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**BEHAVIOURAL AND  
NEUROBIOLOGICAL CHANGES  
MEDIATED BY TNF- $\alpha$   
SIGNALLING IN THE CNS**

Thesis Submitted by  
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April 2014

For the degree of  
**Doctor of Philosophy**

School of Medicine and Dentistry,  
James Cook University, Townsville QLD

## ACKNOWLEDGEMENT

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## STATEMENT OF CONTRIBUTION OF CO-AUTHORS


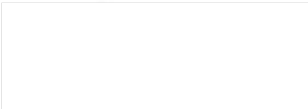
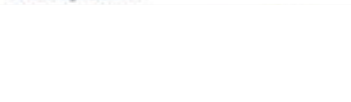


**Table 1.1: Contribution of Candidate and Co-authors**

Chapter	Contribution
<b>Chapter 1:</b> <b>Introduction</b>	The candidate (Camara) formulated and drafted this chapter with supervision from Prof. Baune and Dr Jawahar. Dr Anscomb provided editorial input when framing this chapter for the thesis.
<b>Chapter 2:</b> <b>Methods</b>	The candidate (Camara) was responsible for writing this chapter and articulating the rationale for the methodologies used in this thesis with editorial feedback from Dr Anscomb.
<b>Chapter 3:</b> <b>TNF-<math>\alpha</math> and its Receptors Modulate Complex Behaviours and Neurotrophins in Transgenic Mice</b>	This study was based on an NHMRC grant awarded to Prof Bernhard Baune. The candidate (Camara) performed all experiments used in this study including the behavioural testing of mice, collecting tissue and performing ELISA and histochemical analysis on the tissue, after receiving training for the various techniques from Dr Jaehne and Dr Corrigan. Camara analysed the data and interpreted the results. Camara wrote the manuscript with guidance from Dr Jaehne and Dr Corrigan and supervision from Prof. Baune. Dr Anscomb provided editorial input when Camara arranged this chapter for the thesis.
<b>Chapter 4:</b> <b>TNF-<math>\alpha</math> and its Receptors Govern Adult Behavioural Phenotypes</b>	This study was based on an NHMRC grant awarded to Prof Bernhard Baune. The candidate (Camara) set up and managed the breeding colonies of mice used in this study and performed all experiments described in this study, including the behavioural testing of mice, tissue collection and analysis (ELISA and histology). Camara analysed the data and interpreted the results, and wrote the manuscript with editorial involvement from Dr Jaehne and Dr

	Corrigan, and supervision from Prof. Baune. Dr Anscomb provided editorial input when Camara prepared this chapter for the thesis.
<b>Chapter 5: Centrally Administered Etanercept on Behaviour Following a Peripheral Immune Challenge</b>	The candidate (Camara) designed this study and developed the aims, hypothesis and rationale for this study. Camara performed all experimental procedures described in this chapter, including stereotactic surgeries (trained by Dr Corrigan), behavioural testing of mice and histological analyses. Camara analysed all data, interpreted the results and wrote up the manuscript for publication with feedback from Dr Jaehne and Dr Corrigan and Supervision from Prof. Baune and Dr Anscomb.
<b>Chapter 6: Characterisation of a TNF-<math>\alpha</math> over-expressing strain</b>	This study was based on an NHMRC grant awarded to Prof Bernhard Baune. The candidate (Camara) managed the breeding colonies of mice used in this study and performed all experimental procedures described in this study including the behavioural testing of mice, collecting tissue and performing ELISA and histochemical and RT-qPCR analysis (trained by Dr Jawahar) on the tissue. Camara analysed the data and interpreted the results. The appendix was written by Camara with supervision from Dr Anscomb
<b>Chapter 7: Discussion</b>	The candidate (Camara) was responsible for writing out this chapter after inferring the results of this thesis with editorial input and supervision from Dr Anscomb.

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## DECLARATION ON ETHICS

This research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition, 2004 and the QLD Animal Care and Protection Act, 2001. The proposed research study received animal ethics approval from the JCU Animal Ethics Committee.

***Approval Number: A1644***

This research was also carried out in part at the University of Adelaide, SA. All experimental protocols were approved by the ethics committees of the University of Adelaide in accordance with the guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council. The proposed research study received animal ethics approval from the University of Adelaide Animal Ethics Committee.

***Approval Numbers: M-131-11 and M-180-11***

## PUBLICATIONS AND PRESENTATIONS

### Publications

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Translational Neuroscience.

*\*Joint First-Authors*

**Camara, M. L.**, Corrigan F, Jaehne E.J, Jawahar M. C, Anscomb H, Koerner H and Baune B.T (2013). *“TNF-alpha and its receptors modulate complex behaviours and neurotrophins in transgenic mice.”* Psychoneuroendocrinology.

**Camara, M. L.**, F. Corrigan, Jaehne E.J, Jawahar M. C, Anscomb H and Baune B.T (2014). *“Effects of Centrally Administered Etanercept on Behaviour, Microglia and Astrocytes in Mice Following a Peripheral Immune Challenge.”* Neuropsychopharmacology.

### In Preparation

**Camara, M. L.**, Corrigan F, Jaehne E.J, Anscomb H and Baune B.T. *“Effects of knockout of TNF-a and its receptors on behaviour and neurobiology of adult mice, in the absence of an immune challenge”*. Article in Preparation

## **Presentations/Abstracts**

Society for Mental Health Research Scientific Meeting (December 2014)

ORAL: “Effects of centrally administered etanercept on behaviour, histology and *Tnfa* expression in mice following a peripheral immune challenge”

**Camara, M. L.**, Corrigan F, Jaehne E.J, Jawahar M. C, Anscomb H and Baune B.T

Australian Neuroscience Society Annual Scientific Meeting (January 2014)

ORAL: “Signalling Effects of TNF- $\alpha$  and its Receptors in Mood-like Behaviour”

**Camara, M. L.**, Corrigan F, Jaehne E.J, Anscomb H, Koerner H and Baune B.T

Queensland Brain Institute, 4th Brain Plasticity Symposium (September 2013)

SYMPOSIUM: POSTER: “Immune-Mediated Regulation of Neurotrophins: A Role for TNF- $\alpha$ ”

**Camara, M. L.**, Corrigan F, Jaehne E.J, Jawahar M. C, Anscomb H, Koerner H and Baune B.T

Australian Neuroscience Society Annual Scientific Meeting (February 2013)

POSTER: “The Role of Cytokine TNF-alpha on Measures of Cognition-like and Mood-like Behaviour”

**Camara, M. L.**, Corrigan F, Jaehne E.J, Jawahar M. C, Anscomb H, Koerner H and Baune B.T

Mt Lofty Workshop: Frontier Technologies for Nervous System Function and Repair Adelaide (November 2012)

POSTER: “Dual Roles for TNF- $\alpha$  Receptors in Regulating Cognition”

**Camara, M. L.**, Corrigan F, Jaehne E.J, Jawahar M. C, Anscomb H, Koerner H and Baune B.T

Adelaide Centre for Neuroscience Research Expo, University of Adelaide (June 2012)

POSTER: “The Role of TNF- $\alpha$  in Hippocampal Dependent Cognitive Tasks”

**Camara, M. L.,** Corrigan F, Jaehne E.J, Jawahar M. C, Anscomb H, Koerner H and Baune B.T

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POSTER: “Tumor Necrosis Factor Alpha in the Development of the Hippocampus and Hippocampal-dependent Cognitive Tasks”

**Camara, M. L.,** Anscomb H, Koerner H and Baune B.T

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ORAL: “The Effects of TNF Alpha on Cognitive Function”

**Camara, M. L.,** Corrigan F, Jaehne E.J, Jawahar M. C, Anscomb H, Koerner H and Baune B.T

Townsville Hospital Research Symposium (September 2011)

SYMPOSIUM: POSTER: “Mechanisms of Cognitive Dysfunction: The Role of Tumor Necrosis Factor Alpha on Neurotrophin Production and Hippocampal Development”

**Camara, M. L.,** Anscomb H, Koerner H and Baune B.T

North Queensland Festival of Life Sciences (September 2011)

ORAL & POSTER: “The Effects of TNF alpha on Cognitive Function: An Analysis of the molecular Basis of Behaviour”

**Camara, M. L,** Anscomb H, Koerner H and Baune B.T

## ABSTRACT

### Background

TNF- $\alpha$  is a pleiotropic cytokine having a diverse range of functions extending from the immune system to the central nervous system (CNS). It is recruited into the CNS through the blood brain barrier and also produced by astrocytes and microglia, in the CNS. A majority of its actions are governed by its main receptors: TNF-R1 (expressed by nearly all cell types) and TNF-R2 (expressed by immune and endothelial cells). The general consensus over these receptors has been that TNF-R1 is primarily degenerative due to the presence of a death domain; while TNF-R2 is more neuroprotective due to the lack of said death domain. There is active involvement of these members of the TNF- $\alpha$  superfamily in mediating neurobiology and behaviour in the CNS; TNF- $\alpha$  mediates synaptic scaling and facilitates expression of neurotrophins, brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). However in peripheral or central inflammation, an up-regulation of TNF- $\alpha$  upsets this homeostatic balance triggering mechanistic changes in the CNS that can lead to behavioural impairments. Indeed TNF- $\alpha$  is a known biomarker of Alzheimer's disease, multiple sclerosis and major depressive disorder. It is also an active biomarker of rheumatoid arthritis and psoriasis that are comorbid with psychiatric symptoms.

These factors make the need for understanding TNF- $\alpha$  neurobiology and developing targets to modulate TNF- $\alpha$  expression in inflammatory conditions essential. This body of work was therefore conducted to test the behavioural and neurobiological effects of genetic deletion and pharmacological blockade of TNF- $\alpha$  and its receptors in young and mid-adult mice.

## Methods

This body of work was carried out in three parts:

Chapters 3 and 4 were run using similar methodologies in mice of different ages. Mice deficient for TNF- $\alpha$  (TNF<sup>-/-</sup>) and each of its receptors, TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> (n = 14/strain for both studies) were established on a C57BL/6 background. These mice were used to test the consequences of targeting TNF- $\alpha$  and its receptors on behaviour and neurobiology in young and mid-adult mice compared to C57BL/6 mice (WT: n = 21/chapter 3, n = 14/chapter 4). Mice underwent behavioural testing to measure locomotion, cognition-like, social, and emotion-like behaviour. Hippocampal tissue was collected from these mice to measure levels of NGF and BDNF by ELISA and immunohistochemistry performed to test for neurogenesis (chapters 3 and 4) and microglial numbers (chapter 4).

For the third part of this study, only C57/BL6 mice were used and were divided in to 4 treatment groups. 2 groups were challenged with an acute dose of LPS (n = 20; IP administration) while 2 groups received saline (n = 20; IP administration). One group of LPS mice received etanercept via ICV administration (n = 10; LPS-Etan) and a control group received aCSF also via ICV (n = 10; LPS-aCSF). Similarly, one group of saline treated mice (n = 10) received etanercept through ICV (Sal-Etan) while the other group (n = 10) received aCSF also through ICV (Sal-aCSF). 24 hr post-drug treatment, behaviour of mice was tested and hippocampal tissue was collected to measure the number of microglia and astrocytes in the dentate gyrus.

## Results

3 months old TNF<sup>-/-</sup> mice had impaired cognition, while TNF-R2<sup>-/-</sup> mice showed good memory. All strains of mice had normal social behaviour and knockout strains had lower anxiety than WT mice. Analysis of neurotrophin levels revealed TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice to have significantly lower levels of NGF compared to WT mice, suggesting that some of the behavioural changes could be linked to changes in levels of circulating neurotrophins.

At 6 months age, TNF<sup>-/-</sup> and TNF- $\alpha$  receptor knockout mice displayed similar learning to WT mice, but TNF-R1<sup>-/-</sup> mice displayed better memory than all strains of mice. TNF<sup>-/-</sup> mice had impaired social behaviour and exploratory behaviour in TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice was decreased compared to WT mice. It is also interesting to note that both TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice exhibited lower depression-like behaviour than WT mice. Additionally TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice expressed significantly lower levels of BDNF than WT mice and TNF-R2<sup>-/-</sup> mice displayed significantly higher levels of NGF compared to all strains of mice.

Pharmacologically peripheral administration of LPS reduced locomotor activity, impaired cognition-like and increased anxiety-like behaviour. This change was associated with an increase in microglial numbers in the dentate gyrus. Centrally administered etanercept was able to significantly improve LPS induced anxiety-like behaviour, and prevent the increase of microglia, but had no significant effect on cognition-like behaviour.



## Discussion

Taken together, these findings demonstrate that TNF- $\alpha$  plays a complex role in mediating behavioural phenotypes. This role is further influenced by the inflammatory state and life stage of the individual.

Specifically while normal signalling of TNF- $\alpha$  and its receptors is required for cognition in young mice, the absence of TNF- $\alpha$  and its receptors may improve cognition from mid-adulthood. So also from mid-adulthood, lack of TNF- $\alpha$  and its receptors may protect mice from depression-like behaviour, hinting at a possible target to counter depression in older individuals. Despite these positive features of lack of TNF- $\alpha$  signalling in mid-adulthood, it appears that TNF- $\alpha$  is required throughout a lifespan to maintain social and exploratory behaviour. This suggests that modulation of the cytokine rather than blockade may be the direction to follow.

From a pharmacological perspective, peripheral LPS is effective in triggering behavioural and neurobiological changes as there appears to be an active exchange between the peripheral immune system and CNS. Furthermore, etanercept treatment appeared effective in reducing not only anxiety-like behaviour but also number of microglial cells induced by LPS, showing that even with non-specific inflammation, TNF- $\alpha$  is an active participant in CNS pathology. This reinforces the need for targeting TNF- $\alpha$  in inflammatory disorders with psychiatric disruptions

These results set a foundation to understand the complex role of TNF- $\alpha$  in mediating behavioural phenotypes over the course of an individual's life. Likewise TNF- $\alpha$  modulation appears to be the key to alleviating psychiatric conditions enhanced by this cytokine. Moving onwards, future work needs to look at the effects of targeting TNF- $\alpha$  and its receptors in aged animals to understand the

consequences on behaviour and neurobiology specifically neurotrophin production and glial cell activity.

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## LIST OF ABBREVIATIONS

aCSF - Artificial Cerebrospinal Fluid

AD - Alzheimer's disease

ADAM17 - ADAM Metallopeptidase Domain 17

AITRL – Activation-Inducible Tumor Necrosis Factor-Related Ligand

AMPA -  $\alpha$ -Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor

BBB - Blood Brain Barrier

BCSFB - Blood Cerebrospinal Barrier

BDNF - Brain Derived Neurotrophic Factor

BM - Barnes Maze

BSA - Bovine Serum Albumin

C57/BL6 -Strain of Wild Type mouse

CA1 - Cornu Ammonis 1

CA3 - Cornu Ammonis 3

CaCl<sub>2</sub> - Calcium Chloride

CaCl<sub>2</sub>.H<sub>2</sub>O - Hydrated Calcium Chloride

CBA - Cytometric Bead Assay

CD -Cluster of Differentiation

cDNA - Complementary DNA

CSF – Cerebrospinal Fluid

CHAT - Choline Acetyl Transferase

cIAP 1, 2 – Cellular Inhibitor of Apoptosis

Cldn - Claudin

CNS - Central Nervous System

CRP - C Reactive Protein

CXCL - Chemokine

DAB - 3,3'-Diaminobenzidine

DAPI - 4',6-Diamidino-2-Phenylindole

DCX - Doublecortin

DD - Death Domain

dNTP - Deoxyribonucleotide

DTT - Dithiothreitol

E2 (PGE2) - Prostaglandins

EAE - Experimental Autoimmune Encephalomyelitis

ELISA - Enzyme Linked Immunosorbent Assay

EPM - Elevated Plus Maze

EZM - Elevated Zero Maze

FACS -Fluorescence Activated Cell Sorting

FADD - Fas-associated Death Domain

Fc – Fragment of Crystallization

FST -Forced Swim Test

GABA -  $\gamma$  Amino Butyric Acid

GFAP - Glial Fibrillary Acidic Protein

HBE - Hole Board Exploration

HC - Home Cage

HPA - Hypothalamus Pituitary Adrenal Axis

HRP – Horse Radish Peroxidase

IBA1 - Ionized Calcium-Binding Adapter Molecule 1

ICAM - Intercellular Cell Adhesion Molecule

ICV - Intra Cerebroventricular Injection

IFN $\gamma$  - Interferron Gamma

IgG - ImmunoglobulinG

IHC -Immunohistochemistry

I $\kappa$ B - Inhibitor of Kappa B

IKK - Inhibitor of kappa B Kinase

IL-1 $\beta$  – Interleukin 1 Beta

IL-4/6/8/14/12 - Interleukins

IP - Intra Peritoneal

JNK – c-Jun-NH<sub>2</sub> Terminal Kinase

KCl – Potassium Chloride

Ki67 – Cellular Marker of Proliferation

KO - Knockout

LPS - Lipopolysaccharide

LPS-aCSF - Lipopolysaccharide-artificial Cerebrospinal Fluid

LPS-Etan - Lipopolysaccharide-Etanercept

LTD - Long Term Depression

LTP -Long Term Potentiation

MAPK - Mitogen Activated Protein Kinase

MAPKKK - Mitogen Activated Protein Kinase Kinase Kinase

MCP-1 - Monocyte Chemotactic Protein-1

MEKK3 – Mitogen Kinase

mEPSC - mini Excitatory Post Synaptic Current

MDD - Major Depressive Disorder

MgCl<sub>2</sub>.6H<sub>2</sub>O - Hydrus Magnesium Chloride

MHC II - Major Histocompatibility Complex

mIPSC - mini inhibitory post synaptic current

mRNA messenger Ribonucleic Acid

MS - Multiple Sclerosis

NaCl – Sodium Chloride

NaHCO<sub>3</sub> – Sodium Bi Carbonate

NaH<sub>2</sub>PO<sub>4</sub> – Monosodium Phosphate

NF $\kappa$ B – Nuclear Factor Kappa B

NGF – Nerve Growth Factor

NHS – Normal Horse Serum

NMDAR - N-Methyl-D-Aspartate Receptor

NORT – Novel Object Recognition Test

Nrg1 - Neuregulin1

NT3/4 – Neurotrophin 3 and 4

OFT – Open Field Test

P38 – P38 Mitogen Activated Protein Kinase

P55 – Tumor Necrosis Factor Receptor 1

P75 - Tumor Necrosis Factor Receptor 2

P75<sup>NTR</sup> – p75 Neurotrophin Receptor

PCR – Polymerase Chain Reaction

PET - Positron Emission Tomography

PFC – Prefrontal Cortex

PBMC - Peripheral Blood Mononucleated Cell

qPCR – Quantitative Real Time Polymerase Chain Reaction

RA – Rheumatoid Arthritis

RIP - Receptor Interacting Protein

RNA – Ribonucleic Acid

RNAse - Ribonuclease

S100B – S100 (glial specific) Calcium Binding Protein

Sal-aCSF –Saline Artificial Cerebrospinal Fluid

Sal-Etan – Saline Etanercept

SEM – Standard Error of Mean

SGZ – Subgranular Zone

SODD – Silencer of Death Domain

solTNF – Soluble Tumor Necrosis Factor

SPT – Saccharin Preference Test

SVZ – Subventricular Zone

TACE - Tumor Necrosis Factor Converting Enzyme

TBS – Tris Buffered Saline

TGF - Transforming Growth Factor



Tg6074 – Mouse Strain

TH1 – T Helper Cell 1

TH2 - T Helper Cell 1

TMB - 3,3',5,5'-Tetramethylbenzidine

TNF1 –Tumor Necrosis Factor Polymorphism (Guaninie)

TNF2 - Tumor Necrosis Factor Polymorphism (Adenine)

TNF- $\alpha$  – Tumor Necrosis Factor

TNF-R1 - Tumor Necrosis Factor Receptor 1

TNF-R2 - Tumor Necrosis Factor Receptor 2

TNFRS1A - Tumor Necrosis Factor Super Family Receptor 1

TNFRS1B - Tumor Necrosis Factor Super Family Receptor 2

TRADD - Tumor Necrosis Factor Receptor Associated Death Domain

TRAF2 - Tumor Necrosis Factor Receptor Associated Factor – 2

TRAIL - Tumor Necrosis Factor Related Apoptosis Inducing Ligand

TRIS - Tris(hydroxymethyl)aminomethane

TrkB – Tyrosine Kinase B receptor

VCAM - Vascular Cell Adhesion Protein

V > L – Valine > Leucine

WT - Wild Type

# **Chapter 1 : Introduction to TNF- $\alpha$**

## **Neurobiology and Signaling**

## TUMOUR NECROSIS FACTOR ALPHA MEDIATED MECHANISMS OF COGNITIVE DYSFUNCTION

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Translational Neuroscience

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# TUMOUR NECROSIS FACTOR ALPHA MEDIATED MECHANISMS OF COGNITIVE DYSFUNCTION

**Abstract**

**Background:** Tumour necrosis factor - alpha (TNF- $\alpha$ ) is a pro-inflammatory cytokine that combines a plethora of activities in the early stages of an immune response. TNF- $\alpha$  has gained increasing importance given TNF- $\alpha$  up-regulation in multiple brain pathologies like neuropsychiatric conditions such as depression, schizophrenia, as well as neuroinflammatory disorder like multiple sclerosis (MS).

**Aim:** The aim of this review is to critically analyse neurobiological, immunological and molecular mechanisms through which TNF- $\alpha$  influences the development of cognitive dysfunction.

**Principal findings/results:** The review presents several lines of original research showing that the immunological properties of TNF- $\alpha$  exacerbate inflammatory responses in the central nervous system such as microglial and endothelial activation, lymphocytic and monocytic infiltration and the expression of downstream pro-inflammatory cytokines and apoptotic factors. Depression, schizophrenia, and MS all manifest symptoms of activated immune response along with cognitive dysfunction, with TNF- $\alpha$  overexpression as a central clinical feature common to these disorders. Furthermore, TNF- $\alpha$  acts negatively on neuroplasticity and the molecular mechanisms of memory and learning (i.e., long-term potentiation and long-term depression). TNF- $\alpha$  also exerts influence over the production of neurotrophins (i.e., nerve growth factor and brain-derived neurotrophic factor, neurogenesis, and dendritic branching).

**Conclusions/significance:** This review outlines that TNF- $\alpha$  and its receptors have a substantial yet underappreciated influence on the development and progression of neuropsychiatric symptoms across several disease entities. An improved understanding of these underlying mechanisms may help develop novel therapeutic targets in the form of drugs specifically targeting downstream products of TNF- $\alpha$  activation within the central nervous system.

**Keywords**

Tumour necrosis factor alpha • Cytokines • Neuropsychiatry • Neuroinflammation • Depression • Neuroplasticity • Neurotrophins

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## **Introduction: CNS Disorders and the Involvement of the Immune System**

Mental and neurological disorders like schizophrenia and depression constitute 13% of the global disease burden, quickly surpassing the threat by cardiovascular disorders and cancer (Collins et al., 2011). A common link between neuropsychiatric conditions such as major depressive disorder (MDD), schizophrenia, and neuro-inflammatory disorder like MS and RA, which manifest neuropsychiatric symptoms (Lieberman, 1999, Mandolesi et al., 2010, Kupfer et al., 2012), is the presence of exaggerated immune system activation both peripherally and within the CNS (Sharief and Hentges, 1991, Zorrilla et al., 2001, Miller et al., 2009). In the periphery, patients with MDD have impairments in cellular immunity (reduced natural killer cell activity) (Blume et al., 2011), as well increased expression of pro-inflammatory cytokines like interleukin (IL)-1 $\beta$ , IL-6, tumour necrosis factor (TNF- $\alpha$ ) and chemokine IL-8 (Zunszain et al., 2012). This peripheral immune activation is associated with stimulation of resident astrocytes, microglia and neurons within the CNS, which produce inflammatory cytokines (Lieberman et al., 1989, Rock et al., 2004, Tsakiri et al., 2008). Indeed, depression is now thought to be the result of an increased production of pro-inflammatory cytokines that are caused by external or internal stressors, with this known as the cytokine theory of depression (Zunszain et al., 2012). This up-regulation of cytokines leads to a low grade chronic inflammatory state which alters neurotransmitter metabolism, neuroendocrine function, promotes oxidative and nitrosative stress and reduces neuroplasticity resulting in the typical symptoms of melancholia, anhedonia, psychomotor slowing, decreased appetite and fatigue (Zunszain et al., 2012).

While many of these studies highlight the involvement of cytokines in the etiology of psychiatric disorders, several reports have revealed conflicting results. Levine et al (1999), for instance, showed elevated levels of IL-1 $\beta$  and decreased levels of IL-6 with no change in TNF- $\alpha$  in CSF of depressed patients compared to healthy controls. On the other hand while some studies reported similar decreased IL-6 levels in CSF of depressed patients, other studies reported either higher levels of IL-6 (Stubner et al., 1999, Lindqvist et al., 2009) or no difference between patients and controls (Carpenter et al., 2004). Similarly with serum analysis, some studies found TNF- $\alpha$  levels to be elevated in MDD while others reported no change in serum TNF- $\alpha$  levels compared to controls (Brambilla and Maggioni, 1998, Karlovic et al., 2012). This inconsistency in findings was thought to be due to difference in gene expression of cytokines, as well as other confounding factors like prior medication, gender and techniques used to measure cytokines (Haack et al., 1999, Young et al., 2014).

Microarray and gene set analysis of post mortem CNS tissue also showed up-regulation of some cytokines like IL-2, IL-3, IL-10, TNF- $\beta$  (Shelton et al., 2011). Interestingly another microarray study of post mortem brain tissue showed that normal aging was associated with greater changes in inflammatory markers, such as MHC and TLRs, as well as IL-1 $\beta$ , TNF- $\alpha$  and IL-6, than the development of AD (when compared to age matched controls) The authors suggested that inflammation may play a greater role in the early stages of AD rather than the later stages in the brains examined (Cribbs et al., 2012).

As in MDD, reports of peripheral and central immune activation have been documented in schizophrenia. Elevated serum and/or plasma levels of pro-inflammatory factors like prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (Kaiya et al., 1989), C-reactive protein (CRP) (Dickerson et al., 2007), IL-1 $\beta$  and TNF- $\alpha$  (Theodoropoulou et al., 2001) have been found in schizophrenic individuals. Furthermore, high microglial activation in the brain of schizophrenic patients has been reported in post-mortem studies (Bayer et al., 1999, Radewicz et al., 2000). This has been verified by positron emission tomography (PET) studies in patients, particularly in the temporo-limbic portion of the brain (van Berckel et al., 2008, Doorduyn et al., 2009). These activated microglia then release TNF- $\alpha$  and IL-6 (Miller et al., 2009) contributing to the diseased state of the brain in schizophrenia. The presence of an exaggerated immune response and downstream signalling of pro-inflammatory cytokines in MDD and schizophrenia are thought to be the underlying causes of the impairments in learning and memory, along with increased anxiety and sleep disturbances (Niitsu et al., 2011, Zunszain et al., 2012) seen in these psychiatric illnesses. Indeed, neurodegeneration induced by this neuroinflammatory state is seen as a reduction in hippocampal volume in depressed patients which is associated with mild to moderate impairments in cognitive performance compared to healthy norms (Campbell et al., 2004).

The link between inflammation and neuropsychiatric disorders can also be seen in neuroinflammatory condition like MS and systemic autoimmune disorder like RA, which also have neuropsychiatric symptoms. MS patients often suffer from cognitive dysfunction and depression, (Mandolesi et al., 2010) with the presence of an activated immune system especially pro-inflammatory cytokines thought to be the

cause of the neuropsychiatric symptoms observed (Sharief and Hentges, 1991). Indeed this supposition has been demonstrated in animal models (Ziehn et al., 2010), where experimental autoimmune encephalomyelitis (EAE) leads to a reduction in the volume of the CA1 region of the hippocampus, primarily due to activated microglia and infiltrating macrophages that release pro-inflammatory cytokines like TNF- $\alpha$  (Renno et al., 1995, Ziehn et al., 2010). Renno et al (1995), previously verified that microglia (and macrophages) express high amounts of TNF- $\alpha$ . EAE was induced by injecting mice with myelin basic protein, total RNA was extracted from whole brain and RT-PCR performed for Cd3, IFN- $\gamma$  and TNF- $\alpha$ . TNF- $\alpha$  (and Cd3/IFN- $\gamma$ ) levels were increased in EAE mice and correlated to disease progression. FACS analysis of mononuclear cells of the CNS also revealed that TNF- $\alpha$  was expressed by macrophages and microglia. Glial activation (microglial positive cells measured by CD45+ labelling) in the study by Ziehn et al (2010) led to a decrease in volume of the CA1 region of the hippocampus (measured using a Cavalieri estimator), and was verified using NeuN and Nissl staining; these changes were accompanied by a decrease in the number of GABA-ergic inter-neurons and increased number of TUNEL positive cells (apoptotic cells) in the hippocampus, (Ziehn et al., 2010).

RA, on the other hand, though a systemic autoimmune disorder has its own set of neuropsychiatric symptoms. RA patients exhibit deficits in cognitive function (learning and memory) (Appenzeller et al., 2004) as well as increased anxiety (El-Tantawy et al., 2008) and depression (Hider et al., 2009). The cognitive deficits observed in RA patients could be attributed to the high levels of pro-inflammatory cytokine TNF- $\alpha$  in serum (Aloe et al., 1992b, El-Tantawy et al., 2008). Therefore

these high levels of TNF- $\alpha$  may interact with the CNS to impair the expression of neurotrophins like nerve growth factor (NGF) resulting in a loss of neurogenesis and synaptic function (Aloe et al., 1992b).

These findings demonstrate an overarching theme that immune system activation, particularly release of pro-inflammatory cytokines, can affect core functions of the CNS like neuroplasticity and cognition (Stellwagen and Malenka, 2006). The significance in studying the neurobiological functions of TNF- $\alpha$  specifically stem from its unique influence in mediating synaptic scaling, especially  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) dependent plasticity (Stellwagen and Malenka, 2006) as well as its effects of development of hippocampal structures (Golan et al., 2004). The pro-inflammatory cytokine TNF- $\alpha$  is constitutively expressed in the CNS, and has been shown to exert physiological neuroprotective and neurodegenerative effects, depending on whether the TNF-R1 or TNF-R2 receptor is activated. Signalling through the TNF-R1 pathway, initiates the activation of caspases leading to apoptosis, due to the presence of a cytoplasmic 'death domain'. In contrast activation of TNF-R2, promotes cell survival, through inhibition of caspase activation.

