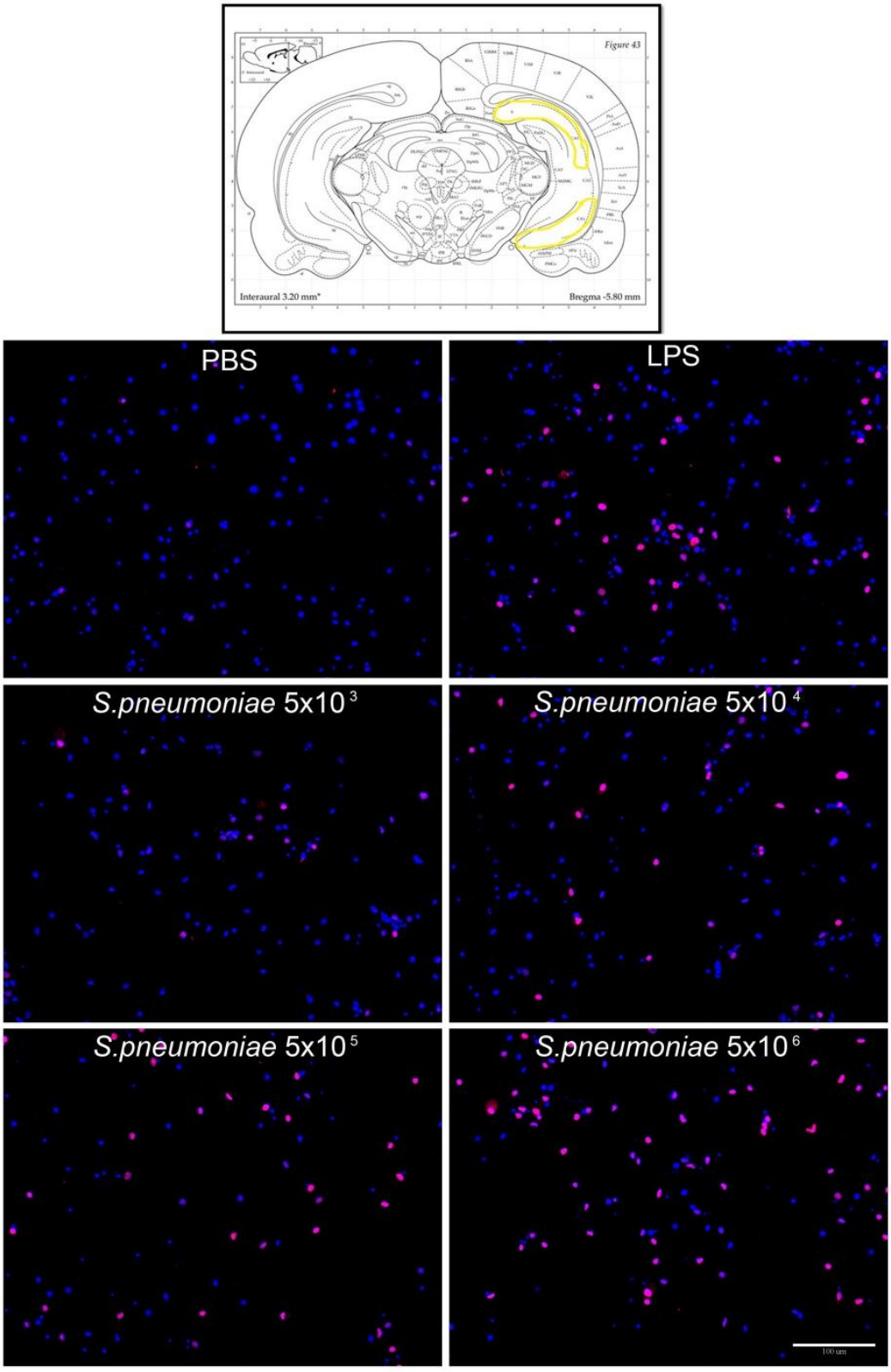


Figure 3.14 Dose-dependent immune reactive response of nuclear NF-IL6 in hippocampal cells after 24 hour stimulation with alcohol-treated *S. pneumoniae*. The expression of nuclear NF-IL6 (pink) induced by alcohol-treated *S. pneumoniae*, LPS and PBS was detected in the CA1 region of the rat hippocampus 24 hours after stimulation. The co-localization of DAPI (blue) staining and NF-IL 6 (red) signals indicate the nuclear origin of the NF-IL6 signal. Scale bar represents 100 um.

Nuclear NF-IL6 (pink) signals are visible in cultures stimulated with $5x10^3$ CFU/ml of alcohol-treated *S. pneumoniae* and the signal strength increases in a dose-dependent manner.

Table 3.2 shows the qualitative rating of NF-IL 6 immunoreactivity present in alcohol-treated *S. pneumoniae* stimulated hippocampal cultures. The strongest nuclear NF-IL6 immunoreactivity was induced by the highest concentration of *S. pneumoniae* bacterial cell wall and the signal strength was comparable to the nuclear NF-IL6 immunoreactivity induced in LPS treated cell cultures. In contrast the nuclear NF-IL6 immunoreactivity induced by the lowest concentration of *S. pneumoniae* bacterial cell walls and the signal strength was comparable to the nuclear NF-IL6 signalling of PBS treated cell cultures.