Thus although increased levels of TNF- $\alpha$  may underlie the neuropsychiatric symptoms of a number of diseases (Fiore et al., 1996), in physiologically unchallenged conditions, this group has shown in a murine model, that during early stages of development basal levels of TNF- $\alpha$  are required in the CNS for memory and learning (Baune et al., 2008). These neuroprotective effects of TNF- $\alpha$  are mediated by its influence on neurotrophin production (Golan et al., 2004) as well as on its ability to influence synaptic scaling (Stellwagen et al., 2005). This chapter



provides an introduction to the neurobiology of TNF- $\alpha$  within the CNS under normal and pathological conditions. A more detailed knowledge of the cellular mechanisms by which TNF- $\alpha$  contributes to the development and progression of neuropsychiatric symptoms, particularly the mechanisms subserving cognition, could be instrumental in developing effective therapeutic strategies.

### **Inter-Relationship between Immune System and CNS Function**

A few decades ago the CNS was regarded as a region devoid of immune reactions (Medawar, 1948, Barker and Billingham, 1977). Thus the CNS, which includes the brain and spinal cord, was believed to be a site of “immune privilege”. However this claim has now been significantly modified and redefined to suggest that the CNS is in fact an area of selective immune reactivity, which results from active processes (Streilein, 1993, Ransohoff et al., 2003).

The immune system plays a very complex role in the maintenance of the central nervous system. The immune system sustains homeostatic equilibrium of the various structures of the brain and constantly surveys the brain ensuring neuroprotection (Tabakman et al., 2004). Furthermore, it also plays a crucial non-immune role in the neurodevelopment, by acting on processes such as neurogenesis, neuronal migration and synaptic plasticity (*For reviews see* (Boulanger and Shatz, 2004, Garay and McAllister, 2010)). The blood brain barrier (BBB) of the CNS is the main system that controls leukocyte trafficking from the periphery into the brain (Zlokovic, 2008, Banks and Erickson, 2010) while the epithelial blood cerebro-spinal fluid barrier (BCSFB) controls the influx of

lymphocytes to brain parenchyma. It is through this interaction between the BBB and the BCSFB that immune cells enter and patrol the CNS (Engelhardt, 2006).

Threat by external stimulus in the form of injury, infection or stress may affect the balance between CNS and the immune system. This results in the trafficking of T and B cells leading to downstream activation and subsequent entry of pro-inflammatory cytokines like TNF- $\alpha$  into the brain via the BBB (Pan and Kastin, 2002, Bossu et al., 2008, Brietzke et al., 2009, Maccioni et al., 2009, Cross and Waubant, 2010). The BBB has cytokine binding sites that either alter intracellular function (receptors) or convey the cytokines across the BBB (transporters) (Banks and Erickson, 2010). Pro-inflammatory cytokines trigger the expression and activation of other inflammatory factors including other cytokines, chemokines and reactive oxygen species, that leads to necrotic and apoptotic neural injury (Leib and Tauber, 1999, Miric et al., 2010). Of these cytokines, TNF- $\alpha$  in particular is shown to be up regulated in CNS inflammatory conditions that involve BBB disruption (Nishioku et al., 2010). Both TNF- $\alpha$  receptors, TNF-R1 (p55) and TNF-R2 (p75) are present on the endothelial cells of the blood brain barrier and function like transporters (Pan and Kastin, 2002). Additionally, TNF- $\alpha$  suppresses the expression of claudins (Cldn), particularly Cldn5 that is an essential molecular component of tight junctions and permeates the CNS (Forster et al., 2008, Aslam et al., 2012).

Apart from peripheral inflammatory cells that traverse the BBB, the CNS has its own immune system. Microglia are the resident immune cells of the CNS (Wake et al., 2012). Resting microglia or those that are activated by an adaptive immune response of Th1 and Th2 cytokines IL-4 and IFN- $\gamma$  promotes neurogenesis (Butovsky et al., 2005). Additionally resting microglia are also thought to induce

neurogenesis through mitogen activated protein kinase (MAPK) signalling pathways (Morgan et al., 2004). Infections or trauma on the other hand cause microglia to be differentially activated and release pro-inflammatory cytokines like IL-6, TNF- $\alpha$  and IL-1 $\beta$ .

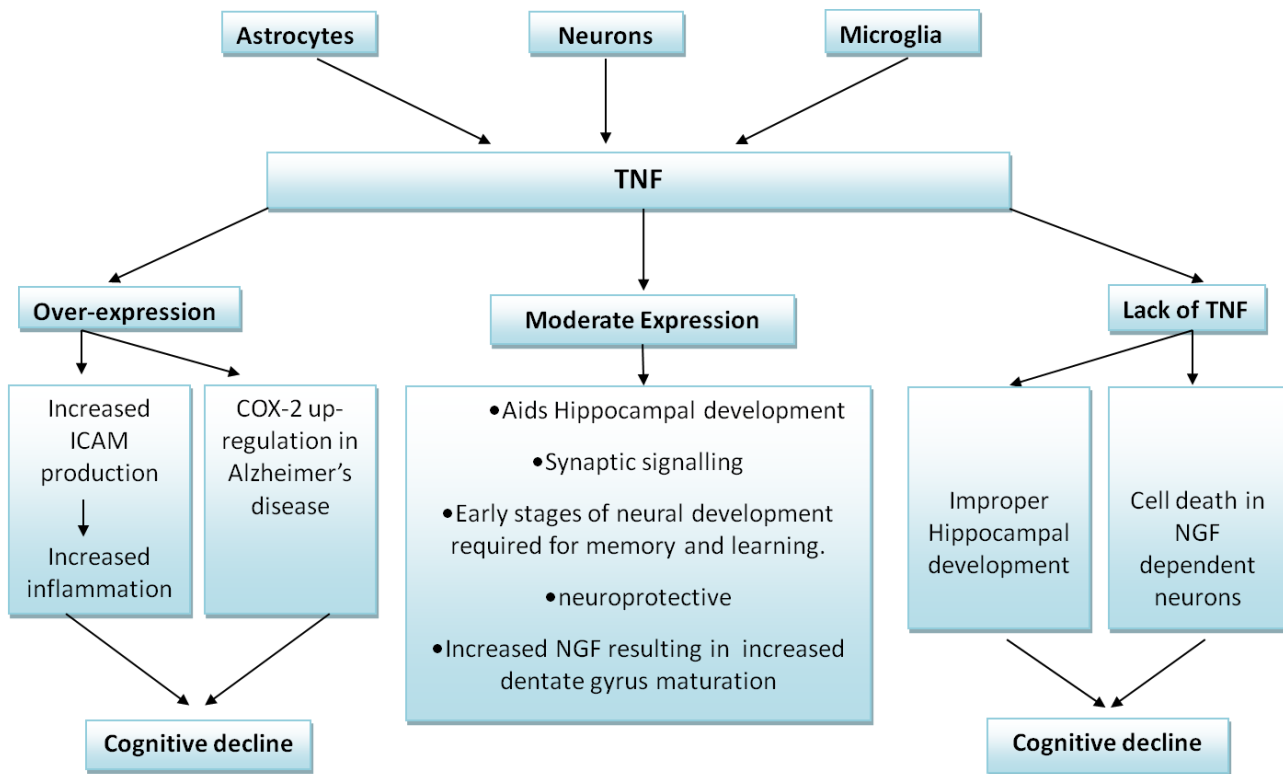
Activated microglia present dual functions – 1) The elimination of damaged neurons and 2) Activation of immune system, by downstream signalling of pro-inflammatory cytokines (Rock et al., 2004). Of the pro-inflammatory cytokines released by microglia, TNF- $\alpha$  is thought to be the main factor behind BBB dysfunction resulting in a feedback loop of continual inflammation between the periphery and the CNS (Nishioku et al., 2010).

TNF- $\alpha$  is a pleiotropic cytokine having both beneficial as well as detrimental inflammatory functions. The inflammatory nature of TNF- $\alpha$  allows for regulation of the immune system and protection against infections by B-cell proliferation. But on the other hand TNF- $\alpha$  can promote tumor growth and is an active biomarker of many illnesses from cerebral malaria to rheumatoid arthritis (Aggarwal, 2003, Clark et al., 2010, Drutskaya et al., 2010, Juhasz et al., 2013).

Regardless of the primary source of TNF- $\alpha$  in the brain, the symptoms that it produces in acute inflammatory conditions are deleterious (Figure 1.1). Animal studies have linked TNF- $\alpha$  over-expression to cerebral malaria (Medana et al., 1997), progression of septic shock syndrome (Tracey, 1991), formation of sickness behaviour (Bielefeldt Ohmann et al., 1989), thrombosis and tissue necrosis (Probert et al., 1993). However despite these negative effects, under physiologically unchallenged conditions TNF- $\alpha$  exerts beneficial actions. In normal conditions it

promotes and regulates neurogenesis (Das and Basu, 2008), plays a role in hippocampal development (Golan et al., 2004), and is essential to learning and memory formation (Albensi and Mattson, 2000, Stellwagen and Malenka, 2006) (Figure 1.1, Table 1.2). This complex and divergent role of TNF- $\alpha$  is mediated by its signalling through its main receptors, TNF-R1 and TNF-R2. TNF- $\alpha$  can function both in a neuroprotective manner as well as in a neurodegenerative manner based on its expression and the receptor through which it signals (Horiuchi et al., 2010). The following sections detail the neurobiology of TNF- $\alpha$  from expression to signalling patterns in physiologically unchallenged as well as pathological conditions.

**Figure 1.1: Illustration of the differential expression of TNF- $\alpha$  by astrocytes and microglia**



**Legend:** The figure demonstrates how TNF- $\alpha$ , produced by astrocytes and microglia is required for cognition at moderate levels by aiding hippocampal development and synaptic signalling. While an over-expression of the protein causes increased intercellular cell adhesion molecule (ICAM) production, resulting in cognitive decline. Similarly a lack of TNF- $\alpha$  causes death of nerve growth factor (NGF) dependent neurons and affects cognition. This shows that basal levels of TNF are required for memory and learning and a disruption in this causes cognitive dysfunction.

## Neurobiology of TNF- $\alpha$ in the CNS

The pro-inflammatory cytokine TNF- $\alpha$  is a member of the TNF- $\alpha$  super family of ligands (Aggarwal, 2003, Juhasz et al., 2013) (Wajant et al., 2003). It is synthesized in its pro form as a monomeric type-2 transmembrane protein (tmTNF, 26kDa) and is inserted into the cell membrane and cleaved to its soluble form (solTNF, 17kDa) by a matrix metalloprotease TNF- $\alpha$  converting enzyme (TACE/ADAM17) (Aggarwal, 2003, Juhasz et al., 2013). Both forms of TNF- $\alpha$  are biologically active and are produced in the CNS by astrocytes, microglia as well as neurons (Lieberman et al., 1989, Li et al., 2008, McCoy and Tansey, 2008). TmTNF acts by cell-to-cell contact whereas solTNF acts at distant sites (Perez et al., 1990). Of note, TmTNF allows bi-directional signalling during an inflammatory response, by transmitting information to other cells by binding as a ligand to TNF- $\alpha$  receptors, but also functioning as a receptor transmitting reverse signals to tmTNF containing cells (Alexopoulou et al., 1997).

TNF- $\alpha$  signals mainly through two receptors, TNF-R1 (TNFRSF1A/p55) and TNF-R2 (TNFSFR1B/p75) (Kassiotis and Kollias, 2001), with sTNF- $\alpha$  preferentially binding to TNF-R1 (Grell et al., 1998) and TmTNF to TNF-R2 (Bazzoni and Beutler, 1996). Both receptors are expressed on a variety of cell types; TNF-R1 is expressed by all nucleated cells including neurons while TNF-R2 is expressed by immune cells, endothelial cells, microglia and cells of the hematopoietic lineage as well as some neurons (Kinouchi et al., 1991, Grell et al., 1998).

**Table 1.1: Studies on TNF<sup>-/-</sup> and TNF receptor knockout mice**

STUDY REFERENCE	MODEL	ANALYSIS	RESULT
(Golan et al., 2004)	C57/BL6 mice and TNF <sup>-/-</sup> mice.	ELISA: Serum protein analysis of NGF, BDNF, TNF- $\alpha$  IHC: Dendritic tree of hippocampal neurons IHC: staining for BDNF and NGF in medial hippocampal slices  Cognitive Testing: Morris Water Maze Open Field Test Elevated Plus Maze Hole Board	Associated with reduced BDNF levels and branching of apical dendrites in CA1 and CA3 regions of hippocampus
(Baune et al., 2008)	C57/BL6 mice, TNF <sup>-/-</sup> mice, TNF -R1 <sup>-/-</sup> and TNF -R2 <sup>-/-</sup> mice	Cognitive Testing: Novel Object Recognition Open Field Test; Barnes Maze  Gene Expression: PCR of brain stem, hippocampus, cerebellum and frontal cortex. CBA for IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12p70	Basal levels of TNF- $\alpha$ under non-inflammatory conditions are required for normal learning and memory
(McAfoose et al., 2009)	TNF <sup>-/-</sup> mice, C57/BL6 mice	Cognitive Testing: Barnes Maze	Absences of TNF- $\alpha$ with increasing age is protective of age-related memory decline
(Kassiotis and Kollias, 2001)	TNF <sup>-/-</sup> mice and C57/BL6 mice, TNF -R1 <sup>-/-</sup>	Flow Cytometry of spleen cells using FACSCalibur	TNF- $\alpha$ is required for down-regulation of T-cell responses against myelin antigens in EAE

**Legend:** The table shows studies conducted on TNF<sup>-/-</sup> and TNF- $\alpha$  receptor deficient mice in understanding CNS disorders.

## **TNF-R1 Signalling Pathway**

TNF-R1 is characterised by the presence of a death domain (DD) that distinguishes it from TNF-R2. The presence of the DD allows TNF-R1 to dissociate silencer of death domain (SODD) and subsequently activates TNF- $\alpha$  receptor associated death domain (TRADD) (Chen and Goeddel, 2002). TRADD activation results in the recruitment and activation of three proteins, Fas-associated death domain (FADD), receptor interacting protein (RIP) and TNF- $\alpha$  receptor associated factor 2 (TRAF2) (Hsu et al., 1995, Hsu et al., 1996) each leading to different signalling cascades (Figure 1.2).

FADD activation leads to recruitment of caspase 8 resulting in activation of an apoptotic pathway (Micheau and Tschopp, 2003). TRAF2 activates a mitogen associated protein kinase kinase kinase (MAPKKK or MAP3K) to ultimately activate c-Jun-NH<sub>2</sub> terminal kinase (JNK) and c-Jun. RIP activation is responsible for the activation of NF $\kappa$ B pathway by activation of MEKK3 a mitogen kinase that degrades inhibitor of kappa B kinase (IKK) (Chen and Goeddel, 2002).

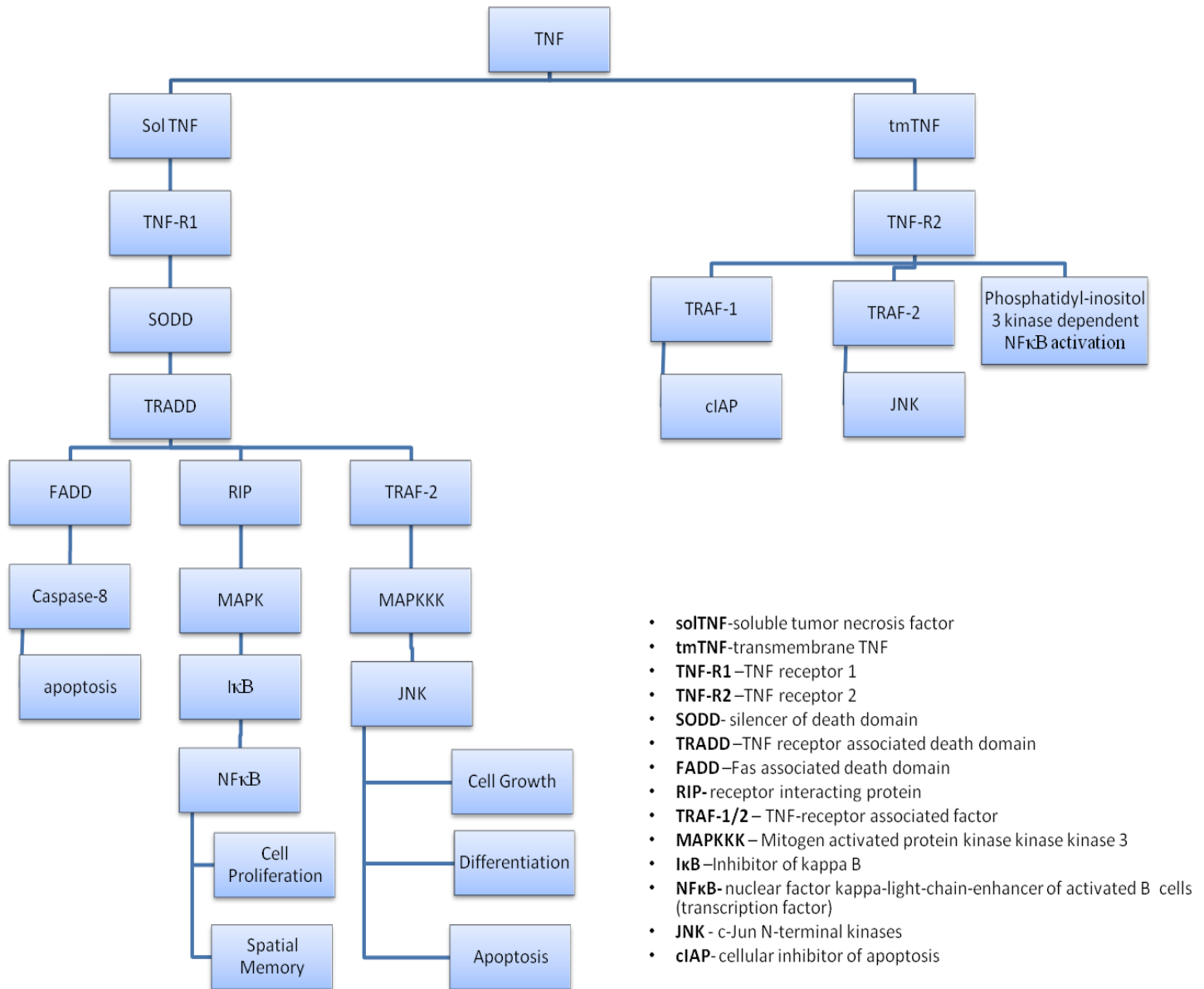
Extensive crosstalk occurs between these three pathways. The recruitment of the TRADD associated pathways results in further recruitment of cellular inhibitor of apoptosis 1 and 2 (cIAP 1, 2) resulting in downstream activation of JNK pathway (Winston et al., 1995, Shu et al., 1996). Acute activation of JNK activation is cytoprotective, however chronic activation of the pathway leads to caspase dependent apoptosis (Tobiume et al., 2001).

NF $\kappa$ B is expressed in the cytoplasm and is maintained in its inactive form by inhibitor of kappa B (I $\kappa$ B). IKKs on activation phosphorylate I $\kappa$ B allowing NF $\kappa$ B to migrate to the nucleus to modulate transcription of genes involved in neuroprotective



or neurodegenerative processes (Ghosh and Karin, 2002). NF $\kappa$ B also regulates genes involved in synaptic plasticity, spatial memory and cell proliferation (*For review see (Camandola and Mattson, 2007)*). It also protects cells from apoptosis but cross talk with the FADD pathway in chronic conditions results in caspase activation causing cleavage and degradation of RIP that is essential to NF $\kappa$ B signalling (Chen and Goeddel, 2002). This demonstrates a complex interaction between the downstream signalling pathways of TNF-R1 activated signalling.

**Figure 1.2: TNF- $\alpha$  signalling in the CNS**



**Legend:** The figure shows the signal transduction pathway for TNF- $\alpha$ . TNF- $\alpha$  acts via two different receptors TNF-R1 and TNF-R2 and exerts either positive or negative neurobiological processes depending on the downstream pathway.

## **TNF-R2 Signalling Pathway**

TNF-R2 due to its presence on limited type of cells (immune, endothelial and microglial cells), demonstrates fewer biological effects than TNF-R1 and does not induce caspase dependent apoptosis (Grell et al., 1998). TNF-R2 is shown to activate TRAF-1 and TRAF-2 proteins resulting in NF $\kappa$ B and cIAP activation respectively, thus showing overlapping effects with TNF-R1 (Rothe et al., 1995). Additionally this receptor also activates phosphatidylinositol 3-kinase dependent NF $\kappa$ B signalling which protects neurons from glutamate induced excitotoxicity, giving evidence to the neuroprotective effects that have been observed with TNF-R2 signalling (Marchetti et al., 2004) (Figure 1.2). Thus the conditions under which TNF- $\alpha$  is produced – normal physiological or pathological - will determine its subsequent binding to the appropriate receptor and dictate its neurobiological functions.

## **TNF- $\alpha$ Signalling and Neuroplasticity**

The neurobiological effects of TNF- $\alpha$  in the CNS extend from its ability to influence synaptic scaling as well as influence neurogenesis to neurotrophin production (Figure 1.3). Long term potentiation and depression, the strengthening and weakening respectively of synaptic connections are a form of Hebbian plasticity and are believed to be the synaptic model of memory and learning processes (Bliss and Collingridge, 1993). Hebbian plasticity is maintained by regulating the strength of synaptic input and neuronal excitation. This is known as homeostatic synaptic plasticity or synaptic scaling and is defined by changes in surface expression of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA $\alpha$ s) at the synaptic junction. (*For review see (Wang et al., 2012)*).

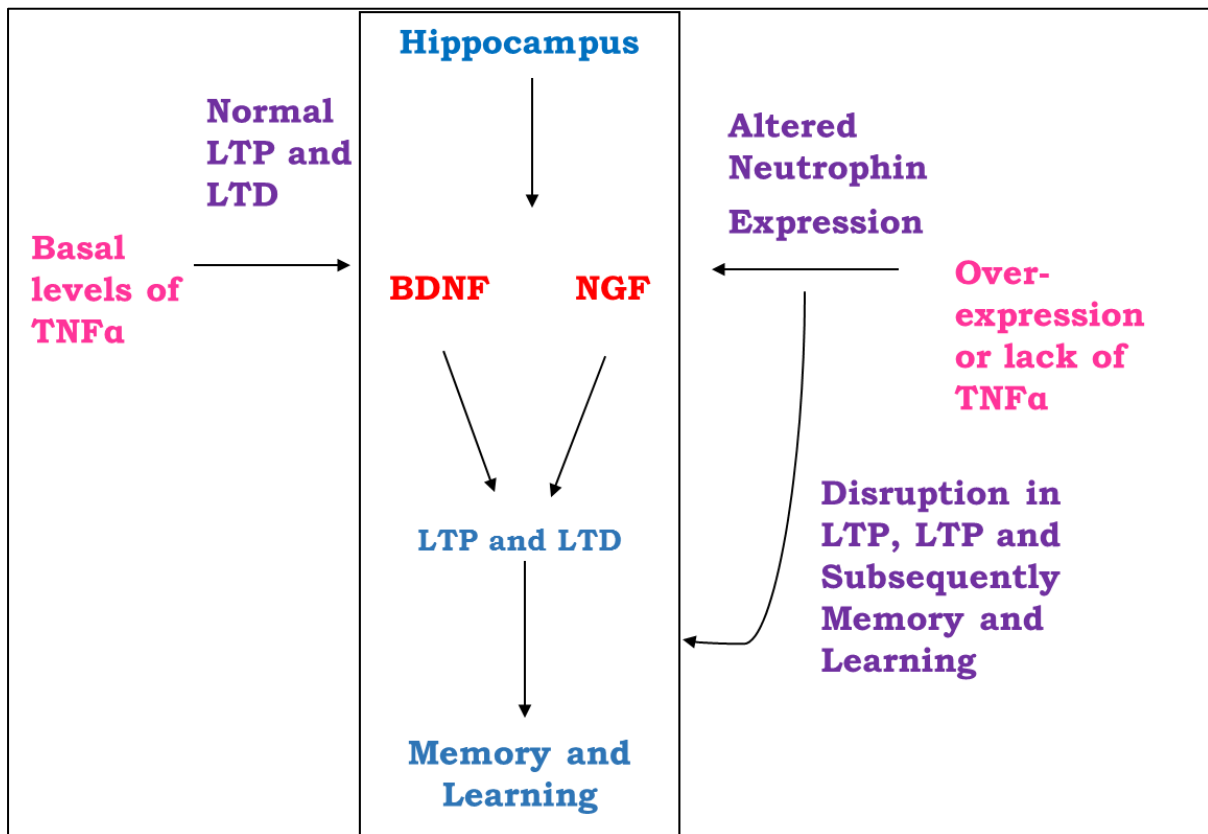
Glutamate is the main excitatory neurotransmitter and is required for memory formation, learning and synaptic plasticity. It mediates this through the action of its receptors AMPARs and N-Methyl-D-Aspartate receptors (NMDARs) (Stellwagen et al., 2005). Additionally glutamate produced NMDAR can activate NF $\kappa$ B (Lipsky et al., 2001). Animal studies in vitro have shown that astrocytic released TNF- $\alpha$  causes trafficking of AMPARs, in primary cell cultures and hippocampal slices (Beattie et al., 2002) by acting directly on neurons as well as by decreasing the expression of inhibitory  $\gamma$  amino butyric acid (GABA) receptors (Stellwagen et al., 2005). Interestingly this effect on AMPARs seems to be specific to TNF- $\alpha$  but not to other cytokines and is mediated through TNF- $\alpha$  receptor, TNF-R1 but not TNF-R2 (Stellwagen et al., 2005). This interaction between TNF- $\alpha$  and glutamate receptors denotes a possible role of TNF- $\alpha$  in AMPAR dependent long term potentiation (LTP) and long term depression (LTD), the underlying mechanisms of learning and

memory (Stellwagen and Malenka, 2006). Furthermore TNF- $\alpha$  increases amplitude of miniature excitatory post-synaptic currents (mEPSC) and decreases miniature inhibitory post synaptic currents (mIPSC) (Beattie et al., 2002, Stellwagen et al., 2005). However, chronic increase in levels of TNF- $\alpha$ , in pathological conditions, inhibits LTP in the CA1 region and dentate gyrus of the hippocampus (Butler et al., 2004). TNF- $\alpha$  inhibits synaptic scaling through activation of NF $\kappa$ B pathways (Albensi and Mattson, 2000, Bolshakov et al., 2000), c-Jun N-terminal kinase (JNK) (Wang et al., 2004) and p38 MAPK pathway (Bolshakov et al., 2000). The p38 MAP kinase pathway is a signal transduction pathway that regulates inflammatory processes of cell death as well as playing a role in growth and differentiation (Ono and Han, 2000), *For review see* (Pickering et al., 2005). These results demonstrate how chronic inflammation causes the neuroprotective effects of TNF- $\alpha$  to switch to neurodegenerative.

The influence of TNF- $\alpha$  in the CNS further extends to neurotrophin production and expression. Nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) all derived from a common ancestral gene, are similar in sequence and structure, and are therefore collectively named neurotrophins (Hallbook, 1999). Neurotrophic factors are involved in growth, maintenance and differentiation of neurons (Huang and Reichardt, 2001). They are important regulators of neural survival, development, function, and plasticity (Lewin and Barde, 1996, Sofroniew et al., 2001). NGF and other neurotrophins are synthesized in nerves and are believed to be essential for survival and the regeneration of sensory and sympathetic neurons (Henderson, 1996) as well as of injured neurons (Huang and Reichardt, 2001). TNF- $\alpha$  has the ability to act in a

positive or negative manner on neurotrophin synthesis in a dose dependent manner (Aloe et al., 1999).

**Figure 1.3: Role of TNF- $\alpha$  in neurotrophin production and hippocampal memory and learning**



**Legend:** The figure shows how basal levels of TNF- $\alpha$  are required for the production of brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). BDNF and NGF in turn are required for long-term potentiation (LTP) and long-term depression (LTD), the molecular basis of memory and learning. An over-expression of TNF- $\alpha$  causes LTP and LTD to be altered, consequently altering memory and learning.

NGF exerts its effects by binding to two receptors, the p140<sup>TrkA</sup> receptor that is specific to NGF and the p75<sup>NTR</sup> receptor that is common to all neurotrophins. The p75<sup>NTR</sup> receptor is a member of the TNF- $\alpha$  receptor super family and can bind to the

p140<sup>TrkA</sup> receptor to enhance the responsiveness of NGF. In the absence of p140<sup>TrkA</sup>, p75<sup>NTR</sup> can induce apoptosis. In the CNS, inflammation causes glial cells and immune cells to produce NGF (Takei and Laskey, 2008). The close association with TNF- $\alpha$ , glia, and neurons, led to the proposal of a positive feedback loop of NGF synthesis; NGF expression by glia could lead to the production of TNF- $\alpha$ , similarly TNF- $\alpha$  produced by neurons and glia could further activate other inflammatory cells to release NGF (Takei and Laskey, 2011). In the presence of NGF, TNF- $\alpha$  activates TNF-R2 however in the absence of NGF, TNF-R1 is activated (Takei and Laskey, 2011). However it was shown that in the absence of TNF- $\alpha$  (TNF<sup>-/-</sup> mice), the expression of NGF was increased compared to WT mice; Additionally this increase in the levels of NGF was accompanied with better hippocampal development characterised by higher cell density in the hilus of the dentate gyrus, suggesting that in the absence of TNF- $\alpha$  other pro-inflammatory cytokines could possibly activate microglia to produce NGF (Golan et al., 2004). TNF- $\alpha$  further influences the production of BDNF, a modulator of synaptic plasticity and neuronal morphology (Golan et al., 2004, Saha et al., 2006a). An in vitro study showed that astrocytic TNF- $\alpha$  causes BDNF levels to increase through activation of NF $\kappa$ B transcription factor (Saha et al., 2006a). In support of this finding it was shown in mice that TNF- $\alpha$  deprivation in the hippocampus caused BDNF levels to be low resulting in decreased dendritic branching in the CA1 and CA3 region of the hippocampus leading to improper development of the hippocampus and subsequently cognitive dysfunction (Golan et al., 2004). These studies demonstrate the complex interaction between TNF- $\alpha$  receptor signalling and the effects that downstream proteins, like NF $\kappa$ B have on neurotrophins production. This relationship between TNF- $\alpha$  and neurotrophins

demonstrates the direct or indirect role that TNF- $\alpha$  plays in hippocampal development and neurogenesis.

### **TNF- $\alpha$ Signalling in the Progression and Development of Cognitive Functions in Disorders Linked to the CNS**

Neuropsychiatric conditions could originate from and often result in neural degeneration and increased inflammation in the CNS as well as in the periphery (Streit et al., 2004). An exaggerated expression of TNF- $\alpha$  is found to be a common pathological feature in diseases with cognitive impairments such as depression, schizophrenia and multiple sclerosis (Sharief and Hentges, 1991, Potvin et al., 2008, Khairova et al., 2009). The following sections focus more elaborately on studies that demonstrate how this up-regulation of TNF- $\alpha$  acts on the progression and development of these conditions and the possible mechanisms through which TNF- $\alpha$  influences the cognitive deficits seen in the mentioned illnesses. Table 1.3 summarises some important studies providing evidence for the effect of increased or reduced and no expression of TNF- $\alpha$  within the CNS respectively.