Table 3.2. Qualitative rating of NF-IL6 signal strength present in alcohol-treated *S. pneumoniae* stimulated hippocampal cell cultures.

	Nuclear NF-IL6 immunoreactivity to:					
	PBS	$5x10^3$	$5x10^4$	$5x10^5$	$5x10^6$	LPS
	+	+	++	++(+)	+++	+++

A five point rating was used to rate the immunoreactivity: +++ = high density of nuclear signal, ++ (+) = moderate to high density of nuclear signal, ++ = moderate density of nuclear signal, + = low density of nuclear signal, - = no nuclear signal.

Figure 3.15 depicts the phenotype of cells from the CA1 region of the hippocampus that produced nuclear NF-IL6 signals in response to alcohol-treated *S. pneumoniae* ($5x10^6$ CFU/ml). Nuclear NF-IL 6 signals are visible in microglia (d) and astrocytes (e) but not in neurons (f) present in the hippocampus.

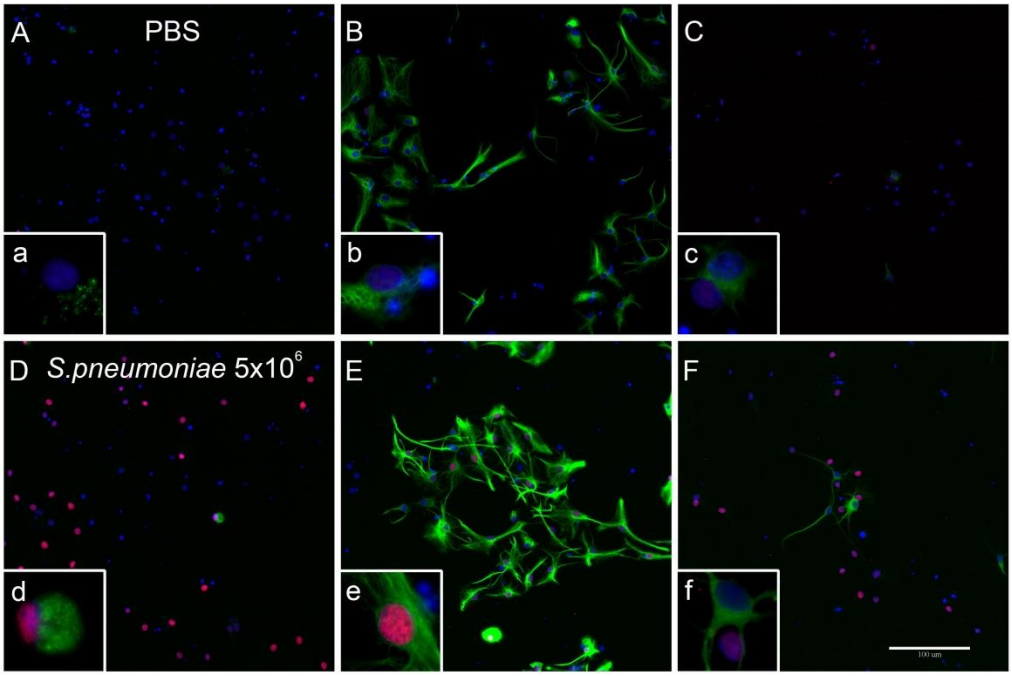


Figure 3.15 Phenotypic characterisation of cells from the CA1 region of the hippocampus after a 24 hour stimulation with alcohol-treated *S. pneumoniae*. Cells stimulated with $5x10^6$ CFU/ml of alcohol-treated *S. pneumoniae* (D, E, F) or PBS (A, B, C) were triple stained for cell marker proteins (green), NF-IL 6 (red) and cellular nuclei (DAPI, blue). Distinct nuclear NF-IL 6 signalling was found in microglia (insert d; Primary antibody = ED1, secondary fluorescent antibody = Alexa- 488) and astrocytes (insert e; Primary antibody = GFAP, secondary fluorescent antibody = Alexa- 488) but not neurons (insert f; Primary antibody = MAP 2a+b, secondary fluorescent antibody = Alexa- 488) of hippocampal cell cultures. In contrast minimal expression of nuclear NF-IL 6 was detected in hippocampal cell cultures stimulated with PBS (a, b, c). Scale bar represents 100 um.

Chapter 4

Discussion

Through i.p. administration of *S. pneumoniae* I have successfully established a model of haematogenous meningitis in neonatal Sprague-Dawley rats. Neonatal rats that were injected (i.p.) with *S. pneumoniae* (mean ± SD: 46 ± 35 CFU) developed physical signs of having an infection (pale skin, decreased activity and decreased feeding) and hypothermia (a decrease in skin temperature from 36 °C to temperatures between 34 °C and 30 °C) 16 -29 hours after receiving the injection. The severity of the infection in neonatal rats (used in experiment 2, to establish a model of haematogenous meningitis) was confirmed by the presence of a high concentration of bacteria in the blood (± $1x10^8$ CFU/ml), liver (± $1x10^8$ CFU/ml), lungs (± $1x10^7$ CFU/ml), spleen (± $1x10^8$ CFU/ml) and brain (± $1x10^6$ CFU/ml) (see Figure 3.7). Moreover the severity of infection and the spread of *S. pneumoniae* from the peritoneum to the blood, liver, lungs, spleen and brain did not differ between male and female rats, indicating that gender did not seem to play a role in the development of haematogenous meningitis in rats used in this study.

In addition, haematogenous meningitis was replicated in a second group of rats (that were used in experiement 3) to investigate whether an early life episode of haematogenous meningitis induced by *S. pneumoniae* would result in learning and memory deficits during adolescence. The high concentration of bacteria seen in the blood ($1x10^7$ CFU/ml) and CSF ($1x10^5$ CFU/ml) of neonatal rats and the skin temperature decreases from 36 °C to 34 °C (see Figure 3.6) confirmed the development of severe neonatal haematogenous meningitis in this group of rats. Severe neonatal haematogenous meningitis did not affect learning and memory in adolescent rats as assessed by the Morris water maze and contextual fear conditioning in this study.

Performance in the Morris water maze showed that spatial learning (measured using the latencies, distances and speeds to the escape platform, see Figure 3.10) was comparable

between adolescent rats that had severe neonatal haematogenous meningitis and adolescent rats that did not have a history of neonatal haematogenous meningitis. Similarly spatial memory (measured using the time taken to find the former platform position, see Figure 3.11A) was comparable for all adolescent rats. In addition all adolescent rats spent more than 25 % of their swim time searching the target quadrant therefore their time spent in the target quadrant could not be attributed to chance (see Figure 3.11B).

Results from contextual fear conditioning showed that all adolescent rats were able to associate an adverse stimulus (foot shock) with a conditional stimulus (the contextual cage) when tested using contextual fear conditioning. Adolescent rats which had severe neonatal haematogenous meningitis displayed a high percentage of freezing (± 70% for males and ± 30% for females) which was similar to the percentage of freezing exhibited by rats which were treated with saline as neonates (see Figure 3.12).

Thus results from my study appear to suggest that an episode of severe neonatal haematogenous meningitis induced by *S. pneumoniae* serotype 2, may not affect hippocampal-dependent spatial or associative learning and memory in adolescence. As mentioned earlier, there are no studies which have investigated the effects of severe neonatal haematogenous meningitis induced by *S. pneumoniae* serotype 2 on cognitive function later on in life, in humans or animals however, my findings are contradictory to existing literature which suggests that a *S. pneumoniae* infection induced by other serotypes produces neuroinflammation which leads to hippocampal damage (Loeffler *et al*., 2001; Leib *et al*., 2003; Barichello *et al*., 2010a; Barichello *et al*., 2014).

Although I measured the amount of bacteria that was present in the blood and CSF of neonatal rats at the time of infection, I did not quantify the level of inflammation within the brain (by measuring the concentration of pro-inflammatory cytokines produced) during the

time of infection. However, using hippocampal cells obtained from neonatal rats I was able to show that neonatal hippocampal cells exposed to high concentrations of alcohol-treated *S. pneumoniae* expressed NF-IL6 particularly in astrocytes and microglia (see Figure 3.15). NF-IL6 immunoreactivity was increased in hippocampal cells that were stimulated with alcohol-treated *S. pneumoniae* in a dose dependant manner (see Figure 3.14). Therefore it is possible that the presence of a greater number of *S. pneumoniae* CFU in the brain during neonatal life could result in a greater activation of astrocytes and microglia. This increased activation of astrocytes and microglia could then produce a level of inflammation that is capable of inducing hippocampal damage and long-lasting hippocampal-dependant memory impairment.

Therefore one explanation for the absence of learning and memory impairments seen in my study could be that the dose of *S.pneumoniae* administered may not have induced a level of inflammation that was capable of producing hippocampal damage. There are a number of other factors that could explain the differences seen in my study and existing studies. These factors relate to (1) the model of meningitis, (2) behavioural tests used and (3) the role of the immune system in learning and memory and in normal brain development. In the section below I will discuss each of these factors in more detail.

4.1 Animal models of meningitis

To my knowledge only four studies have reported neurocognitive deficits related to learning and memory in rodents that had neonatal meningitis induced by *S. pneumoniae* (Loeffler *et al*., 2001; Leib *et al*., 2003; Barichello *et al*., 2010a; Barichello *et al*., 2014). A comparison between these four studies (Loeffler *et al*., 2001; Leib *et al*., 2003; Barichello *et al*., 2010a; Barichello *et al*., 2014) and mine show three major methodological differences. These methodological differences could explain the different effects in learning and memory noted between my study and theirs.