Table 1.2: Studies on the effects of over-expression and under/no expression of TNF- $\alpha$  in the CNS

STUDY	MODEL	EXPRESSION	ANALYSIS	RESULT
(Akassoglou et al., 1997)	Transgenic mice; GFAP- TNF- $\alpha$ <sup>+/+</sup>	Over-expression of TNF- $\alpha$ within the brain (Astrocyte specific)	IHC and histological staining: of astrocytes in the brain and spinal cord.	Mice developed neuronal disease. Increased ICAM and VCAM production
(Selmaj et al., 1991)	Human: post-mortem MS tissue.	-	IHC: of MS plaque tissue samples with GFAP antibody	TNF- $\alpha$ detected in acute MS lesions with astrocyte, endothelial and macrophage morphology
	PBMC isolates from patients with progressive and relapsing MS.		FACS and Immunoflorescent: staining of PBMC for TNF	Higher levels of TNF- $\alpha$ in PBMC FACS analysis in patients compared to controls. (not significant)
(Fiore et al., 1996)	Tg6074 mice (over-produce TNF- $\alpha$ ) and C57/BL6 mice	Over-expression of TNF- $\alpha$	Cognitive Testing: Open Field Test Passive Avoidance Test Hot Plate Test	Kyphosis, ataxia, seizures, poor learning and memory in transgenic mice
(Dean et al., 2010)	Human, post-mortem study	-	Western Blot Analysis: solTNF and tmTNF in BA24 and BA46	High concentration of transmembrane TNF- $\alpha$ in dorsolateral prefrontal cortex, BA46 (area associated with cognition.)

<b>(Grassi-Oliveira et al., 2009)</b>	Patients clinically depressed; plasma analysis	-	ELISA: Plasma analysis of TNF- $\alpha$ , TNF-R1 and TNF-R2	High levels of TNF- $\alpha$ and its receptors in plasma of patients with major depressive disorder compared to controls
<b>(Golan et al., 2004)</b>	C57/BL6 mice and TNF <sup>-/-</sup> mice	Low/no expression of TNF- $\alpha$	ELISA: Serum protein analysis of NGF, BDNF, TNF- $\alpha$	High NGF in young mice compared to wild type mice
			IHC: Dendritic tree of hippocampal neurons; staining for BDNF and NGF in medial hippocampal slices	
			Cognitive Testing: Morris Water Maze, Open Field Test, Elevated Plus Maze, Hole Board	
<b>(Baune et al., 2008)</b>	C57/BL6 mice, TNF <sup>-/-</sup> mice, TNF -R1 <sup>-/-</sup> and TNF -R2 <sup>-/-</sup> mice	Low/no expression of TNF- $\alpha$ and its receptors TNF-R1 and TNF-R2	Cognitive Testing: Novel Object Recognition, Open Field Test, Barnes Maze  Gene Expression: PCR of brain stem, hippocampus, cerebellum and frontal cortex.  CBA for IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12p70	Poor memory and learning in TNF <sup>-/-</sup> adult mice compared to wild type adult

(Kassiotis and Kollias, 2001)	C57/BL6 mice, TNF <sup>-/-</sup> mice, TNF-R1 <sup>-/-</sup> mice	Low/no expression of TNF- $\alpha$ and its receptor TNF-R1	Flow Cytometry of spleen cells using FACSCalibur	Prevented demyelination but cause an exaggerated T-cell response in mice.
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(McAfoose et al., 2009)	TNF <sup>-/-</sup> mice, C57/BL6 mice	Low/no expression of TNF- $\alpha$	Cognitive Testing: Barnes Maze	Cognitive decline in early stages (8-12 weeks of age) with absence of TNF.
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**Legend: Studies illustrating the effects of over-expression and low/no expression of TNF- $\alpha$  in the CNS**

## **Cognitive Dysfunction as Seen In Depression: Relation to TNF- $\alpha$ Expression**

MDD or clinical depression is a neuropsychiatric condition characterised by anhedonia, depressive mood and cognitive dysfunction. Patients with MDD have shown increased levels of inflammatory markers and macrophage activation (Khairova et al., 2009, Schmidt et al., 2011) in the periphery as well as in the CNS (Zorrilla et al., 2001, Miller et al., 2009). It is suggested that chronic stress and its association with inflammation (Eyre and Baune, 2011) is found to suppress adult neurogenesis (Mirescu and Gould, 2006) and is thought to be another precursor for the onset of depression (Leonard and Myint, 2009).

Increase in peripheral blood cytokine levels induced by lipopolysaccharide (LPS) or *Salmonella typhi* vaccination in healthy individuals caused symptoms of depression as well as fatigue, psychomotor slowing (Brydon et al., 2008) and mental confusion (Reichenberg et al., 2001, Miller et al., 2009). These findings are consistent with animal studies demonstrating that the up-regulation of cytokines is related to sickness-like behavioural symptoms of anhedonia and dysfunction in cognition like behaviour (Dantzer et al., 2008, Miller et al., 2009). Both pre-clinical and clinical studies have demonstrated that cytokines, particularly TNF- $\alpha$  and IL-6 are up-regulated in MDD (Howren et al., 2009, Miller et al., 2009, Dowlati et al., 2010, Schmidt et al., 2011). Under normal conditions, TNF- $\alpha$  is required for neurogenesis and the development of memory and learning, as shown by murine studies (Bernardino et al., 2008, Miller et al., 2009). However, continual exposure to these cytokines results in decreased neurogenesis, apoptosis and cognitive dysfunction (Tilleux and Hermans, 2007, Li et al., 2008, Miller et al., 2009).

A recent animal study showed that TNF- $\alpha$  administered through the intracerebroventricular region (ICV) produced depressive-like behaviour in mice (Kaster et al.). In another animal study which tested the effects of stress and depression on cytokine expression, higher levels of TNF- $\alpha$  and IL-1 $\beta$  mRNA were found in the cortex, spleen and hippocampus of stressed rats while anti-inflammatory cytokine IL-10 and transforming growth factor (TGF) and neurotrophins such as BDNF were reduced (You et al., 2011). Once at extremely low concentrations, BDNF causes depolarization and modulates LTP of hippocampal neurons (Golan et al., 2004) (Kafitz et al., 2000).

TNF- $\alpha$  can further influence astrocytes and microglia to release nitrogen and oxygen reactive species that cause oxidative stress to neurons and glial cells (Tilleux and Hermans, 2007, Gavillet et al., 2008, Li et al., 2008, Miller et al., 2009). Such oxidative damage is particularly noticeable in the prefrontal cortex (PFC) and the amygdala (Ongur et al., 1998, Hamidi et al., 2004, Miller et al., 2009), both of which are areas relevant to cognitive and emotional processing and are impaired in depression. Increased microglial activation was also observed in the PFC and hippocampus in post-mortem analysis of suicide patients compared to control subjects (Steiner et al., 2008); lending further evidence to the role of TNF- $\alpha$  in depression.

The role of various cytokines involved in MDD can be explained by the strong inter-relationships between both, anti and pro-inflammatory cytokines. Cytokines respond in an autocrine and paracrine manner to trauma and infection and have strong inter-relationships with each other. Pro-inflammatory cytokines like TNF- $\alpha$  and IL-1 $\beta$  are among the first to be released to the site of infection,

this causes IL-6 to be activated, which in turn regulates both pro and anti-inflammatory functions (Radtke et al., 2010). Some of the anti-inflammatory functions of IL-6 are, that it does not induce cyclooxygenase activity and does not produce metalloproteases that can cause tissue degradation (Barton, 1998). In chronic inflammatory conditions however, IL-6 can act in a pro-inflammatory and can induce the production of acute phase proteins as well as development of humoral and cell mediated immunity by stimulating B cell and T cell production (Gabay, 2006). IL-6 signalling in-turn is negatively regulated by suppressor of cytokine signalling (SOCS3) (Starr et al., 1997). Interestingly TNF- $\alpha$  can induce SOCS3 in macrophages while IL-1 $\beta$  can counteract IL-6 signaling by reducing its gene expression by acting on target promoters (Bode et al., 1999, Zhang and Fuller, 2000), suggesting that TNF- $\alpha$  and IL-1 $\beta$  can both promote and suppress IL-6 expression depending on the situation. While IL-2 can also induce IL-6 production in monocytes (Musso et al., 1992). IL-2 can also negatively regulate Il6ra (encoding IL-6 receptor alpha) and Il6st (encoding IL-6 signal transducer gp130) and consequently decrease IL-6 responsiveness (Liao et al., 2011). IFN- $\gamma$  on the other hand does not induce IL-6 despite being able to modulate the expression of genes in monocytes (Radzioch and Varesio, 1991). Interestingly IFN- $\gamma$  can inhibit IL-6 production when induced by IL-1 $\beta$  but not IL-2 (Musso et al., 1992). IFN- $\gamma$  can also induce TNF- $\alpha$  production in macrophages (Winston et al., 1999).

## **Cognitive Impairment Seen in Schizophrenia and Its Association with TNF- $\alpha$**

Schizophrenia is regarded as a neurodegenerative disorder originally termed “dementia praecox” (Lieberman, 1999). The symptom complexes are distinguished into positive symptoms (hallucinations, delusions, and movement and thought disorders) and negative symptoms (anhedonia, listlessness) as well as cognitive dysfunction like impairment in working memory, attention and social problem solving (Niitsu et al., 2011). Cognitive impairments usually precede the onset of positive and negative symptoms (Arguello and Gogos, 2010).

Recent evidence points to increased inflammation occurring in the CNS as well as the periphery of schizophrenic patients suggesting a role of cytokines like IL-1, IL-6 and TNF- $\alpha$  in schizophrenia (Muller et al., 2000, Drzyzga et al., 2006, Potvin et al., 2008). Serum cytokine levels of patients with chronic schizophrenia to normal healthy controls showed significantly higher levels of TNF- $\alpha$  in patients (Theodoropoulou et al., 2001) higher concentrations of soluble TNF-R1 and TNF-R2 (Coelho et al., 2008). Studies have shown that polymorphisms in the –G308A TNF- $\alpha$  gene is possibly involved in the aetiology of schizophrenia (Wilson et al., 1997, Boin et al., 2001, Schwab et al., 2003). Furthermore, TNF- $\alpha$  is located on the short arm of chromosome 6 (6p21.1-21.3), a locus that is predisposed to schizophrenia (Shi et al., 2009, Ingason et al., 2011).

A possible mechanism through which cytokines including TNF- $\alpha$  exert effects in schizophrenia is via Neuregulin 1, which is considered to be involved in the pathophysiology of schizophrenia (Stefansson et al., 2002). Under normal conditions, the Neuregulin1 gene (Nrg1) along with its receptor erbB4 are essential

for myelination, plasticity as well as LTP formation by acting on post synaptic NMDARs and AMPARs (Stefansson et al., 2002, Li et al., 2007), thereby underlining an important role in cognitive function. Studies of plasma cytokine levels in families having the Nrg1, V>L mutation (Valine to Leucine mutation is associated with a higher risk for schizophrenia), showed that high levels of IL-1 $\beta$ , IL-6, IL-10 and TNF- $\alpha$  were associated with Nrg1 mutation suggesting a relationship between Nrg1 mutation and peripheral cytokine expression (Marballi et al., 2010).

There have been conflicting results regarding the association between TNF- $\alpha$  polymorphisms and TNF- $\alpha$  expression. Among the different variants of the TNF- $\alpha$  polymorphisms, the polymorphism located on the single nucleotide polymorphism (SNP) -308 (G/A) has been most extensively studied (Wilson et al., 1997, Elahi et al., 2009). This biallelic base-exchange polymorphism contains 2 variants – one with a guanine, G (TNF1) and the less common one with an adenine A (TNF2), both at the -308 positions. The TNF2 allele has in some studies been associated with increased expression of TNF- $\alpha$  whilst others report no difference between levels of transcription of TNF- $\alpha$  with either the -308A or -308G alleles (Galinanes et al., 2008, Taudorf et al., 2008). A significant increase in the frequency of TNF2 allele has been noted in schizophrenic patients compared to healthy controls, along with homozygosity of TNF2 but not TNF1 (Boin et al., 2001). In MDD, some studies have reported higher recurrence of the GG genotype of the -308 polymorphism (Omrani et al., 2009, Cerri et al., 2010, Kim et al., 2013) but not the AA genotype, while others report a higher relationship between AA alleles of TNF -308 SNP and MDD (Jun et al., 2003)



A BDNF associated pathway could be another avenue through which TNF- $\alpha$  may exert effects in schizophrenia. A post-mortem analysis of hippocampal tissue revealed lower BDNF levels in schizophrenia patients compared to controls (Durany et al., 2001). The reduced expression of BDNF in the hippocampus could be due to the effects of TNF- $\alpha$  exerts on hippocampal BDNF levels (Golan et al., 2004). Since BDNF in turn is required for pyramidal cell morphogenesis in the CA1 and CA3 regions of the hippocampus (Golan et al., 2004), it can be suggested that increased TNF- $\alpha$  in schizophrenia has effects on the cognitive symptom spectrum of the disease. These findings are consistent with recent a meta-analysis showing reduced serum BDNF levels in chronic untreated schizophrenia patients (Green et al., 2011).

## **TNF- $\alpha$ Associated Cognitive Dysfunction in MS**

MS is an autoimmune neurodegenerative diseases characterized by demyelination and axonal loss of neurons in the central nervous system as well as gliosis of oligodendrocytes (Lassmann et al., 2001). This demyelination affects the ability of neurons to communicate with each other resulting in a loss of sensory and motor function. The disease is associated with a large spectrum of symptoms that include muscle weakness, ataxia, dysarthria (caused by demyelination of neurons in the spinal cord), optic neuritis as well as significant cognitive impairment, especially memory impairments (Mandolesi et al., 2010). MS pathology is associated with inflammation which is characterized by increased expression of pro-inflammatory cytokines, particularly of TNF- $\alpha$  – both in the CNS as well as the periphery (Sharief and Hentges, 1991). In humans, higher levels of TNF- $\alpha$  were observed in the cerebrospinal fluid (CSF) of patients with chronic progressive MS as opposed to patients with stable MS. Furthermore, these elevated cytokine levels correlated with the degree of disability (Sharief and Hentges, 1991). In animal model of MS (experimental autoimmune encephalomyelitis, EAE), TNF- $\alpha$  is predominately produced by infiltrating T cells and macrophages as well as resident microglial cells and a neutralization results in amelioration of the neurological symptoms such as paralysis (Ruddle et al., 1990, Renno et al., 1995, Korner and Sedgwick, 1996, Korner et al., 1997b). This observation together with high numbers of TNF- $\alpha$  positive astrocytes and microglia gave reason to assume that TNF- $\alpha$  is directly involved in the progression of MS (Kuroda and Shimamoto, 1991, Selmaj et al., 1991). Nevertheless, an essential role for TNF- $\alpha$  was also shown in down-regulating and inactivating detrimental autoimmune T cell response against myelin antigens and

protecting from chronic EAE (Barten and Ruddle, 1994). Interestingly, high levels of TNF- $\alpha$  caused inactivation of deleterious auto-specific T cell responses but resulted in progression of disease and excessive demyelination, whereas lower TNF- $\alpha$  levels prevented excessive demyelination but caused exaggerated T cell responses (Kassiotis and Kollias, 2001). The availability of gene-deficient mice (TNF $^{-/-}$ ) allowed experiments that demonstrated that TNF- $\alpha$  is required during the initial stages of the disease. MOG-immunized mice showed delayed onset and a milder course of the disease compared to WT mice suggesting that the cytokine may be required for leukocyte activation during initial stages rather than having a specific role in the progression of the disease. This hypothesis was strengthened by findings that at the peak of EAE, inflammation in WT and TNF $^{-/-}$  mice was comparable and both strains exhibited a perivascular and sub-meningeal infiltration of CD45 $^{+}$  cells as well as microglial activation (Korner et al., 1997c). Additionally, a dual role for TNF- $\alpha$  receptors was established in the EAE model. TNF-R2 $^{-/-}$  mice showed increased demyelination compared to TNF-R1 $^{-/-}$ , and TNF- $\alpha$  double knockout mice (TNF-R1/R2 $^{-/-}$ ) mice, along with increased infiltration of CD4 $^{+}$  and MHC II cells, supporting a neuroprotective role of R2 receptor (Suvannavejh et al., 2000).

In EAE learning and memory is impaired (D'Intino et al., 2005). Post-mortem hippocampal analysis of the mice in this study (D'Intino et al., 2005) revealed a decrease in the levels of choline acetyl transferase (ChAT) and NGF expression in the hippocampus, especially in the late phase of the disease. Animal studies using the Barnes Maze (cognitive testing tool to measure spatial memory and learning in mice) could correlate impairment in cognition like behaviour with morphological changes in these mice such as low CA1 volume and high apoptosis of CA1

hippocampal neurons measured by increased levels of TUNEL cells (apoptotic cells) as well as high microglial activation (Ziehn et al., 2010). The cognitive deficits observed in these mice which were potentially caused by the effects of the auto-inflammation on the hippocampus and other limbic structures was linked to the effects of TNF- $\alpha$  dependant de-regulation on neurotrophic expression as well as on LTP and LTD formation. Neuroimaging studies have supported these findings and have revealed that MS lesions are associated with a decrease in volume of hippocampal structures as well as cingulate gyrus and prefrontal cortex (Roosendaal et al., 2010).

## **TNF- $\alpha$ Contributes to Neuropsychiatric Symptoms in Rheumatoid Arthritis**

Rheumatoid arthritis (RA) is a systemic autoimmune disease that manifests itself by inflammation of the synovial joints (Cuzzocrea et al., 2005). Additionally, the disease is also characterised by cognitive impairments and depression (Hider et al., 2009). An involvement of the peripheral nervous system (Lanzillo et al., 1998) has been reported however there is still debate about the involvement of the CNS (Ando et al., 1995).

Studies have demonstrated that there is an up-regulation of TNF- $\alpha$  in the synovial fluid, as well as over-expression of S100B protein (produced by astrocytes) and neuron specific enolases; all of which are thought to be correlates of neurodegeneration and brain injury (Hamed et al., 2011). The up-regulation of these proteins coincides with poor cognitive performance; cognitive testing of women with RA correlated low mini-mental state examination scores with high levels of S100B protein (Hamed et al., 2011). The cognitive deficits observed in RA were further clarified by another study which demonstrated RA patients exhibiting significantly poorer performance in verbal fluency, logic memory and short term memory than healthy controls (Appenzeller et al., 2004).

The impairments in cognition could be related to the pain associated with RA. In a rat model of adjuvant arthritis, rats showed increased sensitivity to pain and in parallel, poor performance in the Morris water maze compared to controls (Skurlova et al., 2010). Human studies assessing the effects of RA pain on cognition also found that pain was associated with poorer performance in executive functioning tasks (Roldan-Tapia et al., 2007, Abeare et al., 2010). Alternatively, progression of the disease and cognitive dysfunction could be attributed to the effects of TNF- $\alpha$ .

Animal studies have shown that TNF- $\alpha$  over-expression causes advancement of the disease state. Mice deficient in secreted soluble TNF- $\alpha$  but over-expressing transmembrane TNF- $\alpha$ , demonstrated a faster disease progression compared to WT mice. The transgenic mice developed deep dermal or subcutaneous inflammation with the appearance of activated macrophages in synovial fluid (Edwards et al., 2006).

The behavioural deficits seen in RA could also be attributed to a combination of the actions of TNF- $\alpha$  and NGF. Studies conducted by Aloe et al have shown high levels of NGF in the synovial joints of human RA sufferers (Aloe et al., 1992a) and murine models of RA (Aloe et al., 1992b). In Tg197 mice that carry a TNF- $\alpha$  transgene and develop symptoms of RA, anti-NGF treatment was carried out by intra peritoneal (IP) injection, a decrease in the thickness of the synovial cell layer was demonstrated, but anti-NGF treatment also resulted loss of weight and ultimately death, which was not observed in mice that underwent anti-TNF- $\alpha$  therapy (Aloe et al., 1993). Flow cytometry analysis revealed an increase of expression of NGF in CD3+ and CD14+ cells, as well as TNF-R2 mRNA and TrkB receptor of NGF suggesting lymphocytes and myeloid cells are both source and target of TNF- $\alpha$  in RA (Barthel et al., 2009).

Recent studies by Chen et al (Chen et al., 2010) and Uguz et al (Uguz et al., 2009), showed that anti- TNF- $\alpha$  therapy improved cognitive dysfunction in patients suffering from RA. Therefore, anti- TNF- $\alpha$  therapy could potentially be used as a therapeutic method to reverse the deficits in cognition experienced by RA patients.

## Conclusions and Future Directions

Though the major factors that play a role in TNF- $\alpha$  signalling have been identified, the exact mechanisms of their actions are yet to be determined. Further work needs to be done to elucidate the exact mechanisms and signalling pathways that involve TNF- $\alpha$  dependent production of neurotrophins.

Due to the up-regulated pro-inflammatory environment developed in the above mentioned conditions, the use of non-steroidal anti-inflammatory drugs is gaining some momentum as a treatment modality. A range of biological antagonists is available such as Infliximab, a chimeric bivalent IgG1 monoclonal antibody composed of a human constant region and murine variable regions, Adalimumab a humanized bivalent mouse IgG1 monoclonal antibody, and etanercept a fusion protein comprised of human IgG fused to a dimer of the extracellular regions of TNF-R2. Additionally anti-TNF- $\alpha$  therapy is being considered as an option in improving post-operative cognitive dysfunction (Terrando et al., 2010). The outcome of anti-TNF- $\alpha$ , etanercept treatment further extend to anti-depressant effects in non-inflammatory conditions (Bayramgurler et al., 2013a, Bayramgurler et al., 2013b, Krugel et al., 2013)

However, there are two problems with these techniques. First the BBB prevents a deep penetration into the CNS tissue and second, while basal levels of TNF- $\alpha$  are still required for normal functioning, animal models have shown that the complete lack of TNF- $\alpha$  due to genetic modification results in cognitive impairment (Baune et al., 2008). This is possibly due to the influence that TNF- $\alpha$  exerts on neurotrophins - NGF and BDNF. An imbalance in TNF- $\alpha$  causes subsequent de-regulation in these neurotrophins ultimately leading to morphological changes in the

hippocampus such as decreased arborisation of pyramidal neurons (Golan et al., 2004).

Additionally, the role TNF- $\alpha$  plays in LTP and LTD formation by up-regulation of AMPA receptors (Beattie et al., 2002) and endocytosis of GABA receptors (Stellwagen et al., 2005) is crucial in the development of synaptic neuroplasticity related memory and learning (McAfoose and Baune, 2009). Therefore, though complete blockage may prove to be initially beneficial, long-term negative effects may manifest and make further research into these effects necessary.

The complex signalling pathways of TNF- $\alpha$  and its receptors and the duality of its function in being both neuroprotective and neurodegenerative make for a compelling argument against the validity and long-term benefits of anti-TNF- $\alpha$  therapies. TNF- $\alpha$  may both exacerbate and attenuate cognitive dysfunction depending on the physiological context (Peschon et al., 1998, Longhi et al., 2008). Clearer perspectives into TNF- $\alpha$  signalling and pharmacological interventions that can target specific apoptotic factors in the TNF- $\alpha$  receptor associated pathways (Figure. 1.2), rather than complete blockage of TNF- $\alpha$  or of its receptors would make for more effective therapeutics in treatment of neurological disorders in which TNF- $\alpha$  is an active participant.



## Thesis Hypothesis and Aims

In order to better understand the implications of lack and over-expression of TNF- $\alpha$  on various behavioural phenotypes, and to add to the ever-increasing body of literature surrounding TNF- $\alpha$  signalling, this thesis was developed with the following hypothesis in mind:

***De-regulation in the expression of TNF- $\alpha$  by genetic alterations (up-regulation and down-regulation) of the genes coding for TNF- $\alpha$  and its receptors will result in behavioural and neurobiological changes within the CNS***

To test this hypothesis, several aims were developed and were tested in mice.

1. To study the effects of genetic deletion of TNF- $\alpha$  and its receptors on behavioural phenotypes as well as neurotrophin expression in young adult mice
2. To study the effects of knockout of TNF- $\alpha$  and its receptors on behaviour and neurobiology of mid-adult mice
3. To determine the behavioural and neurobiological effects of pharmacological blockade of TNF- $\alpha$  in the CNS of young adult immune activated mice
4. To determine the effects of over-expression of TNF- $\alpha$  in a mouse model of CNS specific TNF- $\alpha$  up-regulation, on the behaviour and neurobiology of mice

Each of these aims will now be described in the chapters that follow.

## **Chapter 2 : Materials and Methods**

## Background on Methodology

The use of animal models to study various ailments and disorders is extensive, and mice share many physiological characteristics with humans spanning from the anatomical to the molecular level. Behavioural phenotypes in mice appear to be similar to humans and mice demonstrate similar responses to humans in regards to anxiety, social interaction, cognition, sexual behaviour as well as circadian rhythms (Van Meer and Raber, 2005, Baker, 2013). These factors make it easier to draw parallels and translate animal research to humans. The relatively short life cycle and the easy handling, as well as low costs, make mice the perfect candidates for studying behaviour not only in pathological and immune challenged conditions but also under normal physiological conditions.

Due to the homologous nature of human and animal genomes much work has focussed on altering the genomes of mice to study a variety of disorders (Abbott, 2010). The use of transgenic mice in medical research has gained popularity (Houdebine, 2007) in the study of disorders on the central nervous system such as mouse models of schizophrenia (O'Donnell, 2013, Suh et al., 2013) and Alzheimer's Disease (Gotz et al., 2004, Spires and Hyman, 2005). The use of knockout mice has also gained impetus in the last few decades and has helped focus research to understanding signalling patterns of genes or proteins of interest (Austin et al., 2004).

My studies were centred on understanding the implications of up-regulation and down-regulation of TNF- $\alpha$  and its receptors, on behavioural and neurobiological phenotypes within the CNS. There appears to be a highly conserved homology between murine and human TNF- $\alpha$  genes. In humans there are 18 gene encoding proteins comprising a conserved carboxy-terminal domain with 150

amino acids (Reed et al., 2003). This domain is called the TNF- $\alpha$  homology domain, and plays a role in ligand trimerization and receptor binding. Comparisons of TNF- $\alpha$  ligand cDNA between human and murines show that all ligands belonging to TNF family are conserved between both species with the exception of AITRL (Reed et al., 2003). Similarly in the case of TNF- $\alpha$  receptor family, humans have 29 genes encoding the TNF- $\alpha$  receptors of which 25 were found in mice that were homologous to humans. Mice lacked the TRAIL receptor DR4, TRAIL decoy receptor DcR1 and DcR2 and the FasL decoy receptor DcR3 (Reed et al., 2003)

To carry out the specific aims that were detailed in the introductory chapter of this thesis, I have made use of various strains of mice that either have deletions of genes coding for TNF- $\alpha$  and its receptor action, or mice strains that involve an up-regulation of TNF- $\alpha$  expression (*see appendix*). For studies detailed in chapters 3 and 4 I have used genetically altered mice having deletions of TNF- $\alpha$  as well as mice having genetic deletions of each of its main receptors (TNF-R1 and TNF-R2). These mice were subject to a comprehensive behavioural testing battery to understand the implications of such a deletion on various behavioural phenotypes. Post behavioural analyses, mice were sacrificed and their hippocampal tissue was collected for protein and immunohistochemical analysis to better understand the neurobiology behind the observed behaviour.

Mice in chapter 3 were tested at 3 months of age to assess how early developmental behavioural phenotypes were affected by lack of normal signalling of TNF- $\alpha$  and its receptors, while in chapter 4, mice of the same strains were aged to 6

months in order to understand the implications of altering TNF- $\alpha$  signalling on behavioural and neurobiological phenotypes in mid-adult mice.

Encouraging results in chapters 3 and 4 showed how a lack of TNF- $\alpha$  and its receptor signalling may be beneficial in improving cognition-like behaviour as well as decreasing depression-like behaviour in adult mice. This prompted the need for moving on to a translational research approach and the effect of blocking TNF- $\alpha$ . It was anticipated that blocking the actions of TNF- $\alpha$  temporarily by pharmacological means, could potentially have benefits in alleviating some of the symptoms that have been seen in disorders of the nervous system that involve a component of TNF- $\alpha$  activation (Baune et al., 2012c, Menachem et al., 2012, Pukhalsky et al., 2012, Luleyap et al., 2013).

Experiments in chapter 5 were carried out by treating groups of the common wild type strain of mice (C57BL/6) with an anti-TNF- $\alpha$  drug, etanercept, following a systemic immune challenge by a bacterial endotoxin (Lipopolysaccharide, LPS). Etanercept is currently being used as a therapeutic to rheumatoid arthritis (Wakabayashi et al., 2011, Morgan et al., 2013) and ankylosing spondylitis (Senabre-Gallego et al., 2013), and is also being tested in Alzheimer's disease (Tobinick et al., 2006, Tobinick, 2010). Most recently the focus of etanercept treatment has shifted to understanding the implications on mood-like behaviour, including anxiety-like and depression-like (Kekow et al., 2011, Haji et al., 2012). Promising results in the use of etanercept in decreasing the symptoms of these behaviour (Bayramgurler et al., 2013a) prompted the development of the study detailed in chapter 5, that tested the efficacy of etanercept on anxiety and cognition-like behaviour in mice receiving a peripheral immune challenge.

Due to the quick turnover of cerebrospinal fluid (CSF) (Saunders et al., 1999), mice were sacrificed immediately post behavioural testing and within 24 hr of etanercept treatment. Neurobiological evaluations extended to histochemical analyses of astrocytes and microglia to test the hypothesis that LPS treatment, despite being peripherally administered, would activate both astrocytes and microglia whilst etanercept treatment would help reduce these effects of LPS.

The details of the methodology, as well as the rationale for use of each test are detailed below. Furthermore, specific details of the methodology used to investigate each of the aims are specified in each chapter.

## Animal Ethics

All experimental protocols were approved by the ethics committees of the University of Adelaide in accordance with the guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council.

## Mouse Strains

The C57BL/6 (WT) mice (Jackson stock number: 000664) were used as control mice for experiments detailed in chapters 3 and 4. For experiments detailed in chapter 5, all tests were conducted on this strain of mouse and the mice were allocated different groups based on specific drug treatments. These mice were purchased from the University of Adelaide breeding facility, Adelaide SA.

To understand how targeting TNF- $\alpha$  expression effects behaviour in young and older mice gene-targeted C57BL/6 mouse strain deficient for TNF- $\alpha$  (TNF<sup>-/-</sup>) were generated on a genetically pure C57BL/6 background as previously described (Korner et al., 1997a) and backcrossed for over 10 generations on a C57BL/6 background. This strain was used for studies described in chapters 3 and 4.