Firstly, Loeffler, Leib and Barichello administered *S. pneumoniae* serotype 3 to infant rodents. As previously mentioned (see Chapter 1, section 1.3) *S. pneumoniae* serotypes differ based on the chemical composition of the capsule (Catterall, 1999). Thus the capsular composition of serotype 3 is significantly different to serotype 2 (Kelly *et al*., 1994). In addition studies have shown that serotype 3 is less virulent than serotype 2 (Kelly *et al*., 1994; Orihuela *et al*., 2003; Hammerschmidt *et al*., 2005). The concentration of bacteria in the lungs, blood and CSF of mice 48 hours after an intra-nasal inoculation with *S. pneumoniae* serotype 3 was lower than the concentration of bacteria found in lungs, blood and CSF of mice that were inoculated with a comparable concentration of *S. pneumoniae* serotype 2 (Orihuela *et al*., 2003).

No study has compared the level of neuroinflammation produced by serotype 3 with the level of neuroinflammation produced by serotype 2. However, experimental meningitis in rabbits showed that *S. pneumoniae* serotypes with similar virulence produced different levels of neuroinflammation (Engelhard *et al*, 1997). Serotypes 6B, 14, and 23F produce higher concentration of CSF inflammatory markers (leukocyte counts, lactate and protein concentrations) in rabbits than serotypes 1, 5 and 7F (Engelhard *et al*, 1997). Therefore the different *S. pneumoniae* serotypes used in our studies may explain the different learning and memory outcomes observed by our studies. It is possible that *S. pneumoniae* serotype 3 (used by Loeffler, Leib and Barichello) produced a higher level of neuroinflammation that resulted in learning and memory deficits whereas the level of inflammation produced by *S. pneumoniae* serotype 2 (used in my study) was unable to induce learning and memory deficits.

Secondly, Loeffler, Leib and Barichello induced experimental meningitis via direct inoculation into the brain. As previously mentioned, experimental induction of meningitis can

also be achieved via the direct inoculation of bacteria into the frontal lobe, ventricular system or the cisterna magna (Shapiro *et al*., 2000; Gerber *et al*., 2001; Klein *et al*., 2007). A direct inoculation with $1x10^6$ CFU/ml of *S. pneumoniae* serotype 3 into the cisterna magna of infant rats (P11) resulted in $1x10^7$ CFU/ml of *S. pneumoniae* serotype 3 in the brain 18 hours after infection (Leib *et al*., 2003). In my study i.p. administration of *S. pneumoniae* serotype 2, strain D39 in doses greater than $1x10^2$ CFU/ml produced a higher concentration of bacteria in the CSF ($1x10^7$ CFU/ml) 18 hours after infection but also resulted in the death of rats.

To my knowledge no research group has compared the concentration of bacteria found in the brain after a direct inoculation into the cisterna magna with the concentration of bacteria found in the brain after an intraperitoneal injection. However, literature suggests that the body of a rat given an intraperitoneal injection with bacteria would succumb to systemic shock before the bacteria had a chance to replicate to high concentrations ($\geq 1x10^7$ CFU/ml) in the brain (Koedel et al., 2002). Thus the route of administration that I used may have restricted the level of neruoinflammation produced in my study. Inducing experimental meningitis via direct inoculation of *S. pneumoniae* into the cistern magna (like Loeffler, Lieb and Barichello) would allow me to administer a higher concentration ($\geq 1x10^7$ CFU/ml) of bacteria. A higher concentration of bacteria within the brain may induce a level of neuroinflammation capable of producing long-term learning and memory deficits.

Thirdly, the culturing procedure that is used to grow *S. pneummoniae* differed between studies thus there is an inconsistency in the viability of the bacteria used to induce meningitis. The inconsistency in the viability of the bacteria may explain the different results seen between our studies. *S. pneumoniae* like other bacteria display three phases of growth (Zwietering *et al*., 1990). The first phase of growth is the lag phase in which the least amount of growth occurs. The lag phase is followed by the logarithmic phase. During the logarithmic

phase bacteria replicate exponentially. The last growth phase, the stationary phase, denotes the point at which bacteria have reached their maximal growth potential and start to autolyse.

S. pneumoniae used in my study was grown to the early logarithmic phase which allowed for the replication of bacteria *in vivo*. However, in other studies *S. pneumoniae* was grown to the late logarithmic phase which restricted the replication of bacteria *in vivo* (Leib *et al*., 2003; Barichello *et al*., 2010a). No research group has investigated whether *S. pneumoniae* grown at different points of the logarithmic phase would produce different levels of neuroinflammation *in vivo*.

However, literature indicates that a solution (of Todd-Hewitt broth, yeast extract and glycerol) containing *S. pneumoniae* which was grown to the late logarithmic phase has a higher concentration of bacteria than a solution containing *S. pneumoniae* which was grown to the early logarithmic phase (Zwietering *et al*., 1990). Thus the inoculum grown by Loeffler, Leib and Barichello may have produced a high level of neuroinflammation due to the high concentration of bacteria in the inoculum administered. Although the inoculum used in my study was grown to the early logarithmic phase which allowed for the replication of bacteria *in vivo*, results from my study show (see Figure 3.6) that bacteria that entered the CSF ($1x10^5$ CFU/ml) had not replicated to the high concentrations ($1x10^7$ CFU/ml) reported by Leib (2003).

Methodological differences related to the model of meningitis used are not the only factors that could explain the different outcome between our studies. Detection of learning and memory deficits may also depend on factors that are related to the type of behavioural tests used and the manner in which the tests are conducted.

4.2 Behavioural testing

The first factor that can influence results from behavioural tests are the testing conditions and testing procedures (Vorhees and Williams, 2006; Morellini, 2013). The successful implementation of the Morris water maze test requires an experimental room which is featureless to discourage the use of visual cues outside of the maze as a navigation tool (Vorhees and Williams, 2006). In addition rats must be placed in the pool from random entry points to avoid using internal cues to navigate towards the escape platform (that is judging the route based on previous body placement or previous swim duration) (Morellini, 2013). The size of the pool used and the temperature of the water in the pool may also influence the results produced (Vorhees and Williams, 2006).

Similarly the contextual fear conditioning of rats is also sensitive to testing conditions and testing protocol (communication with Prof Ruth Barrientos, a collaborator from the University of Colorado who has in-depth knowledge on contextual fear conditioning and was instrumental in setting up the protocols that I used for this study). When testing for contextual memory in rats the conditions must remain the same for both conditioning and testing. In addition, the observers must be sufficiently trained to distinguish movement from freezing. I validated the testing conditions and testing protocols that I used in the Morris water maze and in contextual fear conditioning using a pharmacological agent, scopolamine hydrobromide, that induced temporary amnesia in adolescent rats. The testing conditions and testing protocols for both behavioural tests were able to detect learning and memory impairments in rats that received an injection of scopolamine hydrobromide (Figures 3.2, 3.3. and 3.4). Thus I am certain that the absence of learning and memory impairments seen in adolescent rats that were neonatally exposed to *S. pneumoniae* cannot be attributed to the testing conditions or protocols used in my study.

However hippocampal dependant learning and memory impairments may have been detected had I conducted additional testing such as employing spatial reversal in the Morris water maze. Spatial reversal refers to an additional set of trials conducted once rats have learnt the location of the escape platform. Spatial reversal involves the relocation of the platform to assess the cognitive flexibility of rats (Vorhees and Williams, 2006). Cognitive flexibility refers to the ability of rats to use the same visual cues to learn a different swim path (Vorhees and Williams, 2006). Adult rats that were infected with *Escherichia coli* on P4 had the ability to find the platform position after the first set of trails. However when the platform was relocated adult rats were unable to find the new platform position (Williamson and Bilbo, 2014). Thus a neonatal infection with *S. pneumoniae* may spare some cognitive functions while disrupting others.

The use of other behavioural tests which assess cognitive functions that are governed by different brain regions may have also provided more insight into the cognitive effects of an early life exposure to *S. pneumoniae*. Animal studies have shown that *S. pneumoniae* can induce neuronal loss in the cortex, pre-frontal cortex and hippocampus of adult rats (Barichello *et al*., 2009a). Thus testing rats in a cross maze can assess place and response learning which is associated with the pre-frontal cortex in rodents (Ragozzino *et al*., 1999).

Moreover the use of multiple behavioural tests can provide a comprehensive assessment of cognitive function. For example, the use of six different behavioural tests was used to assess anxiety-like behaviour and depressive-like behaviour in adult rats 10, 30 and 60 days after experimental meningitis was induced with *S. pneumoniae* (Barichello *et al*., 2010b). Interestingly, behavioural deficits were seen up to 30 days after the induction of meningitis but could no longer be detected 60 days after the induction of meningitis (Barichello *et al*., 2010b).

Thus the second factor that can influence results from behavioural tests is the age at which testing takes place. Most studies which investigate the neurocognitive effects of an early life infection assesses the behaviour during adulthood (Pletnikov *et al*., 1999; Bilbo *et al*., 2005; Spencer *et al*., 2005; Spencer *et al*., 2006; Harrè *et al*., 2008; Galic *et al*., 2009; Kenter *et al*., 2010; Williamson and Bilbo, 2014). For example, adult rats that were given a neonatal (P5) injection of LPS showed learning and memory impairments when tested in the Morris water maze and contextual fear conditioning (Harrè *et al*., 2008). Similarly adult rats that were exposed to LPS neonatally (P5) showed memory impairments in the passive avoidance test and decreased anxiety in the elevated plus maze (Wang *et al*., 2013).

Moreover assessing neurobehavioural development throughout the postnatal period may have provided more insight into the cognitive effects of an early life exposure to *S. pneumoniae*. The neurobehavioral assessment of neonatal rats that were given an LPS injection on P5 showed altered development during the postnatal period (Fan *et al*., 2005). Neurobehavioral development was assessed from P6 to P21 using the surface righting reflex, negative geotaxis, swimming development, wire hanging behaviour and cliff avoidance response (Fan *et al*., 2005). The postural reflexes of LPS treated rats were significantly impaired. Therefore learning and memory deficits may have been detected in my study if I had conducted behavioural testing throughout the postnatal period and at different stages of life (adolescence, adulthood and senescence).