Chapters 3 and 4 also required the use of animal models that targeted each of the TNF- $\alpha$  receptors. TNF-R1<sup>-/-</sup> (Jackson stock number: 003242) (Peschon et al., 1998) and TNF-R2<sup>-/-</sup> mice (Jackson stock number: 002620) (Erickson et al., 1994) were both obtained from Jackson Laboratories (Bar Harbor, ME) and were also established on a C57BL/6 background and backcrossed for over 10 generations; these strains were used in previous published studies conducted within this research group (Baune et al., 2008). These mice were used to test the consequences of

targeting individual TNF- $\alpha$  receptors on behaviour and biology of the brain in both, young and adult mice.

All mice strains were inbred in house for over 10 generations on a C57BL/6 background. Mice were housed in groups of three to six mice per cage during the experimental period, with food and water available *ad libitum* and were handled prior to behavioural testing to minimize anxiety during the testing period. Ambient temperature of the housing and testing rooms was  $22 \pm 1^\circ\text{C}$ . Mice were housed under a 12 hr light-dark cycle, lights on at 0700 hr, and all behavioural testing was conducted between 0800 hr and 1600 hr. Details of the strains and number of mice used in each study have been outlined in Table 2.1

For the experiments detailed in chapter 3, 3 month old mice of all strains were used. This age group was selected in order to understand the early developmental effects of altered TNF- $\alpha$  and TNF- $\alpha$  receptor signalling. The experiments outlined in chapter 4 were conducted as an expansion study on an older subset of the same strains used in chapter 3 (6 month old mice). This was done in order to assess whether ageing mice would alter any of the behavioural and neurobiological phenotypes that were observed in the younger subset of mice (3 month old).

The short study carried out and detailed in chapter 5 was conducted using young adult, 3 month old mice, again to gain an understanding of the implications of pharmacological blockade at a young age.

Due to poor breeding in certain cases within the different strains of mice used in this body of work, as well as attrition of mice, the numbers of mice used were not always similar. Despite this, efforts were made to keep the numbers of mice consistent across studies.



Study (Chapter)	Mouse Strain Used	Number of Mice	Genotype	Phenotype	Age at which Analysed	Pharmacological Treatment
<b>Chapter 3: TNF-<math>\alpha</math> and Its Receptors Modulate Behaviour and Neurotrophins in Knockout Mice</b>	1. TNF-/- 2. TNF-R1-/- 3. TNF-R2-/- 4. C57BL/6	1. 14 2. 14 3. 14 4. 21	1. TNF- $\alpha$ gene deleted 2. TNF-R1 gene deleted 3. TNF-R2 gene deleted 4. Normal wild type mice	1. Do not express TNF- $\alpha$ 2. Do not express TNF-R1 3. Do not express TNF-R2 4. Express all cytokines and receptors normally	3 months of age for all strains.	NO
<b>Chapter 4: TNF-<math>\alpha</math> and its Receptors Govern Adult Behavioural Phenotypes</b>	1. TNF-/- 2. TNF-R1-/- 3. TNF-R2-/- 4. C57BL/6	1. 14 2. 9 3. 14 4. 14	1. TNF- $\alpha$ gene deleted 2. TNF-R1 gene deleted 3. TNF-R2 gene deleted 4. Normal wild type mice	1. Do not express TNF- $\alpha$ 2. Do not express TNF-R1 3. Do not express TNF-R2 4. Express all cytokines and receptors normally	6 months of age for all strains.	NO
<b>Chapter 5: Centrally Administered Etanercept on Behaviour Following a Peripheral Immune</b>	C57BL/6 in 4 groups 1. Sal-aCSF 2. LPS-aCSF 3. Sal-Etan	1. 10 2. 10 3. 10 4. 10	<ul style="list-style-type: none"> <li>All Normal wild type mice- C57BL/6</li> </ul>	<ul style="list-style-type: none"> <li>Express all cytokines and receptors normally</li> </ul>	Analysed at 3 months of age	1. Treated with Saline and Artificial CSF) 2. Treated with LPS and Artificial CSF

<b>Challenge</b>	4. LPS-Etan					3. Treated with Saline and etanercept 4. Treated with LPS and etanercept
<b>Chapter 6: Characterization of a TNF-<math>\alpha</math> over- expressing strain</b>	1. GFAP- TNF+/+ 2. C57BL/6	1. 14/age group 2. N= 21 (3 months), n=14 (7 months	1. Over-expression of astrocytic TNF- $\alpha$ 2. Normal wild type mice	1. Over-expression of astrocytic TNF- $\alpha$ 2. Express all cytokines and receptors normally.	Analysed at 3 and 7 months of age	NO

Table 2.1: Table summarising the different strains and treatment groups of mice used in the studies outlined in chapters 3-6

## **Experimental Procedures**

### ***Anaesthetics***

#### **Isoflurane**

The experiments detailed in chapter 5, required mice to be anaesthetised during administration of etanercept or artificial CSF in the cerebellar ventricles. In order to perform the procedure anaesthesia was induced by placing mice in a transparent plastic induction chamber with Isoflurane (purchased from Laboratory Animal Services at the University of Adelaide) delivered at 2 % in 800 ml/min O<sub>2</sub> for mice. Maintenance was achieved via a nose cone covering the nose and mouth with 1.5 % Isoflurane at 1 % at 800 ml/min O<sub>2</sub> for mice.

#### **Pentobarbital**

For tissue and serum collection from mice, animals were first anaesthetised using Pentobarbital (300 mg/ml) (Troy Laboratories Pty Ltd. NSW). Pentobarbital was purchased from Laboratory Animal Services at the University of Adelaide and stored at room temperature. Animals requiring perfusion and fixation were administered 0.2-0.4 ml of pentobarbital by intraperitoneal injection (IP) via a 25 gauge needle until all responses to pain were abolished. This included checking the animals pedal withdrawal and corneal reflexes.

## **Drugs**

### **Lipopolysaccharide (LPS)**

Cell walls of gram negative bacteria are made up of LPS and it has been used extensively to mimic infection in animal studies (Bilbo and Schwarz, 2012). LPS triggers an immune response activating Toll-like receptors, as well as increasing the expression of chemokines and cytokines like TNF- $\alpha$ , IL-1 $\beta$ , CXCL1 among others, making it an interesting candidate to study immune responses (Golan et al., 2005, Ortega et al., 2011). To induce a systemic inflammatory reaction for experimental procedures detailed in chapter 5, LPS from *Escherichia coli* (Sigma Chemical Co., St. Louis, MO; 0111:B4) was diluted to 1 mg/kg in saline and injected (IP) at 1400 hr. Control mice were injected with saline only by IP injection. The numbers of mice that were treated with LPS are detailed in chapter 5.

### **Etanercept**

To understand the effects of pharmacological blockade of TNF- $\alpha$  on the behaviour and neurobiology of mice an anti-TNF- $\alpha$  drug (etanercept) was used in the study detailed in chapter 5. Etanercept is a fusion protein of TNF- $\alpha$  receptor type 2 (TNF-R2) coupled to the Fc fragment of human IgG (Grattendick et al., 2008, Tan et al., 2012) (Etanercept; Enbrel, Wyeth-Ayerst laboratories). Etanercept can bind to both sTNF as well as tmTNF in addition to members of the lymphotoxin family (Thalayasingam and Isaacs, 2011). It is currently being used in the treatment of rheumatoid arthritis (Kekow et al., 2011). Clinical studies into the effects of this drug as a treatment to Alzheimer's disease are presently underway (Tobinick et al., 2006). In the study detailed in chapter 5, I sought to understand not only the implications of

this treatment on cognition-like behaviour, but also on the lesser explored area of anxiety-like behaviour, as well as understanding the implications of TNF- $\alpha$  blockade on expression of TNF- $\alpha$  and its receptors. Due to the large size of etanercept it is unable to cross the blood brain barrier, as such the study required the use of surgery to inject the drug directly into the cerebellar ventricles. Etanercept (25 mg/Kg) was diluted 1:10 in artificial cerebrospinal fluid (aCSF – Composition detailed in Table 2.2) and a total volume of 2  $\mu$ l was injected by intracerebroventricular injection (ICV) as previously established (Nilsberth et al., 2009).

**Table 2.2: Composition of artificial cerebrospinal fluid**

	<b>WORKING CONC (mm)</b>	<b>1X 1LITER</b>	<b>10X 1LITER</b>
<b>NaCl</b>	125	7.305	73.05
<b>KCl</b>	2.5	0.186	1.86
<b>MgCl<sub>2</sub>.6H<sub>2</sub>O</b>	1	0.2033	2.033
<b>NaH<sub>2</sub>PO<sub>4</sub></b>	1.25	0.15	1.5
<b>CaCl<sub>2</sub>.2H<sub>2</sub>O</b>	2	0.294	2.94
<b>NaHCO<sub>3</sub></b>	25	2.1025	21.025
<b>Glucose</b>	25	4.505	45.05
<b>pH with NaOH to 7.3</b>			

**Legend:** The table details the composition of artificial cerebrospinal

18 hr post LPS/saline administration, mice were anaesthetised with isoflurane (1%), mounted in a stereotaxic frame, and kept at 37°C through a feedback controlled heating pad. A 0.3 mm hole was made at the point relative to Bregma: 1 mm to the right and 0.5 mm posterior to allow injection into the lateral ventricle. A 33 gauge needle connected to a 5 µl Hamilton syringe was then lowered 2.5 mm and either etanercept or an equal volume of aCSF (2 µl) injected at a rate of 0.5 µl/min. The needle was then left in place for 2 min, before it was removed and the skin sutured. Mice were then placed in their home-cage on a heat pad to recover, with all animals awake within 5 min after the injection. Once mice had regained normal mobility they were allowed unlimited access to food and water and checked regularly for 24 hr to ensure there were no adverse effects from surgery. Due to the quick turnover of the cerebrospinal fluid (Saunders et al., 1999), behavioural testing commenced 24 hr post-surgery and mice were given up to 2 hr of rest between each test (as each test is run for 5-10 min). Mice were sacrificed immediately post behavioural testing and tissue was collected immediately. Table 2.3 details the experimental schedule.

**Table 2.3: Weekly dosing and testing schedule for experiments detailed in chapter 5**

Day 1	Day 2	Day 3
LPS/ Saline Dosing	etanercept /aCSF	
	Treatment by ICV	OFT (9-10am)
	injection	
		EZM (11-12 am)
		Y maze (1-3pm)
		Tissue Collection

**Legend:** The table lists the testing schedule for experiments in chapter 5

## **Behavioural Analysis**

All studies conducted within this PhD were carried out by first, conducting a behavioural assessment on all mice and then subsequent neurobiological assessments were carried out on tissue collected from these mice. Behaviour was evaluated to understand the effects of genetic deletion (chapters 3 and 4) and pharmacological blockade (chapter 5) on normal behavioural phenotypes such as locomotor activity, social behaviour and exploration as well as on more specific phenotypes such as cognition-like behaviour and emotion-like behaviour.

A comprehensive behavioural battery was carried out to assess baseline motor activity, anxiety-like behaviour, depressive-like behaviour, sociability and cognition-like behaviour. Mice were given at least one day off to rest and recover between tests and to reduce anxiety from continuous handling by experimenters. Mice were returned back to their home cage on completion of the test (most tests were run for 5-10 min, details explained in Table 2.6). The testing schedule for experiments detailed in chapters 3, 4 and 6 is explained in Table 2.4. Table 2.3 explains the testing schedule of the study examined in chapter 5. Tests were conducted in order of least to most stressful, to minimise any effects of previous testing (Lad et al., 2010). A single test was not repeated in the same mouse.



**Table 2.4: Behavioural testing schedule for chapters 3, 4 and 6**

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday/Sunday
<b>Week 1</b>	Home Cage	Rest/Recovery Day	OFT	Rest/Recovery Day	Y maze	Rest/Recovery Day
<b>Week 2</b>	Hole Board Exploration	Rest/Recovery Day	Elevated Zero Maze	Rest/Recovery Day	Sociability Test	Rest/Recovery Day
<b>Week 3</b>	Barnes Maze Pre-training	Barnes Maze training Day 1	Barnes Maze training Day 2	Barnes Maze training Day 3	Barnes Maze training Day 4	Barnes Maze Probe trial x 2 Small room
<b>Week 4</b>	Forced Swim Test	Tissue Collection				

**Legend:** The table shows the behavioural testing schedule for experiments detailed in chapters 3, 4 and 6. Mice were tested in groups of 21 over a period of 4 weeks.

Equipment for all tests was purchased from Stoelting Co. (USA) and all movements were tracked using AnyMaze video tracking Stoelting Co. (USA). Parameters that were of interest to each test were generally tracked by the software and raw data was generated by AnyMaze in the form of distance travelled (cm), time spent in the various zone of the apparatus (s) or as latency to particular zones of interest (s). Some tests however required additional manual recording, such as number of head pokes for HBE and time spent in various behaviour such as head dipping and stretch/attend postures for the EZM (s), due to low sensitivity of the software. Table 2.5 summarizes the parameters that were tracked by AnyMaze or that were manually recorded.

**Table 2.5: AnyMaze measures tracked for all behavioural tests**

<b>Test</b>	<b>Parameters Measured</b>	<b>Automatic Tracking by AnyMaze</b>	<b>Manual Recording by Experimenter</b>
<b>Home Cage</b>	Distance Travelled	Yes	
<b>Open Field Test</b>	Distance Travelled	Yes	
<b>Hole Board Exploration Test</b>	Number of Head Pokes		Yes
<b>Elevated Zero Maze</b>	1. Distance Travelled 2. Time In Open Arms 3. Time In Stretch/Attend Posture 4. Time In Head Dipping Behaviour	1. Yes 2. Yes	3. Yes 4. Yes
<b>Y maze</b>	Time In Arms	Yes	
<b>Barnes Maze</b>	Latency To Escape Box	Yes	
<b>Sociability</b>	Time Spent Interacting With Stranger/Familiar		Yes

**Legend:** The Table summarises the measures that were automatically tracked using AnyMaze and those that were recorded by hand is also depicted.

### ***Baseline Locomotor Activity***

#### **Home Cage Locomotor Activity**

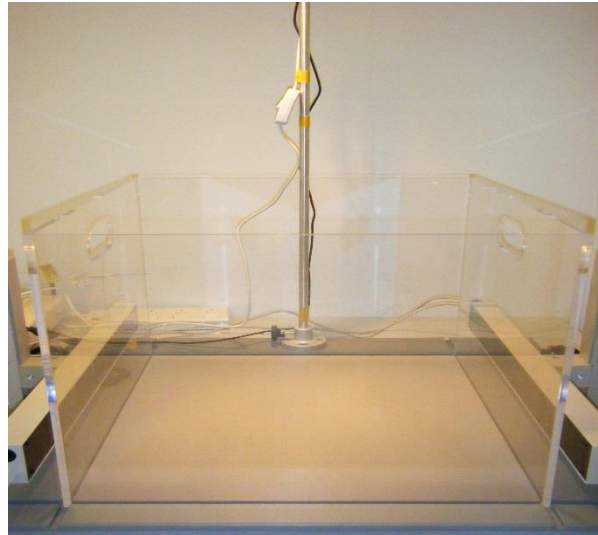
Chapters 3 and 4 detail the use of the home cage test. This test is used to track the movements of mice in a familiar setting (Simen et al., 2006). The test is used as a baseline assessment of locomotor activity, and is conducted to verify that differences observed in any tests measuring behavioural phenotypes are true results and not merely a difference in locomotor activity of the groups being tested (Baune et al., 2008, Camara et al., 2013).

Mice were individually tested for general locomotor activity in home cages with two day old bedding under basal non-stressful conditions according to previous published results within this research group (Baune et al., 2008, Camara et al., 2013). The total distance covered by mice over a 5 min period was measured and test mice were compared to WT mice. This was done with the specific aim of determining whether locomotor activity of test mice was normal in the context of the experimental design (Camara et al., 2013).

### Open Field Test (OFT)

The open field test was used as a second measure of baseline locomotor activity conducted under more stressful conditions (Simen et al., 2006) and documented in chapters 3-5. The Open Field Test is used to determine normal locomotor activity levels in mice and was used as a second measure to validate the observations recorded in the Home Cage test (Camara et al., 2013).

**Figure 2.1: Pictorial representation of the open field.**



**Legend: Mice are allowed to move freely within the chamber for 5min and behaviour is recorded.**

Mice were placed at the centre of a brightly lit plexiglass box (40 X 40 cm) and movements were tracked over a 5 min period, a protocol that was previously established within this research group (Baune et al., 2008). AnyMaze was used to track distance travelled by mice within this apparatus and test mice were compared to controls, to gauge normal locomotor behaviour.

## Cognition-like Behaviour

To understand the effects of imbalanced expression of TNF- $\alpha$  on cognition-like behaviour, two robust methods were incorporated – The Barnes Maze used as a measure of spatial learning and memory and the Y maze as a measure of short-term recognition memory.

### Spatial Memory and Learning:

Figure 2.2: Barnes maze

#### Barnes Maze (BM)

The Barnes Maze is an extensive test, conducted over a 5 day period, and measures spatial learning as well as spatial memory. The brightly lit chamber in which the test is conducted encourages the mouse to seek out the escape box, aided by visual spatial cues. The latency to the mouse over the 4 day training phase indicates the extent of



**Legend:** The picture shows the Barnes maze setup of circular table with holes at the periphery. In the above photograph. The mouse is seen entering the escape box.

spatial learning, where lower latency to the escape box is an indicator of better learning. The probe trial is the second stage of the test and this measures the spatial memory of mice by changing the original position of the escape box. The latency of mice to the original position is taken as a measure of memory consolidation. Baseline testing run in the Home Cage and OFT tests, rule out the possibility of

differences in motor activity in this test so as to not confound cognitive measures as merely a difference in locomotion.

The Barnes maze consists of a bright, circular white platform (91 cm diameter) with 20 holes, which contain either false boxes, or one hidden escape box. The false boxes remove visual cues that might be observed through an open hole. BM procedures were carried out over a 6 day period and run in 3 stages. This protocol was previously established within this group and the details of the methodology are outlined below (Baune et al., 2008).

***Pre-training (Day 1):*** This first phase was run to teach mice to navigate the BM apparatus and locate the escape box and to facilitate learning the training phase. Mice were pre-trained to enter the escape box, first by placement of the mouse into the escape box for 2 min, then by guidance to the escape box before being left for 2 min in the escape box, and finally placement outside the escape box within a glass chamber for up to 3 min, at the end of which the mice were again placed into the escape box for 2 min.

***Training (Day 2-5):*** This second phase of the BM was run over 4 days and was used to measure the level of spatial learning in mice. Mice were first placed in the centre of the maze under a removable chamber, which was then lifted and the mice given 3 mins to locate the escape box. Mice that failed to enter the escape box within 3 min were guided to the box and placed there for 2 min prior to returning to their home cage. Each mouse was subjected to 4 trials per day, separated by 15 min, for 4 days. The average of the time taken to find the escape box across the 4 day period was then calculated. Mice that failed to enter the escape box during the trial period were given a test time of 180 s during analysis, to demonstrate that they

failed to locate the position of the escape box. Lower latencies to the escape box are a representation of learning.

***Probe trial (Day 6):*** This final stage of the test was conducted to measure spatial memory in mice. In the probe trial the escape box rotated is rotated 90° from its original position. Each mouse was given two probe trials of 3 min duration each and latency to the old and new escape locations was recorded. Spatial memory for the (known) old escape box was considered as the tendency of the mice to explore the old escape box in the probe trial instead of exploring the new (unknown) box location. Thus, short latencies to locate the old escape location were considered to indicate spatial memory retention for the original location.

## Spatial Recognition Memory: Y

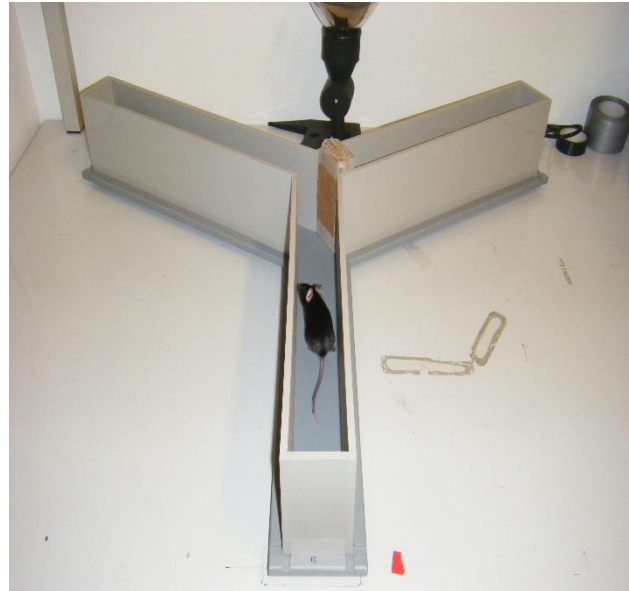
### Maze

This was another test used to measure spatial recognition memory. The Y maze is used specifically to assess hippocampal spatial recognition memory. The apparatus for this test consists of a three-armed chamber, with the arms at a 120° angle from each other. Each arm is 35 cm long, 5.0 cm wide and 10 cm high (Choy et al., 2008). The test was run in 2 phases to measure spatial recognition memory and is considered a robust method for measuring this behaviour (Dellu et al., 1992).

**Phase 1:** This initial phase was conducted to habituate mice to the new environment. One arm of the maze is closed off and mice are placed at the bottom of the “start” arm and allowed to explore the two arms for 15 min.

**Phase 2:** This second phase was used to measure the spatial recognition memory in mice. In chapter 3, Phase 2 took place 24 hr after phase one (Camara et al., 2013) to measure the effect on memory over a longer period, whilst in chapters 4 and 5 the second phase was conducted with a 1 hr delay after the first phase. This was done to assess short term spatial memory by following protocols that were

Figure 2.3: Y maze



**Legend:** The mouse can be seen in the start arm, one arm of the maze has been blocked off in this phase and will be made available to the mouse in the second part of the test in order to test spatial recognition memory.



previously established by assessing short term memory after a 1 hr delay (Dellu et al., 2000, Ma et al., 2007).

In this phase mice were again placed at the bottom of the start arm, with all three arms now open to explore over a 10 min period. As mice have a preference for exploring novel environments (Dulawa et al., 1999), mice generally spend more time exploring the novel arm over the familiar arm, indicating normal spatial recognition memory. However, impaired mice will not recognise the familiar arm and spend a similar amount of time in the familiar and novel arms or more time in the familiar arm. As all mice started in the same arm (start arm) time spent in this arm was not analyzed.

### ***Exploratory Activity:***

#### **Hole Board Exploration Test (HBE)**

The hole-board exploration test was used to measure exploratory behaviour in rodents; and the head dipping behaviour taken as an indicator of exploration, is considered independent of general

**Figure 2.4: Hole board exploration apparatus**



**Legend: The photograph shows the mouse poking its head into one of the 16 holes on the apparatus**

locomotion (File and Wardill, 1975a). While this test is more conventionally used to study the effects of drugs on exploration (Takeda et al., 1998, Kliethermes and

Crabbe, 2006), in my studies I have used this test to study the effects of up and down-regulation of TNF- $\alpha$  expression, as well as targeted TNF-R expression on exploratory behaviour. The apparatus of this test is similar to the open field test, and utilises a hole-board insert, which has 16 holes of 1.5 cm in diameter, spaced 6.0 cm apart. Mice were placed into the apparatus and allowed to explore for 5 min. The number of head dips into the holes was manually recorded and taken as an indicator of exploration (File and Wardill, 1975b, Hart et al., 2010).

### ***Social Behaviour:***

#### **Sociability Test**

The sociability test measures general social behaviour and interest in social novelty in rodent models. Normal rodents have a preference for social interaction with other rodents and will also prefer to interact with a novel mouse over a familiar one (Moy et al., 2004). This test was conducted to measure social behaviour in mice with knockout

**Figure 2.5: Sociability test apparatus**



**Legend: The photograph shows the social novelty phase of the test. The test mouse is allowed to move freely between the 3 chambers and either chose to spent time interacting with the familiar or novel mouse or spend time in the empty chamber**

of TNF- $\alpha$  and its receptors, or mice with over-expression of TNF- $\alpha$  against normal WT mice. The apparatus for this test consists of a three chamber rectangular box,

the walls of which are clear plexiglass. Each chamber is 20 cm in length, 40.5 cm in width and 22 cm high and the test runs in three stages (Moy et al., 2004, Camara et al., 2013).

**Habituation:** This phase was run to familiarise the mouse with the environment. The test mouse was placed in the central chamber and allowed to explore for 5 min. Doors to the other chambers were sealed off in this stage.

**Sociability:** This stage was run to measure normal social behaviour and conducted immediately after the habituation stage. A stranger mouse (a WT mouse of the same age and sex as the test mouse) was placed in one of the side chambers (either left or right, this is alternated to remove preference for a particular side) in a small round wire cage that allowed nose contact between the bars but prevented fighting. Doors to all chambers were opened and the test mouse allowed to explore the entire box for 5 min. The time spent by mice interacting with the stranger mouse vs. the empty cage was recorded, and more time spent with the stranger mouse is considered normal social behaviour.

**Social Novelty:** This stage was run to test the willingness of mice to explore socially novel situations. For this, a second novel mouse was placed in the chamber that was empty during the sociability phase (novel mouse is also a WT mouse of the same age and sex as the test mouse). The test mouse was allowed to explore the box for 5 min and the time spent interacting with the novel mouse over the familiar (stranger) mouse recorded. Increased time spent with the novel mouse over the familiar mouse is an indicator of normal social novelty behaviour.

***Anxiety-like Behaviour:*****Elevated Zero Maze (EZM)**

The elevated zero maze is used as a measure of anxiety-like behaviour. The apparatus consists of a grey Perspex circular platform of 50 cm diameter and width of 5 cm, located at a height of 40 cm above ground. The platform is divided into 4 equal quadrants, two closed quadrants with grey Perspex walls (27 cm high) on the inside and outside,

and two open quadrants without walls. Mice were placed at the centre of either one of the open arms and allowed to explore the apparatus for 5 min. Movement and time spent in open and closed quadrants was recorded as a measure of anxiety-like behaviour. Anxiety is measured primarily by comparing the time spent in the open versus closed quadrants with an anxious mouse spending more time in the closed quadrants. However other measures such as distance travelled by mice and the time spent in head dipping behaviour and in stretch/attend postures are also considered measures of anxiety-like behaviour (Cryns et al., 2007).

**Figure 2.6: Elevated zero maze**

**Legend: Mice are placed in the centre of the apparatus and are allowed to move between the open and closed arms. The figure shows a mouse in the closed arm moving towards a stretch posture.**

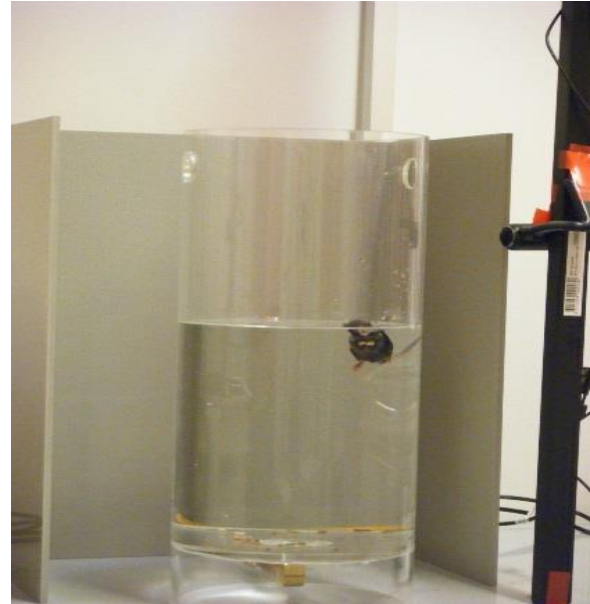
## ***Depression-like Behaviour***

### **Forced Swim Test (FST)**

The forced swim test is a measure of despair and depression-like behaviour, and was conducted according to protocols adapted by Petit-Demouliere et al as described (Petit-Demouliere et al., 2005). Mice were placed in a 4 L cylinder (20 cm diameter) filled up to 20 cm with water (23-24°) for 6 min and their movement was tracked. Time immobile was considered a measure of depression-

like behaviour, and was defined as lack of motion beyond which was required for the mouse to keep its head above water. For analysis, the first 2 min were excluded, since mice are mobile during that time and familiarizing themselves with the environment and any immobility during this time therefore cannot be attributed to despair (Camara et al., 2013).

**Figure 2.7: Forced swim test apparatus**



**Legend: The time spent by mice being immobile is considered as a measure of depression-like behaviour.**

Table 2.6: Measures and duration of all behavioural tests.

Test	Measure	Stages	Duration	Apparatus
<b>Home Cage</b>	Baseline Locomotor Activity	Single stage	5 min	Home Cage
<b>Open Field Test</b>	Locomotor Activity in a stressful environment	Single Stage	5 min	Plexiglass Box
<b>Hole Board Exploration</b>	Exploratory behaviour	Single Stage	5 min	Plexiglass Box with hole insert
<b>Barnes Maze</b>	Spatial learning and memory	<b>Training:</b> 3 stage per day repeated over 4 days  <b>Probe Trial :</b> 2 stages	Each stage is run for 3 min	Circular table
<b>Y maze</b>	Spatial Recognition memory	2 stages <ul style="list-style-type: none"> <li>• <b>Stage 1:</b> 2 arm access</li> <li>• <b>Stage 2:</b> 3 arm access</li> </ul>	<ul style="list-style-type: none"> <li>• <b>Stage 1:</b> 10 min</li> <li>• <b>Stage 2:</b> 5 min</li> </ul>	3 chambered apparatus with arms at 120° angles
<b>Sociability</b>	Social behaviour	3 Stages <ul style="list-style-type: none"> <li>• <b>Habituation</b></li> <li>• <b>Sociability</b></li> <li>• <b>Social Novelty</b></li> </ul>	Each stage runs for 5 min	3 roomed chamber
<b>Elevated Zero Maze</b>	Anxiety-like behaviour	One Stage	5 min	Perspex circular platform
<b>Forced Swim Test</b>	Depression- like behaviour	One Stage	6 min	Glass Cylinder filled with water

**Legend:** The table details the various behavioural tests and their measures used in the different chapters, along with the duration of each test and the apparatus used.

## **Biological Analysis**

Post behavioural assessments, all mice were sacrificed and tissue collected for biological analyses. For experiments detailed in chapters 3-5, a subset of mice were used for ELISA analysis while tissue from another subset of mice were collected for immunohistochemistry (IHC) (explained in Table 2.7).

**Table 2.7: Number of mice used in each of the biological studies described in this thesis**

Study	Mouse Strain/ Treatment Group	No of mice for Protein Analysis	No of mice for IHC	N of mice for qPCR
Chapter 3: TNF- $\alpha$ and Its Receptors Modulate Behaviour and Neurotrophins in Knockout Mice	a. TNF-/- b. TNF-R1-/- c. TNF-R2-/- d. C57BL/6	7 per strain	7 per strain	n/a
Chapter 4: TNF- $\alpha$ and its Receptors Govern Adult Behavioural Phenotypes	a. TNF-/- b. TNF-R1-/- c. TNF-R2-/- d. C57BL/6	7 per strain	a. 5 b. 4 c. 5 d. 5	4 per strain
Chapter 5: Centrally Administered Etanercept on Behaviour Following a Peripheral Immune Challenge	<b>C57BL/6 in 4 groups</b> a. Sal-aCSF b. LPS-aCSF c. Sal-Etan d. LPS-Etan	n/a	5 per group	5 per group
Chapter 6: Characterization of a TNF- $\alpha$ over-expressing strain	a. GFAP-TNF+/+ b. C57BL/6	7 per strain	n/a	4 per strain

**Legend:** The table shows how the subsets of mice that underwent behavioural testing that were then sacrificed for biological analysis.



## ***Protein Analysis***

### **Tissue Processing**

In order to evaluate whether there were any underlying changes in neurotrophin levels and expression of TNF- $\alpha$  within the TNF- $\alpha$  transgenic/knockout mice for the experiments detailed in chapters 3 and 4, a subset of mice of each strain that underwent behavioural testing ( $n = 7$  mice), were used for protein analysis. Mice were sacrificed by Pentobarbital overdose given through IP injection and once all reflex to pain were abolished, mice were decapitated and their brains were rapidly removed. The hippocampal formation and prefrontal cortex (PFC) tissue were isolated and stored fresh frozen at  $-80^{\circ}\text{C}$  until required. The hippocampus and prefrontal cortex were chosen for their role in cognition and emotion-like behaviour. The hippocampus plays a role in spatial cognition (Sweatt, 2004) as well as some emotional responses (Sweatt, 2004), while the PFC plays a role in cognitive control (Miller and Cohen, 2001) and goal oriented emotional processing (Davidson, 2002).

For the experiments detailed in chapter 5, following completion of behavioural testing mice were sacrificed and blood collected by means of cardiac puncture. Serum was then extracted by spinning blood down at 2500 rpm for 15 min and stored at  $-80^{\circ}\text{C}$  until analysis of TNF- $\alpha$  levels by ELISA.

### **Protein Extraction**

Tissue samples were thawed and placed on ice. Samples were then weighed and 10x homogenisation buffer (50 mL tris-buffered saline (TBS), one protease inhibitor tablet (Roche) and 50  $\mu\text{L}$  of Triton-X) was added to the samples in a mortar. Samples were then thoroughly homogenised by a pestle, transferred to an eppendorf

tube and then centrifuged at 8500 rpm for 15 min at 4°C. Supernatant was collected and stored for at -20°C until required.

### **Protein Quantification**

A 15 µl aliquot of sample was used to estimate protein concentration against a standard curve derived from serial dilutions of bovine serum albumin (BSA) (Sigma A21543). 5 µl of supernatant or BSA was added to the recommended amounts of Biorad protein assay reagents (500-0113, -0114 and 0115). Absorbance was read at 620 nm, with each sample run in triplicate and the readings averaged. Each sample was then diluted with TBS to achieve a final concentration of 50 ng protein per 100 µl of TBS.

### **Enzyme Linked Immunosorbent Assay (ELISA)**

To test how neurotrophin expression was altered with up and down-regulation of TNF- $\alpha$  expression as well as by targeted deletion of TNF- $\alpha$  receptors for experiments detailed in chapters 3, 4 and the appendix, NGF and BDNF ELISAs were performed on brain homogenates. E<sub>max</sub> Immuno Assay system (Promega) for NGF and BDNF were used. Protocol according to the manufacturer's instructions was followed (Sei et al., 2000, Gilmore et al., 2003).

To test the peripheral expression of TNF- $\alpha$  post LPS and etanercept treatment, serum from mice that were used in the study detailed in chapter 5, TNF- $\alpha$  ELISA (eBiosciences mouse TNF- $\alpha$  Ready Set Go Kit) was used according to manufacturer's instructions.

## NGF ELISA

To run the NGF ELISA E<sub>max</sub> Immuno Assay system (Promega), Nunc, Maxisorp plates were coated with 100µl/well mixture of carbonate coating buffer and anti-NGF polyclonal antibody, sealed and incubated overnight at 4°C. Plates were then washed once with wash buffer (TBS with 1% Triton X). 200 µl/well of Block and Sample buffer (prepared as per manufacturer's instructions) was added and incubated for 1hr at room temperature. Plates were washed once again and standards added by serial dilution of top standard as per instructions. 100 µl of diluted samples (brain homogenates) were then added in duplicate and incubated for 6 hr in a 37°C oven. Plates were then washed 5 times and plates coated with secondary antibody and incubated overnight at 4°C.

Plates were then washed 5 times the next day and 100µl of HRP Conjugate was added per well and incubated for 2.5 hr at room temperature. After washing 5 times 100 µl/well room temperature TMB One Solution was added. Reaction was stopped with 100 µl/well 1N hydrochloric acid and absorbance read at 450 nm (Millenium Science, BioTek plate reader)

## BDNF ELISA

To run the BDNF ELISA E<sub>max</sub> Immuno Assay system (Promega), Nunc, Maxisorp plates were coated with 100 µl/well mixture of carbonate coating buffer and Anti-BDNF monoclonal antibody sealed and incubated overnight at 4°C. Plates were then washed once with wash buffer (TBS with 1% Triton X). 200 µl/well of Block and Sample buffer (prepared as per manufacturer's instructions) was added and incubated for 1hr at room temperature. Plates were washed once again and

standards added by serial dilution of top standard as per instructions. 100 µl diluted samples (brain homogenates) were then added in duplicates and incubated for 6 hr in a 37°C oven. Plates were then washed 5 times and plates coated with secondary antibody and incubated overnight at 4°C. Plates were then washed 5 times the next day and 100µl of HRP Conjugate was added per well and incubated for 2.5 hr at room temperature. After washing 5 times 100 µl/well room temperature TMB One Solution was added. Reaction was stopped with 100µl/well 1N hydrochloric acid and absorbance read at 450 nm.

### **TNF-α ELISA**

The TNF-α ELISA was performed using the eBiosciences mouse TNF-α Ready Set Go Kit. Nunc, Maxisorp plates were coated with 100 µl/well mixture of coating buffer prepared as per manufacturer's instructions and was sealed and incubated at 4°C overnight. The next morning, wells were aspirated and washed 3 times with wash buffer). 1: The standard curve was prepared by making 2 fold serial dilution of the top concentration. 100 µl of diluted samples (brain homogenates or serum samples) were then added in duplicate and the sealed plates were left to incubate overnight at 4°C. On day 3, the wells were aspirated, washed 3 times and then 100 µl of detection antibody was added to all wells (prepared as per kit instructions). This was allowed to incubate at room temperature for 1hr. Wells were then aspirated and washed 3 times before adding 100 µl Avidin-HRP. This was allowed to incubate at room temperature for 30 min after which a final aspirate-wash step requiring 5 washes was carried out. 100 µl of substrate solution was then added and incubated for 15 min before stopping the reaction with 50 µl H<sub>2</sub>SO<sub>4</sub> stop solution.

Absorbance was then read at 450 nm and amount of TNF- $\alpha$  in each sample calculated by comparing to the standard curve.

### ***Immunohistochemistry (IHC)***

#### **Tissue Fixing**

Animals were injected IP with pentobarbital until all corneal and motor reflexes were absent. The peritoneal cavity was then exposed and diaphragm incised laterally and the thorax cut bilaterally. 10% neutral buffered formalin was then injected into the left ventricle via a 23 gauge needle. Animals were then decapitated 30 min later to minimise damage to blood vessels. The brains were extracted and placed in formalin to be processed after 24 hr.

#### **Tissue Processing**

Mice brains were cut into 3 coronal slices of (4 mm each) and placed in cassettes into neutral buffered formalin. Cassettes were then processed overnight accordingly: 20 min in ethanol baths of increasing concentration (50%, 70%, 80%, 95%, 100%, 100%), followed by 2 xylene baths (90 min) and then paraffin baths of increasing duration (30, 60, 60, 90 min). Brain slices were embedded in paraffin wax and 150  $\mu$ m sections of hippocampal tissue were sectioned with a microtome (Leica RM2245) and placed on to glass slides (Menzel-Glaser, Superfrost plus). Slides were then air dried and stored until required.

## Immunohistochemical Staining

For IHC staining the following steps were carried out;

1. Slides were de-waxed and dehydrated and endogenous peroxidase activity was blocked by incubation with 0.5% hydrogen peroxide in methanol for 30 min.
2. After this slides were washed with phosphate buffered saline (PBS; 2 x 3 min), before undergoing antigen retrieval (TRIS for Doublecortin, Citrate for other antibodies) by heating slides at boiling point for 10 min.
3. Slides were allowed to cool for an hour, washed in PBS (2 X 3 min) and then blocked with 3% normal horse serum (NHS) for 30 min.
4. Primary antibody was added and allowed to incubate overnight (Doublecortin 1:8000, Millipore, Ki67 1:2000, Abcam, IBA1 1:1000, Santa Cruz, GFAP 1:40,000, Dako, IL-1 $\beta$  1: 1000, R&D systems).
5. On the second day slides were washed with PBS (2 X 3 min) and the appropriate species of secondary antibody was added and incubated for 30 min (Abacus, :250) (Doublecortin: anti-guinea pig, Ki67, GFAP: anti-rabbit, IBA1, IL-1 $\beta$ : anti-goat). (Table 2.9)
6. Slides were washed again in PBS (2 X 3 min) and then incubated with streptavidin peroxidase for 1hr followed by another wash in PBS (2 X 3 min).
7. Sections were stained with Diaminobenzadine (DAB) (Sigma) and counter stained with haematoxylin prior to dehydration and mounting with DePex mounting media.

8. After allowing slides to air dry they were digitally scanned using the Nanozoomer (Hamamatsu) and viewed with the associated software (NDP view).
9. Serial images of the CA region of the hippocampus and the dentate gyrus of each section were captured as jpegs. These images were then exported to Image J (NIH) and the number of positive cells counted.

**Table 2.8: The table shows a list of the stains used for immunohistochemistry for the studies outlined in this body of work**

<b>Cells/Proteins of Interest</b>	<b>Primary Antibody</b>	<b>Dilution (In Normal Horse Serum)</b>	<b>Secondary Antibody</b>	<b>Retrieval Buffer</b>
<b>Microglia</b>	IBA1	1:1000	Anti-Goat	Citrate
<b>Astrocytes</b>	GFAP	1:40,000	Anti-Rabbit	Citrate
<b>IL-1<math>\beta</math></b>	IL-1 $\beta$	1:1000	Anti-Goat	Citrate
<b>Proliferating Cells</b>	Ki67	1:2000	Anti-Rabbit	Citrate
<b>Immature Neurons</b>	Doublecortin (DCX)	1:8000	Anti-Guinea Pig	TRIS

**Legend:** Details of the cells of interest, corresponding stains and the relevant secondary antibodies have been outlined. The table also gives details of the concentration of the antibodies required as well as the antigen retrieval solutions used for each.

## Statistical Analysis

Data analyses were carried out using GraphPad Prism software (version 5.01). All data are presented as mean  $\pm$  Standard error of mean (SEM). Data was checked for normal distribution (tested with Kolmogorov-Smirnov test) and based on this comparisons between strains were performed using either parametric measures or non-parametric analyses. ANOVA test (one-way or two-way ANOVA as required) followed by Bonferroni post hoc analysis was used as a parametric measure whilst, Kruskal-Wallis test followed by Dunn's post hoc analysis was used as non-parametric measures. A comparison of 2 parameters was carried out by unpaired T-test (parametric) or Mann-Whitney U test (non-parametric).  $P < 0.05$  was taken as significant for all tests.



# **Chapter 3 : TNF- $\alpha$ and Its Receptors Modulate Behaviour and Neurotrophins in Knockout Mice**

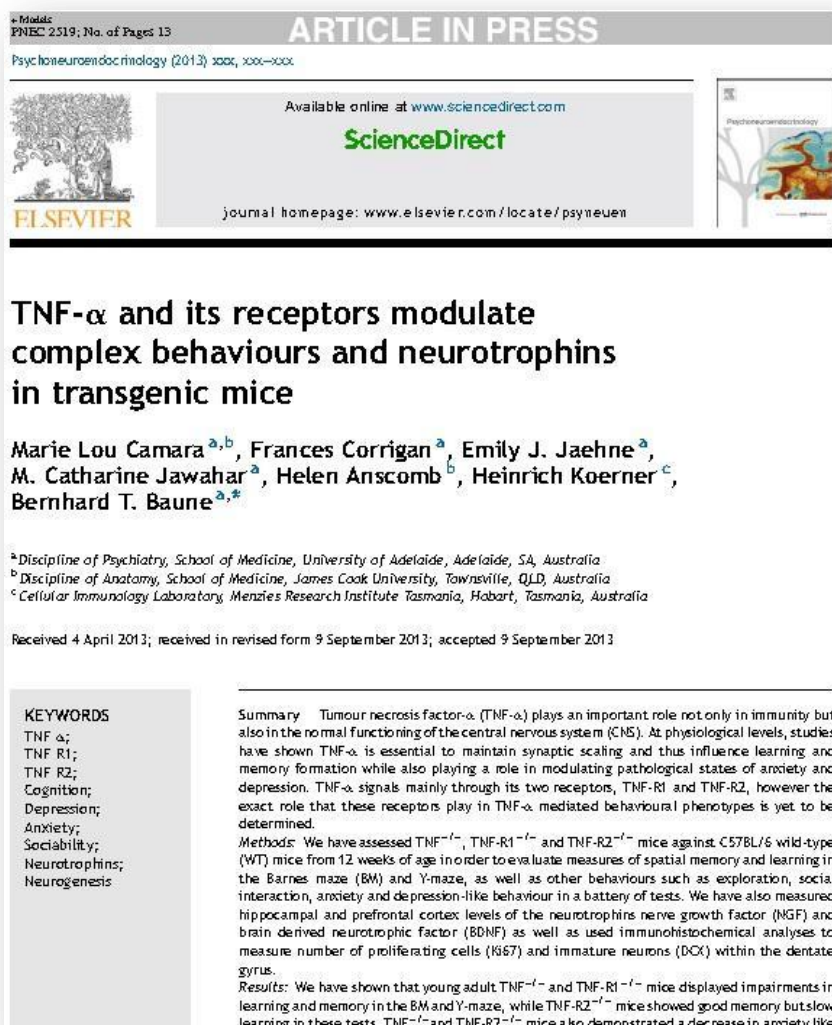
## TNF- $\alpha$ AND ITS RECEPTORS MODULATE COMPLEX BEHAVIOURS AND NEUROTROPHINS IN TRANSGENIC MICE

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## Overview

TNF- $\alpha$  plays an important role not only in immunity but also in the normal functioning of the CNS. At physiological levels, studies have shown TNF- $\alpha$  is essential to maintain synaptic scaling and thus influence learning and memory formation while also playing a role in modulating pathological states of anxiety and depression. TNF- $\alpha$  signals mainly through its two receptors, TNF-R1 and TNF-R2, however the exact role that these receptors play in TNF- $\alpha$  mediated behavioural phenotypes is yet to be determined. Therefore this study was carried out to

**AIM 1:** measure the effects of genetic deletion of TNF- $\alpha$  and its receptors on behavioural phenotypes as well as neurotrophin expression in young adult mice.

TNF<sup>-/-</sup>, TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice were tested against C57BL/6 wild-type (WT) mice from 12 weeks of age in order to evaluate measures of spatial memory and learning in the Barnes Maze (BM) and Y-maze, as well as other behaviour such as exploration, social interaction, anxiety and depression-like behaviour in a battery of tests. Levels of the neurotrophins nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) were measured in the hippocampal formation as well as the pre-frontal cortex. Additionally immunohistochemical analyses were conducted to measure number of proliferating cells (Ki67) and immature neurons (DCX) within the dentate gyrus.

The results of this chapter illustrate that young adult TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice displayed impairments in learning and memory in the BM and Y-maze, while TNF-R2<sup>-/-</sup> mice showed good memory but slow learning in these tests. TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice also demonstrated a decrease in anxiety like behaviour compared to WT mice. ELISA analyses showed TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice had lower levels of NGF

compared to WT mice. These results indicate that while lack of TNF- $\alpha$  can decrease anxiety-like behaviour in mice, certain basal levels of TNF- $\alpha$  are required for the development of normal cognition. Furthermore the results suggest that both TNF-R1 and TNF-R2 signalling play a role in normal CNS function, with knockout of either receptor impairing cognition on the Barnes Maze.

## Introduction

TNF- $\alpha$  is a pro-inflammatory cytokine showing biological effects in the central nervous system (CNS) (Stellwagen et al., 2005, McAfoose and Baune, 2009, Clark et al., 2010, Baune et al., 2012a, Baune et al., 2012b). In the CNS, TNF- $\alpha$  is produced by the resident immune cells of the CNS, the microglia and astrocytes, as well as by certain neuronal populations (Li et al., 2008, McCoy and Tansey, 2008). This cytokine is shown to cross the blood brain barrier (BBB) in conditions of systemic inflammation (Pan and Kastin, 2002) and is known to play a role in activating the hypothalamic pituitary adrenal (HPA) axis by stimulating the corticotrophin-releasing hormone and increasing levels of corticosterone (Bernardini et al., 1990). High levels of TNF- $\alpha$  in the dorsolateral prefrontal cortex have also been linked to major depressive disorder (Dean et al., 2010) and increased expression of TNF- $\alpha$  were also observed in murine models of depressive like behaviour and chronic mild stress (Kaster et al., 2011, You et al., 2011, Kaster et al., 2012).

Beyond its involvement in various CNS pathologies, TNF- $\alpha$  may be is required for maintaining certain physiological processes within the CNS (Stellwagen et al., 2005). TNF- $\alpha$  has been shown to play a role in mediating synaptic scaling (Stellwagen et al., 2005), and was found to be essential to early cognitive development (McAfoose et al., 2009). Constitutive levels of TNF- $\alpha$  are linked to increased neurogenesis (Iosif et al., 2006) and TNF- $\alpha$  is also shown to influence the expression of neurotrophins, such as nerve growth factor (NGF) (Takei and Laskey, 2008) and brain derived neurotrophic factor (BDNF) (Golan et al., 2004). Indeed, modulation of neurotrophins may explain the finding that young adult mice that lack

TNF- $\alpha$  (TNF<sup>-/-</sup> mice) exhibit cognitive impairment (Baune et al., 2008). Furthermore, TNF- $\alpha$  can play an integral part in modulating anxiety like behaviour as well as in fear conditioning (Yamada et al., 2000, Simen et al., 2006).

TNF- $\alpha$  signals through two main receptors, TNF-R1 and TNF-R2, which are thought to have disparate functions, with suggestions that activation of TNF-R1 is neurodegenerative, whilst activation of TNF-R2 is neuroprotective (Shu et al., 1996, Kassiotis and Kollias, 2001, Fontaine et al., 2002). This is because signalling through the TNF-R1 pathway, which primarily occurs via soluble TNF- $\alpha$ , initiates the activation of caspases leading to apoptosis, due to the presence of a cytoplasmic 'death domain'. In the brain, TNF-R1 is widely expressed by astrocytes, oligodendrocytes and microglia, whereas only microglial cells express TNF-R2 (Dopp et al., 1997). In immunologically unchallenged conditions TNF-R1 appears to be more abundantly expressed by neurons in the brain while basal levels of TNF-R2 are expressed only in the cortex, cerebellum and tegmentum (Bette et al., 2003). In contrast, activation of the TNF-R2 pathway, which primarily occurs via transmembrane TNF- $\alpha$ , promotes cell survival, through inhibition of caspase activation (Horiuchi et al., 2010). Intriguingly, the distinction between these two signalling pathways does not appear to be as clear cut as previously thought, with an earlier study demonstrating that TNF<sup>-/-</sup>, TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice all exhibited cognitive deficits (Baune et al., 2008), suggesting that signalling through the receptors is not exclusively neurodegenerative or neuroprotective.

Therefore, to further explore the role of TNF-R1 and TNF-R2 signalling in behavioural responses, an extensive behavioural battery was performed, assessing cognition-like behaviour (learning and memory), social interactions, exploratory

activity, anxiety-like behaviour and depression-like behaviour in young adult (12 week old) TNF<sup>-/-</sup>, TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> transgenic mice. Furthermore, to determine whether behavioural changes in these transgenic mice relate to underlying changes in neurotrophins, these parameters were also investigated. While previous studies have investigated the effects of genetic deletion of TNF- $\alpha$  and its receptors on cognition-like (Baune et al., 2008) and anxiety-like behaviour (Yamada et al., 2000, Simen et al., 2006), this is the first study to provide a comprehensive description of a wider range of behavioural phenotypes, including cognition-, anxiety- and depression-like behaviour extending into sociability and exploratory behaviour, governed by TNF- $\alpha$  and its receptors, while considering molecular mechanisms of neurotrophins and neurogenesis expression to influence such behaviour.

**Table 3.1: Summary of TNF- $\alpha$  and TNF- $\alpha$  receptor deficient behavioural studies**

Study	Test Mouse Strains (tested against WT mice)	Age and Sex of mice	Behavioural Tests	Findings (compared to WT control mice)
(Simen et al., 2006)	<ul style="list-style-type: none"> <li>• TNF-R1<sup>-/-</sup></li> <li>• TNF-R2<sup>-/-</sup></li> </ul>	<ul style="list-style-type: none"> <li>• Male mice</li> <li>• Age unknown</li> </ul>	<ul style="list-style-type: none"> <li>• Home Cage (HC) (red light)</li> <li>• Open field Test (OFT)</li> <li>• Elevated Plus Maze (EPM)</li> <li>• Forced Swim Test (FST) (15 min protocol)</li> <li>• Sucrose Preference</li> <li>• Fear Conditioning</li> </ul>	<ul style="list-style-type: none"> <li>• No difference in baseline locomotor activity in HC or OFT or in FST.</li> <li>• EPM – Non-significant increase in open arm entries in TNF-R2<sup>-/-</sup> mice.</li> <li>• Fear Conditioning – less freezing in TNF-R1<sup>-/-</sup> mice compared to WT mice.</li> <li>• Sucrose Preference- TNF-R2<sup>-/-</sup> mice showed increased sucrose consumption.</li> </ul>
(Kaster et al., 2012)	<ul style="list-style-type: none"> <li>• TNF-R1<sup>-/-</sup></li> </ul>	<ul style="list-style-type: none"> <li>• Male mice</li> <li>• 6 week old</li> </ul>	<ul style="list-style-type: none"> <li>• FST (6 min protocol)</li> <li>• OFT</li> </ul>	<ul style="list-style-type: none"> <li>• No differences in OFT.</li> <li>• TNF-R1<sup>-/-</sup> mice showed decreased immobility in FST.</li> </ul>



(Yamada et al., 2000)	<ul style="list-style-type: none"> <li>• TNF<sup>-/-</sup></li> </ul>	<ul style="list-style-type: none"> <li>• Unknown sex or age</li> </ul>	<ul style="list-style-type: none"> <li>• Water Finding</li> <li>• Diurnal Rhythm Home Cage (4 day test)</li> <li>• Rotarod</li> <li>• OFT</li> <li>• FST</li> <li>• EPM</li> </ul>	<ul style="list-style-type: none"> <li>• No difference in water finding task.</li> <li>• No difference in home cage activity (day or night).</li> <li>• Less exploration in open field.</li> <li>• Decreased immobility time in FST.</li> <li>• Decreased time in open arm in EPM.</li> </ul>
(Quintana et al., 2007)	<ul style="list-style-type: none"> <li>• TNF-R1<sup>-/-</sup></li> <li>• TNF-R2<sup>-/-</sup></li> </ul>	<ul style="list-style-type: none"> <li>• Unknown sex or age</li> </ul>	<ul style="list-style-type: none"> <li>• Hole Board Exploration (HBE)</li> <li>• EPM</li> </ul>	<ul style="list-style-type: none"> <li>• TNF-R1<sup>-/-</sup> and TN-R2<sup>-/-</sup> mice had increased ambulations in the HBE (outer quadrant only).</li> <li>• TNF-R2<sup>-/-</sup> mice showed increased head dipping and rearing in the hole board.</li> <li>• TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice displayed a trend of decreased time spent in the open arm of EPM but did not reach significance.</li> </ul>

(Gimsa et al., 2012)	<ul style="list-style-type: none"> <li>• TNF-R1<sup>-/-</sup></li> <li>• TNF-R2<sup>-/-</sup></li> <li>• TNF-R1/R2<sup>-/-</sup></li> </ul>	<ul style="list-style-type: none"> <li>• Male mice</li> <li>• 6 weeks old</li> </ul>	<ul style="list-style-type: none"> <li>• EPM</li> </ul>	<ul style="list-style-type: none"> <li>• TNF-R2<sup>-/-</sup> and TNF-R1/R2<sup>-/-</sup> mice spent significantly higher time in the open arm compared to WT mice.</li> <li>• TNF-R1<sup>-/-</sup> mice showed a non-significant trend of decreased time in the open arm.</li> </ul>
(McAfoose et al., 2009)	<ul style="list-style-type: none"> <li>• TNF<sup>-/-</sup></li> </ul>	<ul style="list-style-type: none"> <li>• Sex unknown</li> <li>• 3, 6 and 12 month old mice</li> </ul>	<ul style="list-style-type: none"> <li>• Barnes Maze (BM)</li> </ul>	<ul style="list-style-type: none"> <li>• TNF<sup>-/-</sup> mice showed increased latency to the escape box compared to WT mice at 3 months.</li> <li>• At 12 months TNF<sup>-/-</sup> mice showed decreased latency to escape box compared to WT mice.</li> <li>• Spatial learning was decreased in TNF<sup>-/-</sup> mice at 3 months of age but increased at 6 and 12 months of age.</li> </ul>
(Baune et al., 2008)	<ul style="list-style-type: none"> <li>• TNF<sup>-/-</sup></li> <li>• TNF-R1<sup>-/-</sup></li> <li>• TNF-R2<sup>-/-</sup></li> </ul>	<ul style="list-style-type: none"> <li>• Sex unknown</li> <li>• 8- 12 week old mice</li> </ul>	<ul style="list-style-type: none"> <li>• HC</li> <li>• OFT</li> <li>• Novel Object Recognition (NOR)</li> <li>• BM</li> </ul>	<ul style="list-style-type: none"> <li>• TNF<sup>-/-</sup> mice had decreased exploration in the home cage.</li> <li>• TNF-R1<sup>-/-</sup> mice had significantly decreased mobility in OFT, while TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice showed non-significant trends of decreased mobility compared to WT mice.</li> <li>• TNF<sup>-/-</sup> mice did not show a preference for the</li> </ul>

				<p>novel object in the NOR.</p> <ul style="list-style-type: none"> <li>Non-significant trend of increased retention memory was observed in TNF<sup>-/-</sup> mice and all mice showed impaired spatial learning.</li> </ul>
(Patel et al., 2010)	<ul style="list-style-type: none"> <li>TNF-R1/R2<sup>-/-</sup></li> </ul>	<ul style="list-style-type: none"> <li>Sex unknown</li> <li>Approximately 6 weeks of age</li> </ul>	<ul style="list-style-type: none"> <li>Resident Intruder Test</li> <li>OFT</li> <li>Light-Dark Test</li> </ul>	<ul style="list-style-type: none"> <li>Decreased aggression and non-aggressive exploration in TNF-R1/R2<sup>-/-</sup> mice.</li> <li>TNF-R1/R2<sup>-/-</sup> mice exhibited signs of decreased anxiety with increased time in centre of the open field and increased time in the light portion of the light-dark box.</li> </ul>
(Palin et al., 2007)	<ul style="list-style-type: none"> <li>TNF-R2<sup>-/-</sup></li> </ul>	<ul style="list-style-type: none"> <li>Sex unknown</li> <li>7-10 weeks of age</li> </ul>	<ul style="list-style-type: none"> <li>Social Exploration Test</li> <li>Novel Juvenile Test</li> </ul>	<ul style="list-style-type: none"> <li>No apparent differences in baseline exploration in TNF-R2<sup>-/-</sup> mice was observed (but no direct comparisons were made in this study)</li> </ul>

**Legend: The Table summarizes different studies that have used TNF- $\alpha$  and TNF- $\alpha$  receptor knockout mice to study a range of behavioural phenotypes**

## Methods

### *Mouse Strains*

As described in chapter 2, gene-targeted C57BL/6 mouse strain deficient for TNF- $\alpha$  (TNF<sup>-/-</sup>) (n = 14, 7 male, 7 female mice) was generated on a genetically pure C57BL/6 background (Korner et al., 1997a) and were been bred in-house for over 10 generations. The C57BL/6 (WT) mice (Jackson stock number: 000664) were purchased from the University of Adelaide breeding facility (n = 21, 10 male, 11 female). TNF-R1<sup>-/-</sup> (Jackson stock number: 003242) (Peschon et al., 1998) and TNF-R2<sup>-/-</sup> mice (Jackson stock number: 002620) (Erickson et al., 1994) were obtained from Jackson Laboratories (Bar Harbor, ME) (n = 14 per strain, 7 males, 7 females per strain) and were also established on a C57BL/6 background or backcrossed (Baune et al., 2008) and bred in house for over 10 generations (Table 3.2). Test mice were genotyped.

Mice of 12 weeks of age at the start of behavioural testing were used in all experiments. All mice were housed in groups of three to six mice per cage during the experimental period, with food and water available ad libitum and were handled prior to behavioural testing. Ambient temperature of the housing and testing rooms was 22  $\pm$  1°C. Mice were housed under a 12 hr light-dark cycle, lights on at 0700h, and all behavioural testing was conducted between 0800 hr and 1600 hr. Animal procedures were approved by the University of Adelaide Animal Ethics committee.

**Table 3.2: Details of mice used in chapter 3**

Strain	C57/BL6	TNF-/-	TNF-R1-/-	TNF-R2-/-
<b>Genotype</b>	Wild type mice	Deletion of TNF gene on C57/BL6 background	Deletion of TNF- $\alpha$ receptor1 (p55) gene on C57/BL6 background	Deletion of TNF- $\alpha$ receptor2 (p75) gene on C57/BL6 background
<b>Phenotype</b>	Express TNF- $\alpha$ (and other cytokines) normally	Do not express TNF- $\alpha$	Absence of TNF-R1 signalling	Absence of TNF-R2 signalling
<b>Age at which Analysed</b>	<b>3 months</b>			

**Legend:** The table shows the different strains of mice used for this study, along with their genotype, phenotype and the age at which they were analysed.