Studies investigating the neurocognitive effect of an *S. pneumoniae* infection suggest that the neurocognitive deficits seen are due to brain damage caused by the bacterial itself. *In vitro* studies have shown that properties of *S. pneumoniae* can induce hippocampal cell damage by weakening the stability of the mitochondrial membrane resulting in hippocampal apoptosis (Braun *et al*., 2002; Braun *et al*., 2007) or via caspase dependant apoptosis (see Chapter 1,

section 1.3.6, Mitchell *et al.*, 2004). However, it is possible for the developing brain to regenerate after an early life insult (Daval *et al*., 2004). New-born (P0) rats showed a loss of neurons and increased apoptosis within the CA1 region of the hippocampus a week after the induction of hypoxia. Although by P21 cells within the CA1 region of the hippocampus had regenerated to levels that were comparable to non-hypoxic rats (Daval *et al*., 2004). Therefore an early life infection caused by *S. pneumoniae* (such as severe haematogenous meningitis) may result in hippocampal damage in rats that does not produce long-term impairments as the neonatal brain may have the capacity to repair the damage. Although I did not assess hippocampal damage in my study, I was able to show that hippocampal cells do produce an immune response to *S. pneumoniae* (see Figures 3.14 and 3.15). An alternative explanation for neurocognitive deficits seen after an infection with *S. pneumoniae* is that brain damage may result as a consequence of the activation of the immune system via the disruption of cytokine concentrations.

4.3 The role of the immune system in learning and memory and in healthy brain development

Cytokines, protein signalling molecules that rely information within the immune system, play an important role in learning and memory (Goshen and Yirmiya, 2007). The role of pro-inflammatory cytokines in learning and memory has been described using the inverted U-shaped model (Goshen and Yirmiya, 2007). The inverted U-shaped model suggests that relatively low concentrations of pro-inflammatory cytokines (10 ng/rat) may facilitate memory consolidation (Yirmiya *et al*., 2002) whereas the complete absence of pro-inflammatory cytokines or an overproduction of pro-inflammatory cytokines may result in memory impairment (Oitzl *et al*., 1993; Avital *et al*., 2003; Yirmiya *et al*., 2002).Animal studies have shown that a direct inoculation of *S. pneumoniae* into the brain of rats does

induce an increase in pro-inflammatory cytokines (Barichello *et al*., 2009b; Barichello *et al*., 2010c; Barichello *et al*., 2011).

Moreover cytokines have been shown to play an important role in radial glia cell self-renewal during brain development (Deverman and Patterson, 2009). Radial glia cells are precursor cells that can develop into neurons, astrocytes and oligodendrocytes (Deverman and Patterson, 2009). The transformation of radial glial cells into glial cells is said to be modulated by cytokines during the development of the brain (Deverman and Patterson, 2009).

The activation of the JAK/STAT pathway (cytokine signalling pathway) for example, initiates the development of premature astrocytes in late embryonic life (Bonni *et al*., 1997). In addition chemotactic cytokines (chemokines) regulate the proliferation and migration of cells in various brain regions. One of the regions that depend on chemokines, CXCR4 in particular, is the dentate gyrus (Lu *et al*., 2002). CXCR4 is also responsible for axon pathfinding which allows for the development of neural circuitry (Chalasani *et al*., 2003). These are just three examples from a growing body of evidence that suggests a role for cytokines in normal brain development.

Disruption to cytokine function during the development of the brain can therefore result in long-term deficits. Exposure to pathogens which induce increased secretion of cytokines from microglia within the developing brain is associated with abnormal brain structure and impaired cognitive function (Garay and McAllister, 2010; Bilbo, 2013). Microglia are immune cells that reside in the brain and are responsible for clearing away debris and invading pathogens through phagocytosis. The consequence of microglia activation during periods of neurodevelopment is suspected to cause the long-term cognitive deficits seen in rats (Bilbo, 2013).

Although I did not measure the concentration of cytokines produced in the brain of rats used in my study, I was able to show that *S. pneumoniae* could stimulate NF-IL6 expression in hippocampal astrocytes and microglia in a dose dependant manner (see Figures 3.14 and 3.15). Therefore my results suggest that the concentration of *S. pneumoniae* that I administered may not have resulted in the production of cytokines at concentrations high enough to cause lasting impairments.

Alternatively an early life infection with *S. pneumoniae* may have resulted in the priming of microglia thus a learning and memory impairment would only be detected during a subsequent infection. Microglia which are activated during periods of neurodevelopment may became functionally altered and remain in this altered state from early life through to adulthood (Bilbo, 2013). The process by which microglia become functionally altered is referred to as priming (Bilbo and Schwarz, 2012) thus a functionally altered microglial cell is said to be primed. Subsequent activation of primed microglia in this altered state results in cognitive deficits due to an exaggerated immune response (Bilbo, 2013). Rats exposed to *Escherichia coli* during neonatal life displayed impaired contextual memory as adults only if lipopolysaccharide was administered before testing (Bilbo *et al*., 2005). Thus severe haematogenous meningitis experienced in early life may prime microglia which I may have detected if I had administered a subsequent infection (with LPS or *S. pneumoniae*) before conducting behavioural tests.

4.4 Future studies

Animal-based studies

Results from my study suggest that the extent of cognitive impairment seen is dependent on the severity of the disease i.e. amount of bacteria that gains access to the brain and the subsequent inflammation produced. To further investigate the results from my Masters study

I would suggest that the level of neuroinflammation produced by a model of meningitis induced via direct inoculation into the cisterna magna be compared with the level of neuroinflammation produced by a model of haematogenous meningitis induced via the i.p. administration of *S. pneumoniae*. Results from such a study would be useful in determining if the severity of neonatal meningitis, induced by *S. pneumoniae*, translates into altered neurodevelopment and cognitive functioning in later life.

Based on my research, I hypothesise that different culturing techniques produce different levels of neuroinflammation due to the difference in the viability of bacteria. No researcher has determined if replicating bacteria would cause more brain damage *in vivo* than bacteria that are close to the end of their lifespan. The outcome of such a study would have a great impact on the interpretation and implication of results from existing studies and would have an effect on the design of future studies.

When designing a study that uses infectious agents one should also consider establishing a humane endpoint. The administration of an infectious agent to animals carries the risk of undue suffering and death. Therefore it is important to establish objective criteria that could be used as a humane endpoint (Trammell and Toth, 2011). I used body temperature to determine a humane endpoint for the rats used in my study. The route of administration that I used is associated with a high mortality rate due to the development of septic shock (Koedel et al., 2002). Death due to septic shock is preceded by a characteristic decrease in body temperature i.e. hypothermia in rodents (Spencer *et al.*, 2010). Although the exact mechanism of hypothermia is unknown, it is suggested that the increase in bacterial burden impairs an organism's ability to maintain metabolic heat production, thus leading to hypothermia (Romanovsky *et al.*, 1996). Body temperature measurements can be used to predict the point

of death thus allowing for the humane euthanisation of experimental animals (Kort *et al.*, 1998).

Humane endpoints established from other infectious studies conducted using rodents range from 21°C to 36 °C (Kort *et al*., 1998; Vlach *et al*., 2000; Warn *et al*., 2003; Bast *et al*., 2004; Adamson *et al*., 2013). The humane endpoint is dependent on the method used to measure body temperature, the site at which the measurement is taken, the strain of the animal used and the infectious agent that is investigated (Toth, 2000). Therefore it is suggested that the endpoint temperature be determined for specific models in preliminary tests (Nemzek *et al.*, 2004).

In my study rats that had skin temperatures of approximately 34 °C at the time of the first antibiotic administration survived whereas rats that had skin temperatures of approximately 30 °C at the time of the first antibiotic administration did not survive. Thus I used 34 °C as a humane end point for my study. My study is the first to establish a humane end point temperature of 34 °C for a model of haematogenous meningitis induced by *S. pneumoniae* serotype 2, strain D39.

I used a non-invasive microchip transponder to measure skin temperature of infected rats. Non-invasive transponders were used in previous studies to determine endpoints for the humane euthanisation of animals (Kort *et al*., 1998; Vlach *et al*., 2000; Warn *et al*., 2003; Toth and Hughes, 2006; Hankenson *et al*., 2013). Temperature measurements taken from subcutaneous microchip transponders produced less variable results that were more comparable to rectal temperature readings than intraperioneally implanted microchip transponders (Kort *et al.*, 1998).

Implantation of radiotransmitters would provide a continuous measure of body temperature, however the implantation of radiotransmitters requires invasive surgery and a longer period

of anaesthesia as compare to the implantation of microchips. Neonatal exposure to anaesthesia has been shown to cause learning deficits (Satomoto *et al*., 2009) thus I had to reduce exposure to anaesthesia for the purposes of my study. Rectal temperature measurements were also determined to be unsuitable for my study as the act of taking rectal temperature is a stressful event (Groenink *et al.,* 1994). Insertion of a rectal thermometer into the rectum activates the hypothalamic-pituitary-adrenal (HPA) axis (Groenink *et al.,* 1994). The HPA axis is one of the systems that the body employs during stressful events in order to elicit an adaptive response (Heuser and Lammers, 2003).

Activation of the HPA axis during early life can also lead to altered neurodevelopment. Maternal deprivation in P2 to P14 rats resulted in enhanced anxiety and impaired learning and memory measured using the Morris water maze (Huot *et al.*, 2002). Thus using a stress inducing technique to repeatedly measure neonatal body temperature would have influenced my results in the Morris water maze and contextual fear conditioning. My study is the first study to establish a humane endpoint for *S. pneumoniae* infections using a non-invasive technique.

Although it is not common practice to use body temperature as a tool to monitor disease progression, I would recommend that studies investigating the cognitive outcomes of an episode of early life bacterial meningitis should consider using body temperature in conjunction with physical signs of illness to monitor disease progression regardless of the route of administration used.

When designing future studies I would also recommend that researchers consider the effects of gender on learning and memory. Neonatal male rats (P4) were shown to have more microglia in the hippocampus than female rats (Schwarz and Bilbo, 2012). The difference in the number and morphology of microglia seen between the genders suggests that male rats

are more susceptible to infections and are at a higher risk of developing cognitive impairments (Klein, 2000; Schwarz *et al*., 2012). Male mice were found to be more susceptible to a *S. pneumoniae* infection compared to female mice (see Chapter 1, section 1.2.7; Kadioglu *et al*., 2011). However in my study no gender differences were detected (see Figure 3.7 and Figure 3.8).