### ***Behavioural Analysis***

A comprehensive behavioural battery was carried out incorporating several different behavioural types. Tests were conducted in the following order: (1) Home Cage Locomotor Activity, (2) Open Field Test (OFT), (3) Hole-Board Exploration (HBE), (3) Elevated Zero Maze (EZM), (4) Y-Maze, (5) Sociability, (6) Barnes Maze (BM) and (7) Forced Swim Test (FST) and took 4 weeks to complete. Mice were given at least one day off between tests and tests were conducted in order of least to most stressful, to minimise effects of previous testing (Lad et al., 2010). A single

test was not repeated in the same mouse. Equipment for all tests was purchased from Stoelting Co. (USA) and all movements were tracked using ANY-MAZE imaging software Stoelting Co. (USA).

### **Locomotor Activity: Home Cage Locomotor Activity**

Mice were individually tested for general locomotor activity in home cages with two-day-old bedding under basal non-stressful conditions according to previous published results (Baune et al., 2008). Total distance covered over a 5 min period was measured.

### **Open Field Test (OFT)**

Under more stressful conditions basal locomotor activity was measured. Mice were placed at the centre of a brightly lit plexiglass box (40 X 40 cm) and movements were tracked over a 5 min period as previously published (Baune et al., 2008). Distance travelled and time spent in the outer and inner zones of the box was calculated.

### **Cognition-Like Behaviour: Spatial Memory and Learning: Barnes Maze (BM)**

The Barnes maze consists of a bright, circular white platform (91 cm) with 20 holes, which contain either false boxes, or one hidden escape box. The false boxes remove visual cues that might be observed through an open hole. BM procedures were carried out over a six day period according to published protocols with the time taken to find the escape box recorded (Baune et al., 2008).

**Pre-training (Day 1):** Mice were pre-trained to enter the escape box, first by placement of the mouse into the escape box for 2 min, then by guidance to the escape box before being left for 2 min in the escape box, and finally placement outside the escape box within a glass chamber for up to 3 min at the end of which the mice were again placed into the escape box for 2 min.

**Training (Days 2-5):** Mice were briefly placed in the centre of the maze under a removable chamber and given 3 min to locate the escape box. Mice that failed to enter the escape box within 3 min were guided to the box and placed there for 2 min prior to returning to their home cage. Each mouse was subjected to 4 trials per day, separated by 15 min, for 4 days. The average of the time taken to find the escape box across the 4 day period was then calculated. Mice that failed to enter the escape box during the trial period were given a test time of 3min. Lower escape latencies representing good spatial memory.

**Probe trial (Day 6):** Each mouse was given a probe trial of 3 min duration each, with the escape box rotated 90°. Latency to the old and new escape locations was recorded. Retention memory for the (known) old escape box was considered as the tendency of the mice to explore the old escape box in the probe trial instead of exploring the new (unknown) box location. Thus, short latencies to locate the old escape location were considered to indicate spatial memory retention for the original location.

### **Spatial Recognition Memory: Y Maze**

The Y maze is used to assess hippocampal spatial recognition memory. It consists of a three-armed chamber, with the arms at a 120° angle from each other. Each arm is 35 cm long, 5.0 cm wide and 10 cm high (Choy et al., 2008).

**Phase 1:** One arm of the maze is closed off. Mice are placed at the bottom of the “start” arm and allowed to explore the two arms for 15 min.

**Phase 2:** 24 hr after phase one, mice are tested with all three arms left open. Mice are again placed at the bottom of the start arm and allowed to explore the three arms for 10 min. Mice have a preference for exploring novel environments (Dulawa et al., 1999). Thus normal mice should spend more time exploring the novel versus the familiar arm, indicating normal spatial recognition memory, whilst impaired mice will not recognise the familiar arm and spend a similar amount of time in the familiar and novel arms. As all mice started in the same arm (start arm) time spent in this arm was not analyzed.

### **Exploratory Activity: Hole Board Exploration Test (HBE)**

The hole-board exploration test is set up in an apparatus similar to the open field test, and utilises a hole-board insert, which has 16 holes of 1.5 cm in diameter, spaced 6.0 cm apart. Mice are placed into the apparatus and allowed to explore for 5 min. Head dipping behaviour is manually recorded and is an indicator of exploration (Hart et al., 2010).



### **Social Behaviour: Sociability Test**

The sociability test measures general social behaviour and interest in social novelty in rodent models. Rodents have a preference for social interaction with other rodents and will also prefer to interact with a novel mouse over a familiar one (Moy et al., 2004). The apparatus consists of a three chamber rectangular box, the walls of which are plexiglass. Each chamber is 20 cm in length, 40.5 cm in width and 22 cm high. The test runs in three stages (Moy et al., 2004).

**Habituation:** The test mouse is placed in the central chamber and allowed to explore for 5 min. Doors to the other chambers are sealed off in this stage.

**Sociability:** A stranger mouse is placed in one of the side chambers (either left or right) in a small round wire cage that allows nose contact between the bars but prevents fighting. Doors to all chambers are opened and the test mouse is allowed to explore the entire box. The time spent by mice interacting with the stranger mouse vs. the empty cage is recorded.

**Social Novelty:** A second novel mouse is placed in the chamber that was empty during the sociability phase. The test mouse is allowed to explore the box for 5 min and the time spent interacting with the novel mouse over the familiar (stranger) mouse is recorded. Stranger mice used are C57BL/6 mice of the same age and sex as the test mice.

### **Anxiety-Like Behaviour: Elevated Zero Maze (EZM)**

The elevated zero maze is used as a measure of anxiety-like behaviour wherein anxiety is measured as the time spent in the open arm of the maze. The apparatus consists of a grey Perspex circular platform of 50 cm diameter and width

of 5 cm, located at a height of 40 cm above ground. The platform is divided into 4 equal quadrants, two closed quadrants with grey Perspex walls (27 cm high) on the inside and outside, and two open quadrants without walls. Mice were placed at the centre of either one of the open arms and allowed to explore the apparatus for 5 min. Movement and time spent in open and closed quadrants was recorded.

### **Depression-Like Behaviour: Forced Swim Test (FST)**

The forced swim test is a measure of despair and depression-like behaviour, and was performed according to previously published results (Petit-Demouliere et al., 2005). The apparatus consists of a 4 L cylinder (20 cm diameter) filled up to 20 cm with water (23-24°). Mice were placed in water and movement was tracked over a period of 6 min. Immobility was considered a measure of depression-like behaviour, and was defined as lack of motion beyond which was required for the mouse to keep its head above water. For analysis, the first 2 min were excluded, since mice are mobile during that time and familiarizing themselves with the environment and any immobility during this time therefore cannot be attributed to despair.

### ***Protein Analyses***

#### **Cytometric Bead Array (CBA)**

In order to demonstrate that these behavioural changes were in a non-inflammatory setting, following completion of behavioural testing mice were sacrificed, blood was collected by means of cardiac puncture and serum was

extracted by spinning blood down at 2500 rpm for 15 min. Serum was stored at -80°C until analysis. Serum cytokine levels were measured using the BD Cytometric Bead Array (CBA) Mouse Inflammation Kit for the cytokines IL-6, IL-10, MCP-1, IFN- $\gamma$ , TNF (total, both TNF- $\alpha$  and TNF- $\beta$ ) and IL-12p70 according to the manufacturer's instructions.

## ELISA

In order to evaluate whether there were any underlying changes in neurotrophin levels within the TNF- $\alpha$  transgenic mice, a subset of mice ( $n = 7$  mice), were sacrificed by Pentobarbital overdose and the brain rapidly removed. The hippocampus and prefrontal cortex (PFC) tissue were isolated and stored fresh frozen at -80°C until analysed. These brain regions were chosen for their role in cognition and emotion-like behaviour. The hippocampus plays a role in spatial cognition (Sweatt, 2004) as well as some emotional responses (Sweatt, 2004), while the PFC plays a role cognitive control (Miller and Cohen, 2001) and goal oriented emotional processing (Davidson, 2002). Tissue was homogenised in a solution containing 50 mL TRIS, one protease inhibitor tablet (Roche) and 50  $\mu$ L of Triton-X. Homogenates were then centrifuged at 8500 rpm (Davidson, 2002) for 15 min at 4°C, and the supernatant transferred to 1.5 mL eppendorf tubes. All samples were stored at -80°C until analysis. Amount of protein was quantified using the Bradford method and samples then diluted to contain 50 ng of protein. NGF and BDNF ELISAs were then performed using the E<sub>max</sub> Immuno Assay system (Promega)

according to the manufacturer's instructions and as previously published (Sei et al., 2000, Gilmore et al., 2003).

### ***Immunohistochemistry (IHC)***

The subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles are 2 important areas of the brain that are involved in adult neurogenesis (Biebl et al., 2000, Song et al., 2002). Neural stem cells formed in these regions then migrate to the dentate gyrus where they mature into granular neurons (Zhao et al., 2008). Therefore to assess how TNF- $\alpha$  signalling impacts on neurogenesis and how this correlates with behavioural changes, the dentate gyrus of mice was screened for immature neurons and proliferating cells. A subset of mice (n = 7 mice/ group) were perfused with saline, before the whole brain was removed and placed in OCT and then frozen using liquid nitrogen. For IHC staining, serial 8 $\mu$ m sections of hippocampal tissue were collected at 200  $\mu$ m intervals and post-fixed in formalin. These sections were then immunohistochemically analysed for levels of doublecortin (DCX; Millipore 1:8000, anti-guinea pig), which labels immature neurons, and Ki67 (Abcam 1:2000 anti-rabbit), which labels dividing cells. For immunohistochemistry, endogenous peroxidases were blocked using hydrogen peroxide in PBS. Antigen retrieval was then carried out using TRIS buffer for DCX antibody and citrate buffer for Ki67 antibody and non-specific binding was then blocked using normal horse serum. Sections were incubated overnight with primary antibody. The next day slides were incubated with the appropriate secondary antibody (Abacus) for 30 min, and then with streptavidin peroxidase for 1h. Sections were stained with Diaminobenzadine (DAB) (Sigma) and then counter stained with

haematoxylin prior to dehydration and mounting with DePex mounting media. Slides were then scanned using the Nanozoomer (Hamamatsu) and viewed with the associated software (NDP view). Serial images of the dentate gyrus of each section were captured as jpegs. These images were then exported to Image J and the number of positive cells within the dentate gyrus counted.

### ***Statistical Analysis***

Data analyses were carried out using GraphPad Prism software (version 5.01). All data are presented as mean  $\pm$  SEM. Since all data was not normally distributed, (tested with Kolmogorov-Smirnov test) only non-parametric measurements were used. Comparisons between strains were performed using Kruskal-Wallis test followed by Dunn's post hoc analysis, all columns compared to each other for all tests, as stated throughout the results. Comparisons within strains for the Y maze and Sociability test were performed using the Mann-Whitney U test and  $P < 0.05$  was taken as significant for all tests.

## Results

### ***Mean Weight, Age and Sex***

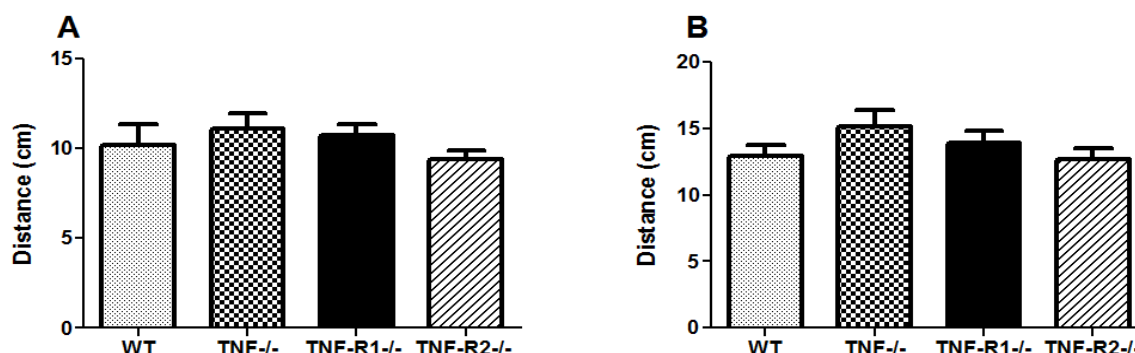
Mean age of all animals used was 12 weeks at the start of behavioural testing. The average weight of mice was  $24.0 \pm 1.4$  g for male mice and  $18.0 \pm 1.5$  g for female mice of all strains and no significant differences were observed between strains as analysed by Kruskal-Wallis test.

To minimise potential gender-specific effects in behaviour that could arise from genetic deletion of TNF- $\alpha$  and TNF- $\alpha$  receptor related genes, roughly equal numbers of male and female mice were used in the present study. No differences in behaviour across gender were noted among strains (data not shown).

### ***Locomotor Activity***

General locomotor activity was measured under non-stressful baseline conditions using the Home Cage locomotor activity test (Figure 3.1A); and was also measured under more stressful conditions, in the novel brightly lit OFT (Figure 3.1B). Since analysis of both tests by Kruskal-Wallis test showed no significant differences in locomotor activity between any of the strains (Home cage  $P = 0.17$ ; OFT  $P = 0.24$ ), indicating similar locomotor activity, all possible behavioural differences noted between strains are most likely related to factors other than locomotor activity.

**Figure 3.1: General locomotor activity**



**Legend:** Distance travelled in A) the home cage, as a measure of baseline locomotor activity and B) the open field. All data represent mean  $\pm$  SEM ( $n = 14/\text{group}$  for TNF<sup>-/-</sup>, TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice;  $n = 21$  for WT mice). Data compared using Kruskal-Wallis test.

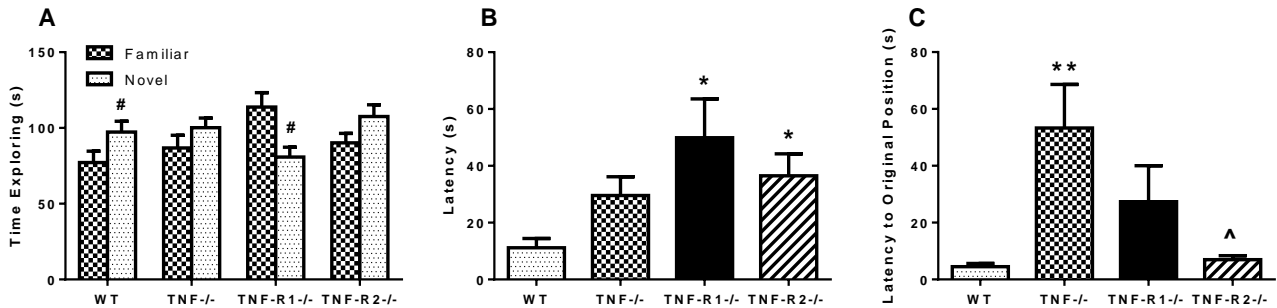
### ***The Role of TNF- $\alpha$ and its Receptors in Cognitive Tasks***

Figure 3.2A shows the time spent in the novel compared to the familiar arm of the Y-maze. As all mice started in the same arm (start arm) time spent in this arm was not analysed. Analysis however showed that all mice spent similar amount of time in start arm. Comparison within strains by Mann-Whitney U test showed that WT mice exhibited a significant preference for the novel arm over the familiar arm, indicating normal spatial recognition ( $P = 0.038$ ). In contrast, the TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice spent a similar amount of time in both the familiar and novel arm (TNF<sup>-/-</sup>  $P = 0.21$ , TNF-R2<sup>-/-</sup>  $P = 0.092$ ), suggesting they do not recognise that this arm is novel, which would indicate impaired spatial recognition memory. As the TNF-R1<sup>-/-</sup> mice demonstrated a significantly higher preference for the familiar arm over the novel arm ( $P = 0.01$ ), it is difficult to assess whether this is due to impaired memory or a preference for a familiar environment.

Figure 3.2B demonstrates that knockout mouse strains had poorer spatial learning compared to WT mice on the BM, as demonstrated by mean escape latency to the escape box over the 4 day training period analysed by Kruskal-Wallis test ( $P = 0.0091$ ). However, Dunn's multiple comparison post-hoc test showed that only TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice had significantly higher latencies to reach the escape box compared to WT mice ( $P < 0.05$ ) suggesting that these mice were poor learners. Spatial recognition memory was tested a day later during the probe trial, and showed significant differences between groups measured by Kruskal-Wallis test ( $P = 0.0049$ ). WT mice exhibited low latency time to locate the old location indicating good spatial retention memory. Interestingly, the TNF-R2<sup>-/-</sup> mice that had performed poorly during the training phase, performed well in the probe trial (Figure 3.2C), indicating that their spatial retention memory was intact. In contrast to TNF-R1<sup>-/-</sup> mice, TNF<sup>-/-</sup> mice had impaired spatial retention memory, with high latencies to reach the location of the old box compared to both WT ( $P < 0.01$ ) and TNF-R2<sup>-/-</sup> ( $P < 0.05$ ) as shown by Dunn's multiple comparison post-hoc test. No differences across strains were noted in the latency to find the new location.



**Figure 3.2: Cognition-like behaviour**



**Legend:** A) Time spent in both the novel and familiar arms during the retention phase of the Y-maze, B) mean latency of mice to find the escape box over the 4 training days of the Barnes maze, and C) latency of mice to find the original escape box position over the new escape box in Probe trial 1 of the Barnes maze. All data represent mean  $\pm$  SEM (n = 14/group for TNF-<sup>-/-</sup>, TNF-R1-<sup>-/-</sup> and TNF-R2-<sup>-/-</sup> mice; n = 21 for WT mice). Data compared using Mann Whitney U test (A) or Kruskal-Wallis with Dunn's multiple post hoc comparison (B, C). #  $P < 0.05$  novel arm vs familiar arm, \*  $P < 0.05$  compared to WT, \*\*  $P < 0.01$  compared to WT, ^  $P < 0.05$  compared to TNF-<sup>-/-</sup>

### **Exploratory Activity**

Exploratory activity in mice was measured using the HBE test, where the number of head dips was regarded as a measure of exploration. No significant difference between the number of head pokes between all strains was noted on the Kruskal-Wallis test ( $P = 0.067$ , Figure 3.3A).

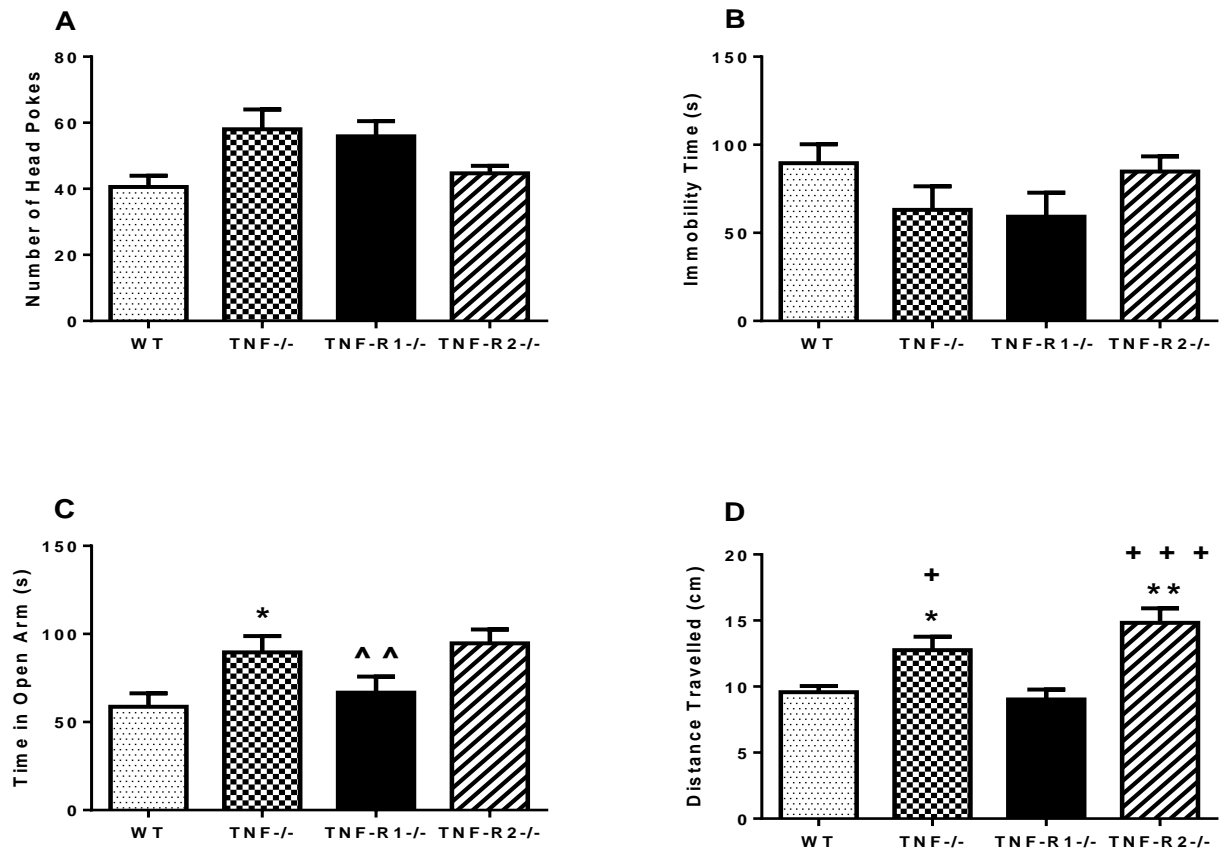
### ***Decreased Anxiety-Like Behaviour in TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> Mice***

The EZM was used as a measure of anxiety. Mice were allowed to move freely between the open and closed arms. Increased time spent in the open arm is believed to be an indicator of lower levels of anxiety-like behaviour, as is higher total distance travelled (Cryns et al., 2007). Analysis by Kruskal-Wallis test showed significant differences between groups ( $P = 0.0054$ ). Comparison of individual strains by Dunn's multiple comparison post-hoc test showed that TNF<sup>-/-</sup> exhibited low anxiety as these mice spent significantly more time in the open arm than WT mice ( $P < 0.05$ ) and TNF-R1<sup>-/-</sup> mice ( $P < 0.01$ ) (Figure 3.3C). A significant difference in overall distance travelled between strains was also noted as measured by Kruskal-Wallis test ( $P < 0.0001$ ). Dunn's multiple comparison post-hoc test revealed an overall greater amount of exploration within the EZM by both TNF-R2<sup>-/-</sup> and TNF<sup>-/-</sup> mice indicating less anxiety in these mice as compared to WT and TNF-R1<sup>-/-</sup> mice (Figure 3.3D).

### ***Depression-Like Behaviour***

To measure depression-like behaviour the FST was employed, where an increase in immobility time is regarded depressive-like behaviour. There were no significant differences in immobility time noted between strains on the FST measured by Kruskal-Wallis Test ( $P = 0.19$ ) (Figure 3.3B), although the TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice showed a trend for lower immobility time than WT mice.

Figure 3.3: Anxiety and depression-like behaviour

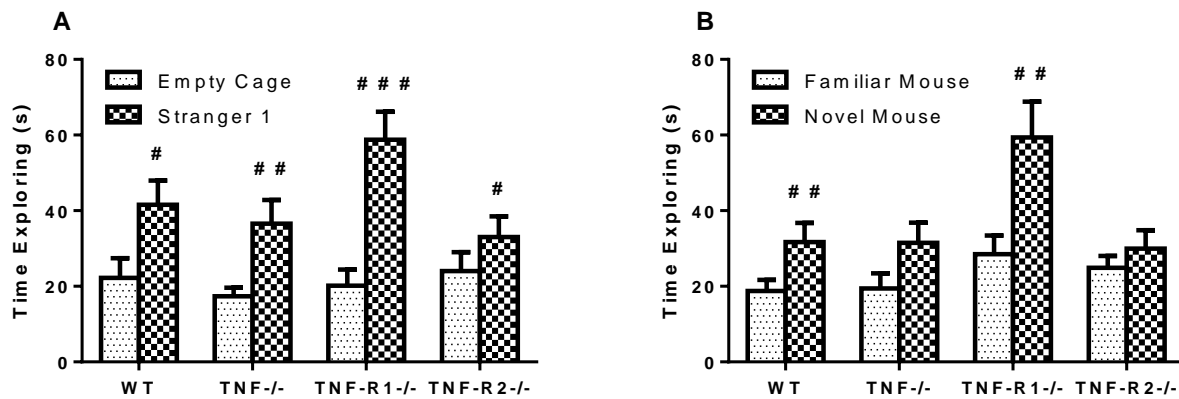


Legend: A) Number of head pokes in to holes, as a measure of exploration, in the Hole Board test, B) Time spent immobile during the last 4 min of the forced swim test, C) Time spent in open arm of the elevated zero maze (EZM), and D) total distance travelled in the EZM. All data represent mean  $\pm$  SEM (n = 14/group for TNF $^{-/-}$ , TNF-R1 $^{-/-}$  and TNF-R2 $^{-/-}$  mice; n = 21 for WT mice). Data compared using Kruskal-Wallis with Dunn's multiple post hoc comparison \* compared to WT,  $^{\wedge}$  compared to TNF $^{-/-}$ , + compared to TNF-R1 $^{-/-}$  (\*  $P < 0.05$ ,  $^+ P < 0.05$ , \*\* $P < 0.01$ ,  $^{\wedge\wedge} P < 0.01$ ,  $^{+++} P < 0.001$ )

### ***Social Behaviour***

The sociability test was performed to assess general social interactions as well as behaviour in novel social situations. In the first phase of this test, social behaviour was measured when mice were allowed to choose between exploring a social situation with a stranger mouse versus an empty cage (Figure 3.4A). All strains demonstrated a preference for the stranger mouse compared to the empty cage (Mann-Whitney U test: WT mice  $P = 0.024$ , TNF<sup>-/-</sup> mice  $P = 0.0079$ , TNF-R1<sup>-/-</sup> mice  $P < 0.0001$ , TNF-R2<sup>-/-</sup> mice  $P = 0.038$ ). A comparison of time spent with the stranger mouse between strains measured by Kruskal-Wallis Test showed no significant difference ( $P = 0.094$ ). In the second phase of this experiment, the preference of mice for a socially novel situation over a familiar social situation was measured (Figure 3.4B) wherein the amount of time spent with a familiar mouse (the stranger mouse from Phase 1) was compared to that of a novel mouse. Only the WT ( $P = 0.0056$ ) and TNF-R1<sup>-/-</sup> mice ( $P = 0.0033$ ) spent significantly more time with the novel over the familiar mice. A comparison of time spent with the novel mouse between strains measured by Kruskal-Wallis Test again showed no significant difference ( $P = 0.65$ ).

**Figure 3.4: Sociability-like behaviour**



**Legend:** A) Time spent interacting with a stranger mouse compared to an empty cage in the Sociability stage, and B) time spent interacting with a familiar and novel stranger mouse in the Preference for social novelty stage of the test. All data represent mean  $\pm$  SEM ( $n = 14/\text{group}$  for TNF<sup>-/-</sup>, TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice;  $n = 21$  for WT mice). Data compared using Mann-Whitney U test. # compared to empty cage (A) or familiar mouse (B) (#  $P < 0.05$ , ##  $P < 0.01$ , ###  $P < 0.001$ )

### ***Differences in Serum Cytokine Levels between Strains***

To test whether there was any change in serum cytokine levels in the young adult mice, a CBA was performed on serum collected at the end of behavioural testing. Low levels of serum cytokines were detected in all strains of mice without significant differences between strains in levels of IL-10, IL-6, MCP-1, IFN- $\gamma$ , TNF, and IL-12p20 as measured by Kruskal-Wallis Test. These results indicate that TNF- $\alpha$  signalling does not affect systemic levels of cytokines under immunologically unchallenged conditions (Table 3.3). It is interesting to note that in TNF<sup>-/-</sup> mice that do not express TNF- $\alpha$ , total TNF levels were still detected. These levels most likely reflect levels of TNF- $\beta$  but further investigation will be needed to confirm this.

**Table 3.3: Serum cytokine analyses for chapter 3**

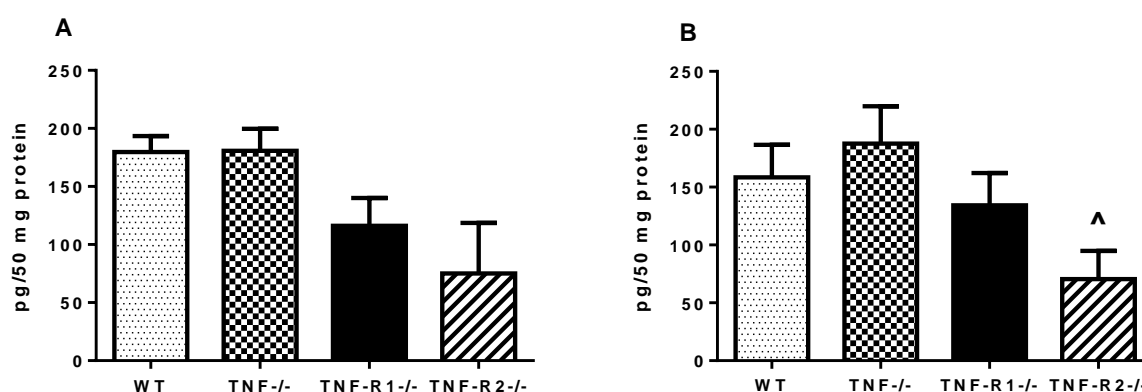
	TNF (total)	IL-10	IL-12p70	MCP-1	IL-6	IFN- $\gamma$
<b>WT</b>	0.31 $\pm$ 0.13	1.14 $\pm$ 0.48	0.76 $\pm$ 0.49	0.53 $\pm$ 0.19	0.24 $\pm$ 0.18	0.10 $\pm$ 0.03
<b>TNF<sup>-/-</sup></b>	0.38 $\pm$ 0.22	0.56 $\pm$ 0.36	1.73 $\pm$ 0.29	0.32 $\pm$ 0.19	0.38 $\pm$ 0.25	0.16 $\pm$ 0.10
<b>TNF-R1<sup>-/-</sup></b>	0.26 $\pm$ 0.14	1.51 $\pm$ 0.75	1.12 $\pm$ 0.56	1.49 $\pm$ 0.86	0.28 $\pm$ 0.25	0.15 $\pm$ 0.05
<b>TNF-R2<sup>-/-</sup></b>	0.39 $\pm$ 0.06	1.25 $\pm$ 0.54	0.91 $\pm$ 0.54	1.17 $\pm$ 0.08	0.45 $\pm$ 0.36	0.10 $\pm$ 0.04

**Legend:** Representation of cytokine expression (across) measured by CBA in all strains of mice. All data represent mean  $\pm$  SEM (n = 14/group for TNF<sup>-/-</sup>, TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice; n = 21 for WT mice). **Levels of TNF (total) in TNF<sup>-/-</sup> mice likely represent levels of TNF- $\beta$ .**

### Levels of BDNF in PFC and Hippocampus

To study how the deletion of genes for TNF- $\alpha$  and its receptors impacted upon neurotrophin production, ELISAs for NGF and BDNF were performed on hippocampal and pre-frontal cortex tissue. Analysis by Kruskal-Wallis test showed no significant differences between strains in the hippocampus (Figure 3.5A) ( $P = 0.39$ ), however BDNF ELISA of prefrontal cortex tissue showed a significant difference between strains (Kruskal-Wallis test:  $P = 0.032$ ) indicating that TNF<sup>-/-</sup> mice had significantly higher levels of BDNF than TNF-R2<sup>-/-</sup> mice (Dunn's multiple comparison post-hoc test:  $P < 0.05$ ) although no significant differences between WT and genetically modified mice was noted (Figure 3.5B).