The different species used in our studies may explain the conflicting outcomes. Certain mouse strains are more susceptible to *S. pneumoniae* infections due to the inability of the mouse to produce antibodies against *S. pneumoniae* (Chaivollini *et al*., 2008). In addition Kadioglu and colleagues (2011) used a higher concentration of bacteria (5×10^4 CFU) to induce illness. The higher concentration of bacteria may have resulted in more severe neuroinflammtion that lead to a detectable gender difference.

Moreover behavioural studies have detected gender differences in Morris water maze performance (Warren and Juraska, 1997; D'Hooge and De Deyn, 2001). Results from these studies suggest that the difference in performance between male and female rats is due to phase of the cycle in which female rats are tested (Warren and Juraska, 1997; D'Hooge and De Deyn, 2001). In my study I did not detect any gender differences in Morris water maze performance.

The rats used in my study were adolescent rats that displayed irregular oestrus cycles however both studies referenced above used adult rats that had a regular, healthy oestrus cycle. The age difference between rats used in our studies could therefore explain the different results reported. I did detect gender differences in contextual fear behaviour that could not be attributed to the phase of the oestrus cycle (see Figure 3.5) or neonatal treatment (see Figure 3.12). Analysis of Figure 3.12 reveals that in the absence of a saline-treated male

and female experimental groups one can claim a treatment effect. Therefore it is crucial that researchers include gender-specific control groups in their experimental design.

Human-based studies

Although animal-based studies provide valuable information on the neurocognitive effect of an early life infection induced by *S. pneumoniae,* it is difficult to apply animal-based results to the human condition. There are a number of longitudinal human-based case-control studies which assess the neurocognitive function of survivors of meningitis. However none of these document the severity of the infection induced by *S. pneumoniae* (by measuring cytokine and leukocyte concentrations) and compares the severity of illness with the degree of neurodevelopment impairments seen in survivors of meningitis induced by *S. pneumoniae.* The documentation of such information would help identify children at risk of developing cognitive impairments and could lead to early intervention programms that either reduce the impact of cognitive impairments or restore cognitive function.

Chapter 5

Conclusion

My study is the first study in our research group to establish a model of haematogenous meningitis in neonatal Sprague-Dawley rats through the intraperitoneal administration of *S. pneumoniae*. Using this model of haematogenous meningitis, I was able to determine that the disease progression and severity of infection to a given dose of *S. pneumoniae* serotype 2, strain D39 is the same for male and female. In addition I was able to show that a neonatal episode of severe haematogenous meningitis induced by *S. pneumoniae* did not result in long-term learning and memory impairments in Sprague-Dawley rats. Moreover I showed the importance of using gender-specific control groups in future studies.

Although results from the Morris water maze and contextual fear conditioning did not show any learning and memory impairments in my study I did demonstrate that *S. pneumoniae* activates microglia in the neonatal hippocampus *in vitro*. Results from the cell culture experiment suggest that there is a dose-dependent effect of *S. pneumoniae* on NF-IL6 expression in the hippocampus. Thus I theorize that the dose of *S.pneumoniae* administered in my study may not have induced a level of inflammation that was capable of producing hippocampal damage.

In conclusion my results suggest that an episode of severe neonatal haematogenous meningitis induced by S. pneumoniae does not affect hippocampal-dependant memory in adolescent Sprague-Dawley rats. However, my results cannot be directly extrapolated to the human condition. Given the high prevalence of bacterial meningitis caused by *S. pneumoniae* it is imperative that research continues in the field.

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Ethical clearance

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2013/03/04

APPLICANT: Ms T Dukhan

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Neonatal exposure to Strptococcus pneumoniae and its consequences on cognitive functioning in adolescent and adult Sprague-Dawley rats

Number and Species

168 juvenile and 18 adult female Sprague-Dawley rats

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20130219. This approval remains valid until 20150218.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

1. **Professor K Erlwanger to be added as co-worker**
2. **Type of barbiturate to be used must be specified**

Signed: _____ (Chairperson, AESC) Date: 4/3/13

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 4/3/13

cc: Supervisor: Dr L Harden
Director: CAS

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Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE
(R 14/16)

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20130101**

BRIEF DESCRIPTION OF APPLICATION:

A neonatal rat model of invasive pneumoccocal disease

APPLICANT: Ms T Dukhan

SCHOOL/DEPARTMENT : Physiology/Braun Function Research Group

DATE CONSIDERED: By circulation

DECISION OF COMMITTEE:

Approved unconditionally

1. This clearance certificate expires on 20180112 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form

DATE: 20130114 **CHAIRPERSON:** _____ (Professor C Tiemessen)

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2013/11/03

APPLICANT: Ms T Dukhan

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Validation of a modified version of the Morris water maze behavioural paradigm, using a Sprague-Dawley rat model

Number and Species

24 male Sprague-Dawley rats, aged 35-45 days

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20130319. This approval remains valid until 20150318.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Signed: _____ (Chairperson, AESC) Date: 26/3/2013

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 27/3/2013

cc: Supervisor: Dr L Harden
Director: CAS

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