Figure 3.5: Levels of BDNF in hippocampus and prefrontal cortex

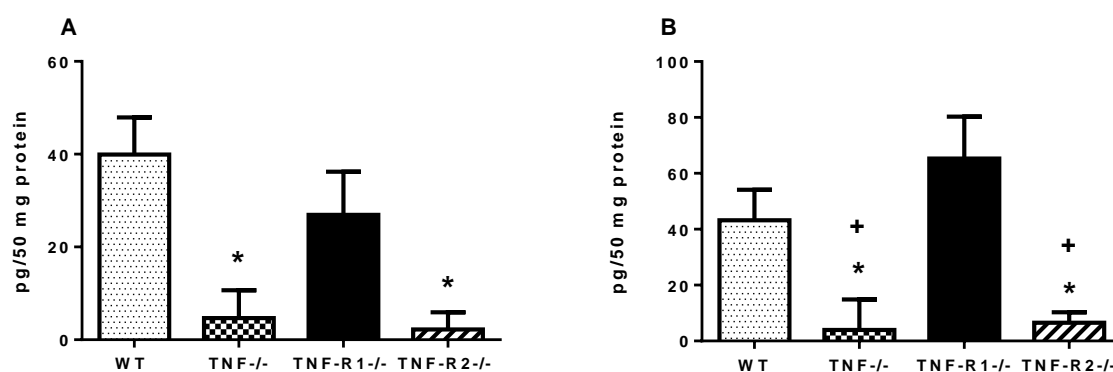


**Legend:** Levels of BDNF in the hippocampus (A) and prefrontal cortex (B), All data represent mean  $\pm$  SEM ( $n = 8$ /group for TNF<sup>-/-</sup>, TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice;  $n = 10$  for WT mice). Data compared using Kruskal-Wallis with Dunn's multiple post hoc comparison. <sup>^</sup>  $P < 0.05$  compared to TNF<sup>-/-</sup>.

### Low Levels of NGF in $TNF^{-/-}$ and $TNF-R2^{-/-}$ Mice

Kruskal-Wallis test showed group differences between strains in the levels of NGF expressed in the hippocampus ( $P = 0.0033$ ). Analysis by Dunn's multiple comparison post-hoc test (Figure 3.6A) showed that  $TNF-R2^{-/-}$  mice ( $P < 0.05$ ) and  $TNF^{-/-}$  mice ( $P < 0.05$ ) had significantly lower levels of NGF than WT mice. Similar results were observed in the prefrontal cortex ( $P = 0.0091$ ) measured by Kruskal-Wallis test (Figure 3.6B) wherein Dunn's multiple comparison post-hoc test showed significantly lower levels of NGF in  $TNF-R2^{-/-}$  mice ( $P < 0.05$ ) and  $TNF^{-/-}$  mice ( $P < 0.05$ ) than WT mice.  $TNF-R2^{-/-}$  mice also exhibited lower levels of NGF compared to  $TNF-R1^{-/-}$  mice ( $P < 0.05$ )

Figure 3.6: Levels of NGF in hippocampus and prefrontal cortex



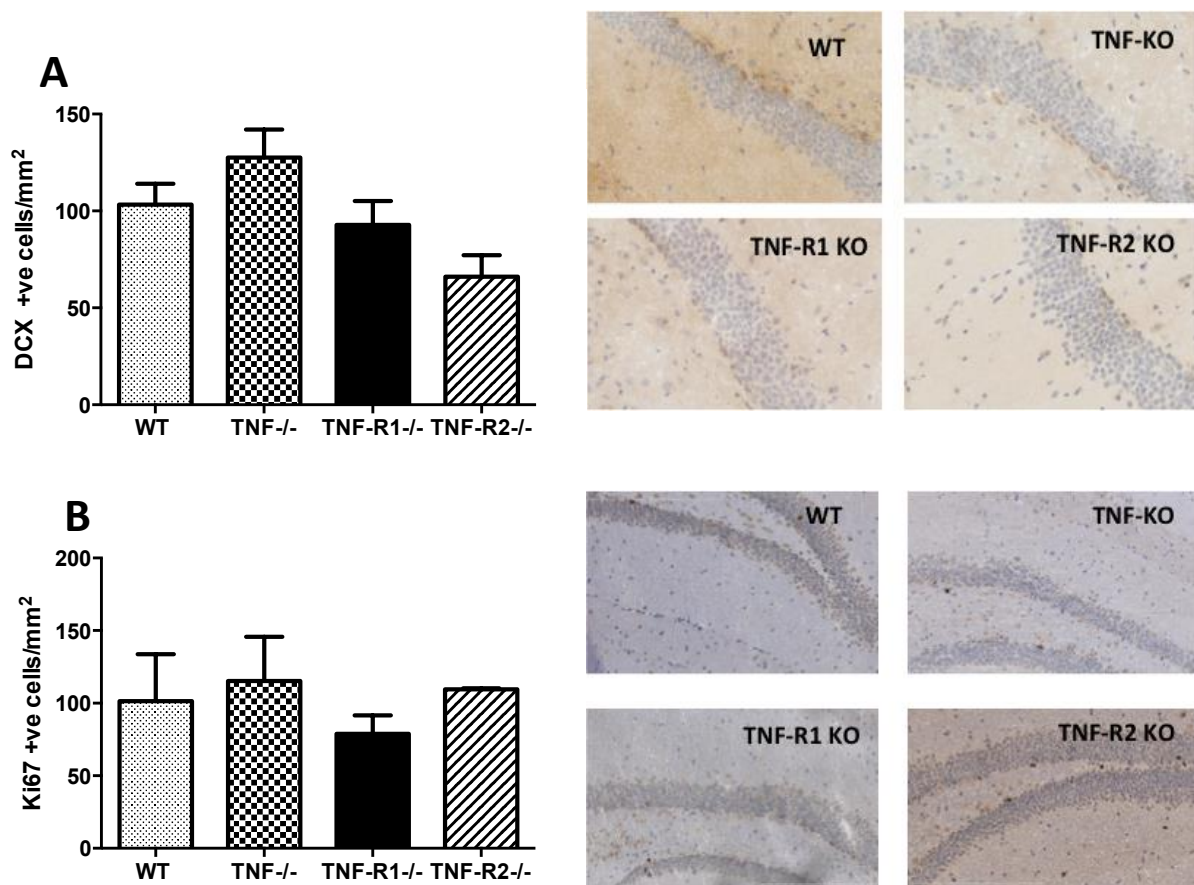
**Legend:** Levels of NGF in the hippocampus (A) and prefrontal cortex (B), All data represent mean  $\pm$  SEM ( $n = 8$ /group for  $TNF^{-/-}$ ,  $TNF-R1^{-/-}$  and  $TNF-R2^{-/-}$  mice;  $n = 10$  for WT mice). Data compared using Kruskal-Wallis with Dunn's multiple post hoc comparison. \*  $P < 0.05$  compared to WT, +  $P < 0.05$  compared to  $TNF-R1^{-/-}$ .



### Evaluation of Neurogenesis in TNF- $\alpha$ Transgenic Mice

To investigate the effects of TNF- $\alpha$  signalling on neurogenesis in the dentate gyrus, IHC techniques were used to stain for numbers of immature neurons utilising doublecortin and proliferating cells in the dentate gyrus with Ki67. Analysis by Kruskal-Wallis test showed no significant differences between groups for either test (DCX:  $P = 0.07$ , Ki67:  $P = 0.47$ , Figure 3.7)

Figure 3.7: DCX and Ki67 counts in the hippocampus



Legend: Number of A) DCX positive cells in the dentate gyrus and B) number of Ki67 positive cells in the dentate gyrus. All data represent mean  $\pm$  SEM ( $n = 3$ ). Data compared using one-way ANOVA. No significant differences were observed between groups.

**Table 3.4: Summary of all behavioural tests**

TESTS	TNF <sup>-/-</sup>	TNF-R1 <sup>-/-</sup>	TNF-R2 <sup>-/-</sup>
HOME CAGE TEST	-	-	-
OPEN FIELD TEST	-	-	-
BARNES MAZE (TRAINING)	-	Impaired Learning	Impaired Learning
BARNES MAZE (PROBE TRIAL)	Impaired Memory	-	-
Y MAZE	Normal Memory	Impaired Memory	Normal Memory
HOLE BOARD EXPLORATION TEST	-	-	-
EZM (TIME IN OPEN ARMS)	Decreased Anxiety	-	-
EZM (TOTAL DISTANCE TRAVELLED)	Decreased Anxiety	-	Decreased Anxiety
SOCIABILITY	Normal Social Behaviour	Normal Social Behaviour	Normal Social Behaviour
SOCIAL NOVELTY	Normal Social Behaviour	Normal Social Behaviour	Normal Social Behaviour
FORCED SWIM TEST	-	-	-
SERUM CYTOKINE ANALYSIS	-	-	-
BDNF ELISA (HIPPOCAMPUS)	-	-	-
BDNF ELISA (PFC)	-	-	-
NGF ELISA (HIPPOCAMPUS)	Lower Expression	-	Lower Expression
NGF ELISA (PFC)	Lower Expression	-	Lower Expression

**Legend:** Table summarizing the differences between the strains compared to WT mice for Home Cage, OFT, BM, EZM, HBE, FST and ELISAs. – represents no significant difference compared to WT mice. Analysis of normal or impaired behaviour within a strain in reference to WT mice has been reported for Y maze and Sociability tests

## Discussion

In this study TNF<sup>-/-</sup> mice displayed impairments in memory in the Barnes maze and Y maze, while TNF-R2<sup>-/-</sup> mice showed good memory but slow learning in these tests. TNF-R2<sup>-/-</sup> and TNF<sup>-/-</sup> mice also showed low levels of anxiety like behaviour. All strains of mice demonstrated a significant preference for social situations over non-social ones, however only WT and TNF<sup>-/-</sup> mice showed a significant preference for social novelty. Analysis of neurotrophin levels in the prefrontal cortex and the hippocampus revealed TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice to have significantly lower levels of NGF compared to WT mice, suggesting that some of the behavioural changes could be linked to changes in levels of circulating neurotrophins. (Summary Table 3.5 and 3.6)

Current literature suggests that TNF- $\alpha$  plays a vital role in the development and functioning of the CNS as well as in cognitive tasks (McAfoose et al., 2009). This group has previously shown that the expression of TNF- $\alpha$  at a young age may be essential to the development of cognition (Baune et al., 2008), but with ageing this beneficial action can change to a more deleterious one (McAfoose et al., 2009), even under physiological conditions. This may relate to an enhancement of the neurodegenerative TNF-R1 signalling, over the neuroprotective TNF-R2 signalling. However previous studies have shown that lack of TNF-R1 has detrimental effects on cognition, suggesting it may exert differential effects in the CNS (Bruce et al., 1996).

Effects of knockout of TNF- $\alpha$  and its receptors on cognition were evaluated utilising the Barnes maze and Y-maze tests. While TNF<sup>-/-</sup> mice demonstrated a slight impairment in the learning phase of the Barnes Maze, the results did not reach

statistical significance. However, the TNF<sup>-/-</sup> mice did exhibit impaired memory compared to WT counterparts in the BM test. These mice were unable to differentiate between the familiar and novel arms on the Y-Maze when compared to their wild type counterparts. The performance of the TNF<sup>-/-</sup> mice is expected given the extensive literature detailing the role of TNF- $\alpha$  in normal cognition (McAfoose and Baune, 2009). A unique characteristic of glial TNF- $\alpha$  is its ability to mediate synaptic scaling (Stellwagen et al., 2005). Studies have shown that exogenous application of TNF- $\alpha$  to hippocampal tissue cultures results in exocytosis of AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid) receptors (AMPA) (Beattie et al., 2002), causing an increase in the number of post synaptic receptors which is accompanied by an increase in mini excitatory post synaptic currents (mEPSC). mEPSCs are a representative of increase in synaptic strength (Beattie et al., 2002). TNF- $\alpha$  also has the ability to decrease inhibitory synapses by causing endocytosis of inhibitory gamma-aminobutyric acid (GABA) receptors (Stellwagen et al., 2005).

As seen in previous studies, both the TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice demonstrated poorer learning on the BM (Baune et al., 2008). This supports the view that signalling via TNF-R1 may not be entirely neurodegenerative. Indeed, it has previously been hypothesised that the poorer learning observed in these mice may be caused by interactions between soluble TNF-R1 and membrane bound TNF-R2 (Baune et al., 2008), although this remains to be investigated. Of note in this study, the TNF-R1<sup>-/-</sup> mice appeared to be more impaired than their TNF-R2<sup>-/-</sup> counterparts, as the TNF-R2<sup>-/-</sup> mice showed good spatial retention memory in the probe trial. Thus, it appears that TNF-R1 signalling may be exerting effects independent of TNF-R2 suggesting that TNF-R1 may play a part in the consolidation of the memory.

Furthermore, the TNF-R1<sup>-/-</sup> mice exhibited a slight preference for the novel arm in the Y-Maze, whilst TNF-R2<sup>-/-</sup> mice did not show a preference for the novel arm. Receptor knockout mice may have different behavioural phenotypes as compared to TNF<sup>-/-</sup> mice most likely due to the effects of enhanced TNF- $\alpha$  signalling through the unaffected receptor. Though this is difficult to determine, an earlier study found no increase in mRNA levels of the other receptor in TNFR1<sup>-/-</sup> or TNFR2<sup>-/-</sup> mice (Simen et al., 2006). However, this does not rule out the potential for enhanced signalling through the unaffected receptors, given the lack of competition for TNF- $\alpha$  binding. In a TNF- $\alpha$  receptor knockout mice we would expect increased activity of the other TNF- $\alpha$  receptor, which would subsequently have different effects on behaviour. Thus a TNF- $\alpha$  receptor knockout mouse, would not be expected to behave exactly like a TNF-KO mouse, as was observed in the present findings. Furthermore, as TNF- $\alpha$  receptors can bind to lymphotoxin- $\alpha$  ligands in addition to TNF- $\alpha$ , It is plausible that some of the effects noted in the present studies in TNF receptor deficient mice may be due to interactions between lymphotoxin- $\alpha$  and TNF receptors (MacEwan, 2002). This would again result in different phenotypes in TNF receptor mice compared to TNF-KO mice. At this stage we are unable to verify these interactions due to limitations of the current studies but these issues should be considerations for future work involving these strains of mice. A similar hypothesis, in that enhanced TNF- $\alpha$  signalling may occur through the unaffected receptor, has been proposed elsewhere (Simen et al., 2006), but further investigation would be needed to clarify this.

**Table 3.5: Comparisons between TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice from chapter 3**

Test	Difference Between Strains	TNF-R1 <sup>-/-</sup>	TNF-R2 <sup>-/-</sup>	TNF-R1 <sup>-/-</sup> vs. TNF-R2 <sup>-/-</sup> (P value)
Home Cage Test	No difference	10.78 $\pm$ 0.57	9.42 $\pm$ 1.73	0.08
Open Field Test	No difference	13.88 $\pm$ 3.59	12.64 $\pm$ 3.40	0.35
Barnes Maze (Training)	No difference	49.89 $\pm$ 47.48	36.48 $\pm$ 26.95	0.82
Barnes Maze (Probe Trial)	No difference	27.40 $\pm$ 12.61	6.95 $\pm$ 1.39	0.86
Hole Board Exploration Test	No difference	55.92 $\pm$ 4.60	44.69 $\pm$ 8.27	0.08
EZM (Time In Open Arms)	Lower anxiety in TNF-R2 <sup>-/-</sup>	66.65 $\pm$ 9.20	94.69 $\pm$ 7.87	0.019
EZM (Distance Travelled)	Lower anxiety in TNF-R2 <sup>-/-</sup>	9.02 $\pm$ 0.75	14.83 $\pm$ 1.01	0.0002
Forced Swim Test	No difference	59.18 $\pm$ 13.58	84.76 $\pm$ 31.2	0.16
BDNF ELISA (Hippocampus)	No difference	116.1 $\pm$ 23.89	75.0 $\pm$ 43.67	0.57
BDNF ELISA (PFC)	No difference	134.2 $\pm$ 27.94	70.52 $\pm$ 24.33	0.17
NGF ELISA (Hippocampus)	Lower expression in TNF-R2 <sup>-/-</sup>	26.94 $\pm$ 9.25	2.24 $\pm$ 3.69	0.030
NGF ELISA (PFC)	Lower expression in TNF-R2 <sup>-/-</sup>	65.30 $\pm$ 14.98	6.56 $\pm$ 3.76	0.015

**Legend:** Representation of the differences between TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice. All data represent mean  $\pm$  SEM and P values (n = 14/group for TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice).

Studies have shown that TNF- $\alpha$  may aid synaptic scaling through long-term potentiation (LTP) formation (Beattie et al., 2002) and may protect neurons from excitotoxicity (Cheng et al., 1994). One possible mechanism by which TNF- $\alpha$  mediates this is through TNF-R1 signalling (Bruce et al., 1996, Stellwagen et al., 2005) and these behavioural results would support the previous findings. TNF- $\alpha$  binds with high affinity to TNF-R1 resulting in the activation of NF $\kappa$ B pathway (Furukawa and Mattson, 1998), NF $\kappa$ B mediates plasticity by inducing calcium currents while decreasing N-Methyl-D-aspartic acid (NMDA) currents (Gary et al., 1998) and is thus integral to TNF- $\alpha$  mediated synaptic transmission.

In this study no significant differences in exploration were observed in TNF- $\alpha$  transgenic strains, suggesting that genetic deletion of TNF- $\alpha$  or its receptors does not impact upon exploratory behaviour in young adult mice. Further studies need to be conducted in order to fully elucidate the roles of TNF- $\alpha$  receptor signalling on exploratory behaviour and whether changes may occur with ageing.

It was also noted that TNF<sup>-/-</sup> mice exhibited significantly less anxiety than the WT mice as evaluated using the EZM. A similar pattern was seen in depressive-like behaviour as assessed in the FST, but this did not reach significance. It should be noted that the present results on anxiety-like behaviour are in contrast to the work done by Yamada et al (Yamada et al., 2000) where TNF<sup>-/-</sup> mice displayed both higher anxiety and depression-like behaviour compared to their wild-type counterparts, although this may relate to the age of the mice tested as it remained unclear at what age the mice were tested in the Yamada study. Despite this contradiction with previous literature, these results seem to be in concordance with the immune-endocrine interaction hypothesis outlining the role that TNF- $\alpha$  plays in anxiety and

depression-like behaviour (Bernardini et al., 1990). This hypothesis builds on the finding that TNF- $\alpha$  stimulates the hypothalamic pituitary adrenal (HPA) axis by activating corticotrophin-releasing hormone and increasing levels of corticosterone (Bernardini et al., 1990). It is also responsible for stimulating anxiety responses as well as promoting depression like behaviour both in humans (Dean et al., 2010) and in murine models (Kaster et al., 2011, You et al., 2011, Kaster et al., 2012), with studies showing that blockage of TNF- $\alpha$  is able to reduce the severity of these effects (Turnbull et al., 1997).

The current results on TNF- $\alpha$  receptors are intriguing as they suggest that the effects of TNF- $\alpha$  on anxiety are mediated via different receptor signalling pathways, whereby TNF-R1 signalling may play a role in reducing anxiety-like behaviour. TNF-R2<sup>-/-</sup> mice that only signal through TNF-R1 travelled greater distance in the EZM than the WT and TNF-R1<sup>-/-</sup> mice, suggesting lower anxiety levels. However no differences between groups were observed in depression-like behaviour on the FST. These results differ from the work done by Simen et al (Simen et al., 2006) that showed less depression-like behaviour in mice deficient for either TNF- $\alpha$  receptor. Furthermore, the Simen study failed to observe any differences in anxiety-like responses in TNF- $\alpha$  receptor knockout mice as was seen here. This could potentially be due to the use of the elevated plus maze in their study as opposed to the use of the elevated zero maze apparatus used in the present study. While both apparatus' measure the same behavioural phenotype the use of the zero maze over the plus maze addresses the issue of ambiguity with time spent in the central square and seems to make the zero maze a more robust apparatus over the plus maze (Shepherd et al., 1994). While it is unclear at what age the mice were tested in the



Simen study, another reason for the differences observed between this and the current study could be due to a difference in the age of mice tested, as the effects of TNF- $\alpha$  on cognition change with ageing (McAfoose et al., 2009). It should be noted that behavioural testing in the present study was conducted during the light cycle and could be another reason for the differences observed between the present study and previous work (see) (Kopp, 2001).

Mice generally have a preference for social interactions with other mice (Moy et al., 2004) and this was observed in our study, with all strains spending more time with a stranger mouse than an empty cage in the first phase of the sociability test. Similarly, a previous study has shown that the deletion of TNF- $\alpha$  receptors decreased aggressive behaviour in mice and promoted social interactions (Erickson et al., 1994). It has been shown that TNF- $\alpha$  administration in the CNS caused a significant decrease in social interaction in WT and TNF-R2<sup>-/-</sup> mice whilst control mice of both strains had levels of social exploration similar, although they were not directly compared (Palin et al., 2007). As these mice have fully functional TNF-R1 it suggests that enhanced TNF-R1 signalling causes the change in social behaviour (Palin et al., 2007). However, the current study demonstrated a trend towards higher social behaviour in TNF-R1<sup>-/-</sup> mice, suggesting that removing this pathway enhanced social interaction. It should be noted that this did not, however, reach significance, with further research needed on the role of TNF-R1 signalling in decreasing social interaction.

The behavioural changes observed in TNF- $\alpha$  transgenic mice as noted in the current study may relate to changes in neurotrophic factor such as BDNF and NGF as levels of neurotrophins within the hippocampus are known to effect a number of

behaviour including the production of depressive-like symptoms (Castren et al., 2007) and cognition (Peng et al., 2005). However, in the current study, despite TNF-R2<sup>-/-</sup> mice having lower levels of BDNF than TNF<sup>-/-</sup> mice, there were no differences in any group compared to WT, suggesting it may not play a major role in the behavioural differences observed.

NGF, on the other hand, did show differences in the present study on TNF- $\alpha$  transgenic mice. NGF is an important factor in the survival and differentiation of neurons and for learning and memory (Sofroniew et al., 2001). In the present study, TNF<sup>-/-</sup> mice displayed lower levels of NGF as compared to WT mice, suggesting an interaction between TNF- $\alpha$  and NGF expression occurring under immunologically unchallenged conditions. Such an interaction has previously been suggested, however, this was found under inflammatory conditions. TNF- $\alpha$  is able to stimulate microglia to produce NGF and vice versa (Takei and Laskey, 2008), and in the absence of NGF, TNF- $\alpha$  can induce neuronal death through TNF-R1 signalling (Barker et al., 2001). The current study has shown that in unchallenged conditions, TNF<sup>-/-</sup> mice along with TNF-R2<sup>-/-</sup> mice displayed lower levels of NGF as compared to WT mice in both the PFC and the hippocampus, suggesting that NGF expression is dependent not only on TNF- $\alpha$  but also on TNF-R2 signalling. This is in accordance with previous literature showing that the effect of NGF on neurons is mediated by TNF- $\alpha$ , (Barker et al., 2001). Furthermore, it is interesting to note that hippocampal spatial memory in the BM and Y maze was impaired in the TNF<sup>-/-</sup> mice. These mice also exhibited low levels of NGF in the hippocampus, which suggests that perhaps TNF- $\alpha$  regulates hippocampal learning and memory through NGF signalling. However, due to the lack of similar results in the TNF- $\alpha$  receptor deficient mice, it

can be assumed that TNF- $\alpha$  receptor activation follows an alternative pathway in hippocampal dependent tasks. Whilst differential expression of the receptors on various cell types could result in behavioural differences, it is difficult to make firm conclusions on the underlying molecular pathways at this stage, because neurobiological adaptations could occur in the genetically modified mice. Further research is needed to address the issue of receptor mediated cognition-like behaviour.

It is also interesting to note that both, TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice that displayed significantly lower levels of NGF in the hippocampus and PFC, travelled the greatest distance in the EZM. Since both these areas play a role in anxiety like behaviour (Davidson, 2002, Sweatt, 2004), it could be suggested that NGF expression may play a role in modulating these behaviour. The use of NGF knockout mice by treating them with TNF- $\alpha$  agonists and antagonists would be effective in teasing out the interactions between TNF- $\alpha$  and NGF. Specifically as NGF knockout mice have been shown to have cognitive impairment compared to WT mice and have displayed pathologies akin to Alzheimer's disease – age dependent amyloid plaques, tau phosphorylation and neurofibrillary tangles, spatial learning impairments (Capsoni et al., 2000, Ruberti et al., 2000).

This is the first study to provide a thorough investigation of the phenotypic and biological changes occurring due to the deletion of the TNF- $\alpha$  gene including the main receptors in mice. Constitutive levels of TNF- $\alpha$  appear to be required for memory, as shown in the probe trial of BM, with a role of TNF- $\alpha$  in this cognition-like behaviour by both TNF-R1 and TNF-R2 signalling pathways, as shown in the training phase of the BM. Furthermore, lack of both TNF- $\alpha$  and TNF-R2 expression appear to

decrease anxiety- like behaviour (as seen in the EZM) and this may be linked to low levels of NGF seen in the TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice.

Understanding of how TNF- $\alpha$  signalling influences neurological function is important when developing therapeutic strategies that aim to inhibit the actions of TNF- $\alpha$  including a reduction of neuroinflammation. Although there have been promising results in the use of anti-TNF- $\alpha$  treatments, for example in Alzheimer's disease (AD) (Tobinick, 2010), it must be noted that while in chronic conditions blocking TNF- $\alpha$  may seem beneficial under inflammatory conditions, it also antagonises other physiologically essential actions of TNF- $\alpha$  in CNS function, potentially offering short term benefits which may not have long-term viability even under pathological conditions such as dementia. Drugs like etanercept are being experimentally tested for the treatment of Alzheimer's disease, and have been found to improve clinical symptoms of AD but it should be noted that use of this drug may increase susceptibility to infections due to a suppressed immune response following TNF- $\alpha$  blockade (Tobinick et al., 2006).

Given the role of TNF-R1 and TNF-R2 signalling may play in normal CNS functions, like cognition, it may be preferable to modulate TNF- $\alpha$  signalling, rather than completely blocking its affects. Complete blockade would prevent both the detrimental and the beneficial effects of TNF- $\alpha$ , but modulation of TNF- $\alpha$  signalling, may allow the beneficial effects to be preserved without the negative effects, thereby improving clinical symptoms.

As such drugs that have the pharmacological properties to modulate the expression and signalling of TNF- $\alpha$  and its receptors as opposed to inducing a

complete blockade should be the focus of future research. While this study provides findings on the effects of genetic deletion of TNF- $\alpha$  and its receptors on behaviour, it should be noted that “flanking gene effects” or “genetic background effects” may act as potential limitations to the study (Eisener-Dorman et al., 2009). However, despite these shortcomings the results of the present study have been consistent with previously published reports concerning TNF- $\alpha$  signalling and cognitive function as well as neurotrophin functions. Further work will need to be done to advance this field and better understand the actions of downstream products of TNF- $\alpha$  receptor signalling. Research comparing both the effects of genetic modification, and pharmacological blockade of TNF- $\alpha$  and its receptors on the behaviour of mice would be the next step in understanding TNF- $\alpha$  mediated CNS functions.

In the present study, the knockout mice were generated to lack total TNF- $\alpha$ . However it is very likely that in the TNF- $\alpha$  receptor knockout mice, the behavioural and neurobiological effects observed were due to a deficiency in both sol and tm TNF- $\alpha$ . As this study did not differentiate between the two types of TNF, it is difficult to assign a particular mechanism of action to each type. To circumvent these shortcomings more thorough investigations into the mechanisms between the different forms of TNF- $\alpha$  will be undertaken in future studies.

## **Chapter 4 : TNF- $\alpha$ and its Receptors Govern Adult Behavioural Phenotypes**

## Overview

TNF- $\alpha$  is a vital component of the immune system and CNS. We previously showed that 3-month-old TNF- $\alpha$  and TNF- $\alpha$  receptor knockout mice had impaired cognition, whilst at 12-months-old mice had better cognition. To extend these findings on possible age-dependent TNF- $\alpha$  effects in the brain, we investigated the behaviour of 6-month-old TNF- $\alpha$  knockout mice and their neurobiological correlates.

**AIM 2:** To study the effects of knockout of TNF- $\alpha$  and its receptors on behaviour and neurobiology of mid-adult mice.

6-month-old TNF<sup>-/-</sup>, TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice were compared to age-matched WT mice and tested for various behaviours. ELISA hippocampal levels of nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) and qPCR mRNA levels of *Tnfa*, *Tnfr1*, *Tnfr2*, *Il10* and *Il1 $\beta$*  were measured. Neurogenesis markers and microglial numbers were assessed by immunohistochemistry within the dentate gyrus.

TNF-R1<sup>-/-</sup> and TNF<sup>-/-</sup> mice were found to have lesser exploratory behaviour than WT mice, while TNF-R1<sup>-/-</sup> mice displayed better memory than WT and TNF-R2<sup>-/-</sup> mice. Both TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice exhibited lower immobility on the depression test than WT mice. Additionally, TNF<sup>-/-</sup> mice expressed significantly lower levels of BDNF than WT mice in the hippocampus while TNF-R1<sup>-/-</sup> mice displayed significantly lower BDNF levels compared to both WT and TNF-R2<sup>-/-</sup> mice. TNF-R2<sup>-/-</sup> mice also displayed significantly higher levels of NGF compared to TNF-R1<sup>-/-</sup> mice.

These results illustrate that TNF- $\alpha$  and its receptors mediate several behavioural phenotypes. Specifically, the absence of TNF- $\alpha$  and TNF-R2 protected mice from depression, suggesting TNF- $\alpha$  may be involved in the pathogenesis of

depression. Finally, BDNF and NGF levels appear to be regulated by TNF- $\alpha$  and its receptors even under immunologically unchallenged conditions.



## Introduction

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), when signalling through its receptors TNF-R1 and TNF-R2 is known to play a vital role in inflammatory processes of the central nervous system (CNS) (Medeiros et al., 2007, Dowlati et al., 2010) as well as the neurobiology of behavioural systems (Albensi and Mattson, 2000, Golan et al., 2004). The two receptors of TNF- $\alpha$  are known to have differential roles in the CNS; whereby TNF-R1 appears to mediate inflammatory processes and activate apoptotic pathways through activation of a death domain (Tartaglia et al., 1993). On the other hand, TNF-R2 signalling leans towards a more neuroprotective pathway in physiological contexts due to the absence of a death domain (Rothe et al., 1995).

Apart from these inflammatory functions, both receptors play a role in behaviour; in receptor knockout mice, the lack of these receptors was linked to cognitive and emotional impairment (Simen et al., 2006, Camara et al., 2013). In young adult mice the expression of TNF- $\alpha$  and its receptors is required for normal cognitive performance however lack of TNF- $\alpha$  and its receptor signalling decreases anxiety-like behaviour (Baune et al., 2008, Camara et al., 2013). This group has also shown that when TNF<sup>-/-</sup> mice were aged to 6 and 12 months, there was a significant improvement in cognition-like abilities in older knockout mice compared to WT mice, indicating lack of TNF- $\alpha$  may be beneficial to cognitive processes as ageing progresses (McAfoose et al., 2009). While work within this group has shown that TNF- $\alpha$  deficient mice had improved cognitive abilities in 12 month and 6 month old mice compared to 3 month old mice (McAfoose et al., 2009), the role of TNF- $\alpha$  and its receptors in emotion-like behaviour is less well understood. A recent study reported a preference of aged (22 month old mice) TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice for

the closed arm of the elevated plus maze, which was attributed to either decreased anxiety-like behaviour or a lack of preference for an arm of the maze (Naude et al., 2014). Lack of these receptors was also linked to decreased anhedonia, depression-like behaviour and fear conditioning in TNF-R1<sup>-/-</sup> mice (Simen et al., 2006). Additionally this group has previously shown that 3 month old TNF- $\alpha$  and TNF- $\alpha$  receptor knockout mice had normal social behaviour like WT mice (Camara et al., 2013) consistent with studies showing TNF- $\alpha$  receptor deficient mice had decreased aggression more and sociability (Erickson et al., 1994).

In addition to the behavioural changes orchestrated by TNF- $\alpha$  and its receptors, these receptors of TNF- $\alpha$  appear to mediate neurotrophin expression. Previous work within this group also showed that genetic deletion of TNF- $\alpha$  and its receptors affects not only behaviour but also neurotrophin expression in the hippocampus of young adult (3 month old) mice. Specifically, TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice both had significantly lower levels of nerve growth factor (NGF) compared to WT mice, while brain derived neurotrophic factor (BDNF) levels in TNF-R2<sup>-/-</sup> mice were lower than TNF<sup>-/-</sup> mice (Camara et al., 2013). This is important as disruptions in neurotrophins like NGF and BDNF are linked to impaired cognition as well as development of depressive phenotypes (Peng et al., 2005, Castren et al., 2007).

TNF- $\alpha$  expression in the CNS is also influenced by microglia, the resident immune cells of the brain (Ransohoff et al., 2003). Stimulated microglia produce TNF- $\alpha$  in the CNS resulting in a positive feedback loop where TNF- $\alpha$  can further stimulate microglia to produce more TNF- $\alpha$  (Nadeau and Rivest, 2000). Additionally stimulated microglia can produce both TNF- $\alpha$  and NGF under inflammatory

conditions (Elkabes et al., 1998). Interestingly microglia are also shown to be more activated in aged individuals (human and rodent studies) without any inflammatory challenge (Perry et al., 1993, Streit and Sparks, 1997). This phenomenon was made clear in another study by this group; where it was found that a single high dose of LPS was effective in increasing microglial numbers in the dentate gyrus, and that treatment with TNF- $\alpha$  antagonist etanercept was effective in reducing microglial numbers (Camara et al., 2014).

While the literature surrounding ageing is quite extensive and we know that ageing is characterised by disruptions in the immune system resulting in a decrease in the number of T cells, along with up-regulation of cytokines like TNF- $\alpha$  (Jiang et al., 2009, Corona et al., 2012), the work done on mid-adulthood is less expansive. We now know that in normal ageing there appears to be a decline in the functionality of the immune system, that makes ageing individuals (commencing from middle age) vulnerable to infections and diseases (Corona et al., 2012), but it is yet unclear when this change begins to occur.

As reported above, the majority of previous studies on the effects of TNF- $\alpha$  and its receptor signalling on behaviour were conducted in young or old mice with age-dependent differential results. It is unclear however, if such an age-dependent change in TNF- $\alpha$  function in the brain occurs gradually over time or more abruptly. This study was therefore conducted in 24 week old mice representing a mid-adult age group. This approach allows a comparison of findings over time between young adult, mid-adult and old age mice of the same genetic background in independent cohorts. Investigated presently are a range of behavioural phenotypes in TNF- $\alpha$  and TNF- $\alpha$  receptor deficient mice of mid-adult age. In addition, I have explored

neurotrophin expression and microglial numbers in the hippocampus in these mice. Finally, I discuss these findings in relation to previously published reports across age groups to better understand the hypothesised age-dependent effects of TNF- $\alpha$  in the brain. I hypothesised that investigation of behaviour in 6-month-old mice, would be a good mid-way point to detect changes in TNF- $\alpha$  mediated behaviour ranging from the deficits seen at 3 months of age (Baune et al., 2008) to the benefits seen at 12 months of age in previous independent studies on cognition in TNF<sup>-/-</sup> mice by this group (McAfoose et al., 2009).

## Methods

### *Mouse Strains*

A gene-targeted C57BL/6 mouse strain deficient for TNF- $\alpha$  (TNF<sup>-/-</sup>) (n=14, 7/male, 7/female mice) was generated on a genetically pure C57BL/6 background (Korner et al., 1997a). C57BL/6 (WT) mice (Jackson stock number: 000664) were purchased from the University of Adelaide breeding facility (n=14, 7/male, 7/female). TNF-R1<sup>-/-</sup> (Jackson stock number: 003242) (Peschon et al., 1998) and TNF-R2<sup>-/-</sup> mice (Jackson stock number: 002620) (Erickson et al., 1994) were obtained from Jackson Laboratories (Bar Harbor, ME) (n= 14 per strain, 7/male, 7/female per strain) and were also established on a C57BL/6 background (Baune et al., 2008). All mice were bred in house for over ten generations. Equal number of male and female mice were chosen for this study to create a comparable study design to our previous study in 3 month old mice, and no difference in behaviour was noted between sexes (Camara et al., 2013). Test mice were genotyped and confirmed to be knockout mice.

Mice of 6 months of age at the start of behavioural testing were used in all experiments. All mice were housed in groups of 3 to 6 mice per cage during the experimental period, with food and water available ad libitum and were handled prior to behavioural testing. Ambient temperature of the housing and testing rooms was  $22 \pm 1^{\circ}\text{C}$ . Mice were housed under a 12 hr light-dark cycle, lights on at 0700 hr, and all behavioural testing was conducted between 0800 hr and 1600 hr. Animal procedures were approved by the University of Adelaide Animal Ethics committee.

**Table 4.1: Mice strains used in this study**

Strain	C57/BL6	TNF <sup>-/-</sup>	TNF-R1 <sup>-/-</sup>	TNF-R2 <sup>-/-</sup>
<b>Genotype</b>	Wild type mice	Deletion of TNF- $\alpha$ gene on C57/BL6 background	Deletion of TNF- $\alpha$ receptor1 (p55) gene on C57/BL6 background	Deletion of TNF- $\alpha$ receptor2 (p75) gene on C57/BL6 background
<b>Phenotype</b>	Express TNF- $\alpha$ (and other cytokines) normally	Do not express TNF- $\alpha$	Absence of TNF-R1 signalling	Absence of TNF-R2 signalling
<b>Age at which Analysed</b>	6 months			

**Legend:** The Table shows the different strains of mice used for this study, along with their genotype, phenotype and the age at which they were analysed.

### ***Behavioural Analysis***

A comprehensive behavioural battery was carried out incorporating several different behavioural types. Tests were conducted in the following order: (1) Home Cage Locomotor Activity, (2) Open Field Test (OFT), (3) Hole-Board Exploration (HBE), (4) Elevated Zero Maze (EZM), (5) Sociability, (6) Barnes Maze (BM) and (7) Forced Swim Test (FST) and run over 4 weeks (Detailed in Table 4.2). Mice were given at least one day off between tests and tests were conducted in order of least to most stressful, to minimize effects of previous testing, similar to previous studies (Camara et al., 2013, Jaehne and Baune, 2014). A single test was not repeated in

the same mouse. Equipment for all tests was purchased from Stoelting Co. (USA) and all movements were tracked using ANY-MAZE imaging software (Stoelting Co., USA).

### **Locomotor Activity:**

#### **Home Cage Locomotor Activity:**

Mice were individually tested for general locomotor activity in home cages with two-day-old bedding under basal non-stressful conditions according to previous published protocols (Baune et al., 2008). Total distance covered over a 5 min period was measured.

#### **Open Field Test (OFT):**

Under more stressful conditions basal locomotor activity was measured. Mice were placed at the centre of a brightly lit plexiglass box (40 X 40 cm) and movements were tracked over a 5 min period as previously published (Baune et al., 2008). Distance travelled and time spent in the outer and inner zones of the box were calculated.

#### **Anxiety-Like Behaviour: Elevated Zero Maze (EZM)**

The elevated zero maze was used to measure anxiety-like behaviour. The apparatus consists of a grey Perspex circular platform of 50cm diameter and width of 5cm, located at a height of 40cm above ground. The platform is divided into four equal quadrants, two closed quadrants with grey Perspex walls (27cm high) on the inside and outside, and two open quadrants without walls. Mice were placed at the

centre of either one of the open arms and allowed to explore the apparatus for 5min. Anxiety was measured by comparing the time spent in the open arm, where an anxious mouse will spend less time (Cryns et al., 2007).

### **Exploratory Activity: Hole Board Exploration Test (HBE)**

The hole-board exploration test is set up in an apparatus similar to the open field test, and utilises a hole-board insert, which has 16 holes of 1.5cm diameter, spaced 6cm apart. Mice were placed into the apparatus and allowed to explore for 5min. Head dipping behaviour is manually recorded and is an indicator of exploration (Camara et al., 2013) .

### **Cognition-Like Behaviour:**

#### **Spatial Memory and Learning: Barnes Maze (BM):**

The Barnes maze consists of a bright, circular white platform (91cm) with 20 holes, which contain either false boxes, or one hidden escape box. The false boxes remove visual cues that might be observed through an open hole. BM procedures were carried out over a 5 day period according to published protocols and time taken to find the escape box recorded (Camara et al., 2013).

**Training** (Days 1-4): Mice were briefly placed in the centre of the maze under a removable chamber and given 3min to locate the escape box. Mice that failed to enter the escape box within 3min were guided to the box and placed there for 2min prior to returning to their home cage. Each mouse was subjected to 3 trials per day, separated by 15min, for 4 days. The average of the time taken to find the escape box across the 4 day period was then calculated. Mice that failed to enter the escape



box during the trial period were given a test time of 3min. Lower escape latencies represent good spatial memory.

**Probe trial** (Day 5): Each mouse was given a probe trial of 3min duration each, with the escape box rotated 90°. Latency to the old escape locations was recorded. Short latencies to locate the old escape location were considered to indicate good spatial memory retention for the original location.

### **Depression-Like Behaviour:**

#### **Forced Swim Test (FST)**

The forced swim test is a measure of despair and depression-like behaviour, and was performed according to previously published results (Petit-Demouliere et al., 2005). The apparatus consists of a 4 L cylinder (20 cm diameter) filled up to 20 cm with water (23-24°). Mice were placed in water and movement was tracked over a period of 6 min. Immobility was considered a measure of depression-like behaviour, and was defined as lack of motion beyond which was required for the mouse to keep its head above water. For analysis, the first 2 min were excluded, since mice are mobile during that time and familiarizing themselves with the environment and any immobility during this time therefore cannot be attributed to despair.

#### **Social Behaviour: Sociability Test**

The sociability test measures general social behaviour and interest in social novelty in rodent models. Rodents have a preference for social interaction with other rodents and will also prefer to interact with a novel mouse over a familiar one (Moy et

al., 2004). The apparatus consists of a three chamber rectangular box, the walls of which are plexiglass. Each chamber is 20 cm in length, 40.5 cm in width and 22 cm high. The test runs in three stages (Moy et al., 2004).

**Habituation:** The test mouse is placed in the central chamber and allowed to explore the entire apparatus for 5 min.

**Sociability:** A stranger mouse was placed in one of the side chambers (left or right) in a round wire cage that allows nose contact between the bars but prevents fighting. Doors to all chambers were opened and the test mouse allowed to explore the entire box. The time spent by mice interacting with the stranger mouse vs. the empty cage was recorded. A preference for the stranger mouse for each test mouse was calculated as follows: Time spent interacting with stranger mouse/ (Time spent with stranger mouse + time spent with empty cage). Values approaching 1 were considered high preference.

**Social Novelty:** A second novel stranger mouse was placed in the chamber that was empty during the sociability phase. The test mouse was allowed to explore the box for 5min and the time spent interacting with the novel mouse over the familiar mouse recorded. A preference for the novel mouse for each test mouse was calculated as follows: Time spent interacting with novel mouse/ (Time spent with novel mouse + time spent with familiar mouse). Values approaching 1 were considered high preference.

Stranger mice used for both phases were C57BL/6 mice of the same age and sex as the test mice.

**Table 4.2: Behavioural testing schedule**

	Monday	Tuesday	Wednesday	Thursday	Friday
<b>Week 1</b>	Home Cage	Recovery Day	OFT	Recovery Day	
<b>Week 2</b>	Hole Board Exploration	Recovery Day	Elevated Zero Maze	Recovery Day	Sociability Test
<b>Week 3</b>	BM training Day 1	BM training Day 2	BM training Day 3	BM training Day 4	Barnes Maze Probe trial x 2
<b>Week 4</b>	Forced Swim Test	Tissue Collection			

**Legend:** The table shows the behavioural testing schedule for experiments. Mice were tested in groups of 21 over a period of 5 weeks.

### ***Protein Analyses***

#### **ELISA**

In order to evaluate whether there were any underlying changes in neurotrophin levels within the TNF- $\alpha$  transgenic mice, a subset of mice ( $n = 7$  mice/strain), were sacrificed by Pentobarbital overdose and the brain rapidly removed. The hippocampus was isolated and stored fresh frozen at  $-80^{\circ}\text{C}$  until analysed. The hippocampus plays a role in spatial cognition (Sweatt, 2004) as well as some emotional responses (Sweatt, 2004). Tissue was homogenised in a solution containing 50 mL TRIS, one protease inhibitor tablet (Roche) and 50  $\mu\text{L}$  of Triton-X. Homogenates were then centrifuged at 8500 rpm (Davidson, 2002) for 15 min at  $4^{\circ}\text{C}$ , and the supernatant transferred to 1.5 mL eppendorf tubes. All samples were stored at  $-80^{\circ}\text{C}$  until analysis. Amount of protein was quantified using the Bradford

method and samples then diluted to contain 50 ng of protein. NGF and BDNF ELISAs were then performed using the E<sub>max</sub> Immuno Assay system (Promega) according to the manufacturer's instructions and as previously published (Sei et al., 2000, Gilmore et al., 2003, Camara et al., 2013).

### **Cytometric Bead Array (CBA)**

In order to demonstrate that these behavioural changes were in a non-inflammatory setting and to examine the effect of knocking out TNF- $\alpha$  or its receptors on other cytokines, following completion of behavioural testing mice were sacrificed, blood was collected by means of cardiac puncture and serum was extracted by spinning blood down at 2500 rpm for 15 min. Serum was stored at -80°C until analysis. Serum cytokine levels were measured using the BD Cytometric Bead Array (CBA) Mouse Inflammation Kit for the cytokines IL-6, IL-10, MCP-1, IFN- $\gamma$ , TNF (total, both TNF- $\alpha$  and TNF- $\beta$ ) and IL-12p70 according to the manufacturer's instructions.

### **Gene Expression Analysis (RT-qPCR)**

A subset of mice was selected for qPCR analysis to measure the expression of *Tnfa*, *TnfaR1*, *TnfaR2*, *Il-10*, *Il-1b* (n = 4/strain). Mice were sacrificed via a lethal dose of pentobarbital via IP injection and hippocampal tissue from mice was collected and stored in RNeasy lysis buffer (Qiagen, Crawley, UK) at -20°C until required for RNA extraction. The hippocampus was analysed as it plays a role in spatial cognition (Sweatt, 2004) as well as some emotional responses (Sweatt, 2004).

Brain tissue was lysed and homogenised using the TissueLyserLT (Qiagen, Australia) with 5mm stainless steel lysis bead (Qiagen, Australia). RNA was then extracted from the homogenised tissue using PureLink® RNA mini extraction kit (Ambion) as per manufacturer's instructions. RNA was then quantified using Nanodrop Lite (Thermo Fischer Scientific). Gene expression analysis for *Tnfa*, *TnfaR1*, *TnfaR2*, *Il-10*, *Il-1b* was performed by RT-qPCR in two steps. First, complementary DNA (cDNA) was synthesised from the total RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen) in a thermal cycler (Kyrtec) following manufacturer's instructions. To standardise the concentration of the products they were diluted to a concentration of 10 ng/ $\mu$ l of cDNA for qPCR.

In the next step qPCR was performed on the cDNA using the Applied Biosystems 7900HT real-time PCR machine (ABI, Life technologies). Expression of *Tnfa*, *Tnfr1*, *Tnfr2*, *Il10*, *Il1b* was quantified relative to *Gapdh*, the endogenous reference gene using the primers listed below;

**Table 4.3: Primer Sequence for qPCR**

Primer Sequence	
<i>Tnfa</i>	Forward: CCACCACGCTCTTCTGTCTA Reverse: AGGGTCTGGGCCATAGAACT
<i>Tnfr1</i>	Forward: GCAGTGTCTCAGTTGCAAGACATGTCGG Reverse: CGTTGGAAGTGGTTCTCCTTACAGCCAC
<i>Tnfr2</i>	Forward: ACAGTGCCCGCCCAGGTTGTCTTG Reverse: GCAGAAATGTTTCACATATTGGCCAGGAGG
<i>Il10</i>	Forward: ACTGCACCCACTTCCCAGT Reverse: ATGTTGTCCAGCTGGTCCTT
<i>Il1b</i>	Forward: CTCATCTGGGATCCTCTCCA Reverse: TGTCAAAAGGTGGCATTTC
<i>Gapdh</i>	Forward: TGTTCTACCCCAATGTGT Reverse: CCTGCTTCACCACCTTCTTG

**Legend:** Details of primers used for qPCR analysis to measure *Tnfa*, *Tnfr1*, *Tnfr2*, *Il10* and *Il1b* gene expression

### **Immunohistochemistry (IHC)**

Animals were injected IP with pentobarbital and perfused via transcardiac injection with 10% neutral buffered formalin, with the brains then processed and embedded in paraffin wax. Serial sections spaced 150  $\mu$ m apart were taken of the entire length of the hippocampus. Sections were then assessed for levels of neuroinflammation (IBA1) and neurogenesis markers Doublecortin (DCX), which labels immature neurons, and Ki67, which labels dividing cells via immunohistochemistry (List of antibodies detailed in Table 4.4). Briefly, slides were de-waxed, dehydrated and endogenous peroxidase activity was blocked, before

undergoing antigen retrieval by heating slides at boiling point for 10 min in citrate solution. Non-specific binding was then specific binding blocked with normal horse serum, before application of the appropriate primary antibody with incubation overnight (Table 4.4). The next day slides were incubated with the appropriate species of secondary antibody (Abacus, 1:250) followed by streptavidin peroxidase. The antigen-antibody complex was then detected with Diaminobenzadine (DAB) (Sigma) followed by counter staining with haematoxylin prior to dehydration and mounting with DePex mounting media.

Slides were digitally scanned using the Nanozoomer (Hamamatsu) and viewed with the associated software (NDP view). Serial images of the CA region of the hippocampus and the dentate gyrus of each section were captured as jpegs. These images were then exported to Image J (NIH) and the number of positive cells manually counted.

**Table 4.4: Immunohistochemistry stains**

<b>Cells/Proteins of Interest</b>	<b>Primary Antibody</b>	<b>Dilution (In Normal Horse Serum)</b>	<b>Secondary Antibody</b>	<b>Retrieval Buffer</b>
Microglia	IBA1	1:1000	Anti-Goat	Citrate
Proliferating Cells	Ki67	1:2000	Anti-Rabbit	Citrate
Immature Neurons	Doublecortin (DCX)	1:8000	Anti-Guinea Pig	TRIS

**Legend:** The table shows a list of the stains used for immunohistochemistry.

### ***Statistical Analysis***

Data analyses were carried out using GraphPad Prism software (version 5.01). All data are presented as mean  $\pm$  standard error of mean (SEM). Data was checked for normal distribution (tested with Kolmogorov-Smirnov test) and based on this, comparisons were performed using 1 way ANOVA with Tukey's post hoc test for comparisons between strains or repeated measures 2-way ANOVA with Bonferroni's post hoc test when comparisons within strains were required, as stated throughout the results. Comparisons of 2 parameters were carried out by unpaired T-test (parametric).  $P < 0.05$  was taken as significant for all tests.



## Results

### *Age and Weight of Mice*

All mice were tested from 6 months of age and testing continued for 5 weeks. Mice were weighed before commencing behavioural testing and were weighed each week until the completion of behavioural testing. Over this time period, male mice of all strains weighed significantly more than female mice (Interaction:  $F_{(3,38)} = 5.14$ ,  $P = 0.0044$ ; Strain:  $F_{(3,38)} = 30.33$ ,  $P < 0.0001$ ; Gender:  $F_{(3,38)} = 127.5$ ,  $P < 0.0001$ ). The average weight of male mice was WT:  $30.06 \pm 0.86$ , TNF<sup>-/-</sup>:  $31.44 \pm 0.22$ , TNF-R1<sup>-/-</sup>:  $22.83 \pm 0.16$ , TNF-R2<sup>-/-</sup>:  $29.58 \pm 0.55$ . While the average weight of female mice was WT:  $25.75 \pm 0.73$ , TNF<sup>-/-</sup>:  $24.74 \pm 0.33$ , TNF-R1<sup>-/-</sup>:  $19.89 \pm 0.11$ , TNF-R2<sup>-/-</sup>:  $21.83 \pm 0.41$ .

One-way ANOVA analysis of combined weight showed a significant difference in weight between strains ( $F_{(3,42)} = 5.20$ ,  $P = 0.0038$ ). Tukey's post hoc test revealed that TNF-R1<sup>-/-</sup> mice weighed significantly less than all other strains of mice (WT:  $P = 0.0043$ , TNF<sup>-/-</sup> mice:  $P = 0.0033$ , TNF-R2<sup>-/-</sup> mice:  $P = 0.031$ ). The mean weight of mice over the 5-week testing period was, WT:  $27.60 \pm 0.79$ , TNF<sup>-/-</sup>:  $27.84 \pm 0.98$ , TNF-R1<sup>-/-</sup>:  $21.07 \pm 0.72$ , TNF-R2<sup>-/-</sup>:  $26.26 \pm 1.12$ .

### *Baseline Locomotor Activity*

Baseline locomotor activity was measured using the HC and OFT test. No differences between strains were observed in the home cage test (One-way ANOVA:  $F_{(3,43)} = 1.84$ ,  $P = 0.15$ ; data not shown). In the more stressful OFT there was also no difference in distance travelled observed between strains (One-way ANOVA  $F_{(3,45)} =$

0.54,  $P = 0.65$ ; Figure 4.1A). These results indicate that differences observed between strains in the subsequent tests are an accurate reflection of differences in behaviour and not merely a difference in locomotor activity. Since the OFT is set in a stressful situation, we also measured the time spent by mice in the inner zone (more stressful environment). Analysis of time spent in the inner zone of the OFT showed a significant difference between strains (one-way ANOVA:  $F_{(3,41)} = 4.38$ ,  $P = 0.0096$ ; Figure 4.1B). Tukey's post hoc analysis indicated that TNF-R2<sup>-/-</sup> mice spent less time in the centre than both WT ( $P = 0.043$ ) and TNF<sup>-/-</sup> ( $P = 0.027$ ), suggesting increased levels of anxiety.

### ***Anxiety-like Behaviour***

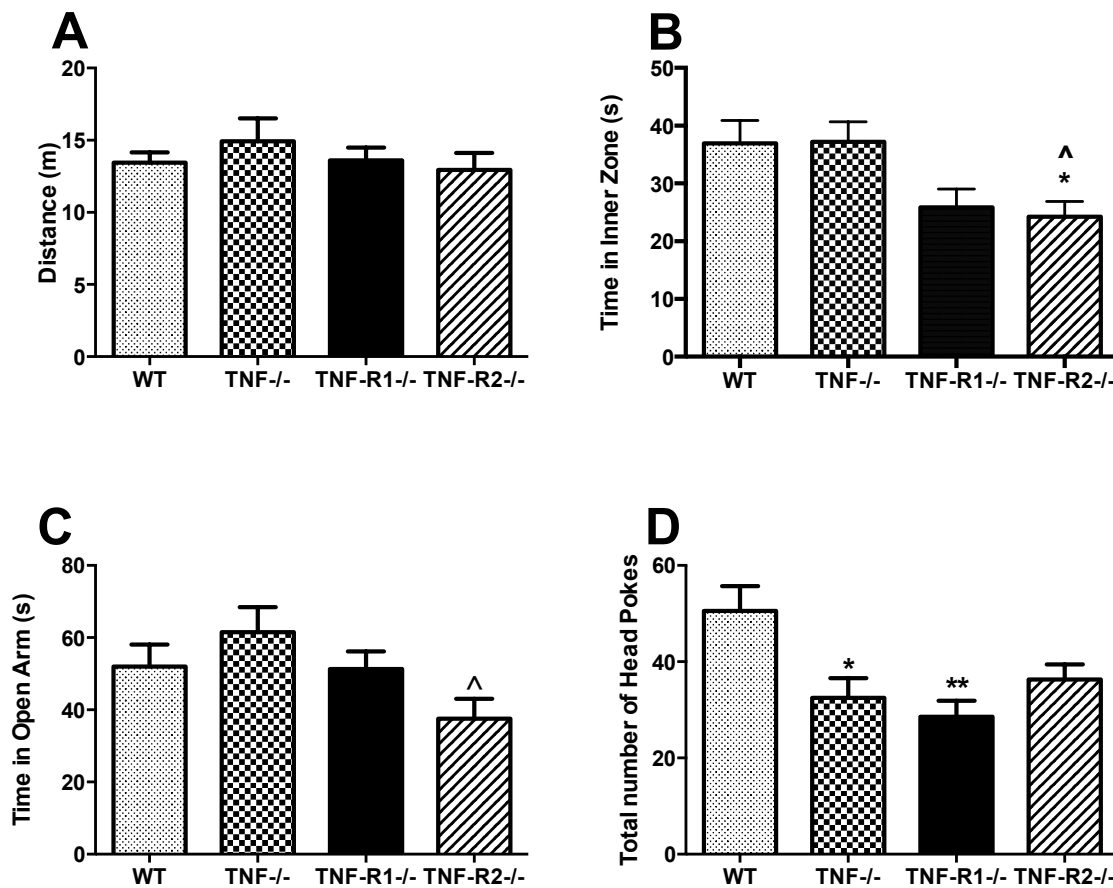
Under more anxious settings time in the open arm of the EZM was also recorded, where less time spent in the open arms was considered higher anxiety-like behaviour. One-way ANOVA of time spent in the open arm showed a significant difference between strains ( $F_{(3,42)} = 3.005$ ,  $P = 0.04$ ; Figure 4.1C) and Tukey's multiple comparison indicated that, TNF-R2<sup>-/-</sup> mice spent significantly less time in the open arm than TNF<sup>-/-</sup> mice ( $P = 0.02$ ), again suggesting higher levels of anxiety in TNF-R2<sup>-/-</sup> mice. However, no strains performed significantly differently to WT.

### ***Decreased Exploratory Behaviour in TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> Mice***

The total number of head pokes was measured in the HBE test and used as an indicator of exploratory behaviour, whereby higher numbers of head pokes indicates greater exploratory behaviour. One-way ANOVA analysis showed a

significant difference between strains ( $F_{(3,45)} = 5.01$ ,  $P = 0.004$ ; Figure 4.1D) and further analysis by Tukey's multiple comparison test showed that TNF $^{-/-}$  ( $P = 0.015$ ) and TNF-R1 $^{-/-}$  mice ( $P = 0.0092$ ) had significantly lower number of head pokes than WT mice, indicating lower exploratory behaviour in these mice.

**Figure 4.1: Baseline locomotor activity, anxiety and exploratory behaviour**



**Legend:** Distance travelled in A) the open field, B) Time in the inner zone of OFT C) Time spent in the open arm of EZM, D) Total number of head pokes in the hole board exploration test. All data represent mean  $\pm$  SEM ( $n = 14$ /group for all strains). Data compared using One-way ANOVA with Tukey's multiple post hoc comparison. (\*  $P < 0.05$  compared to WT, \*\*  $P < 0.01$  compared to WT, ^  $P < 0.05$  compared to TNF $^{-/-}$  mice).

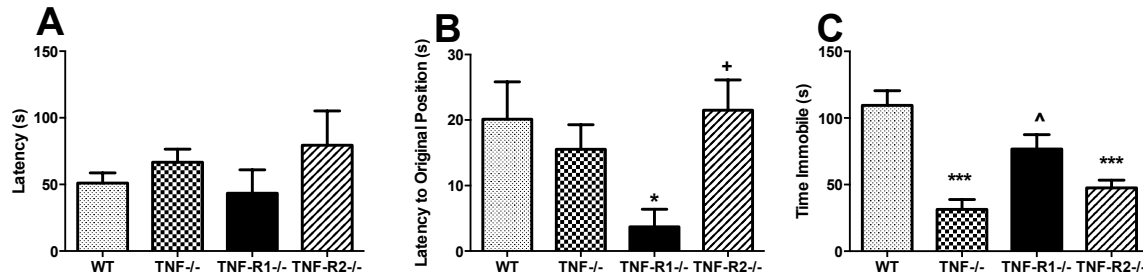
### ***Cognition measured in the Barnes Maze***

The Barnes maze was used to measure spatial learning and memory in the present study. The training phase of the Barnes maze (learning component,) is used to measure learning, wherein a shorter latency to the escape box indicates effective learning. Differences in learning between the strains over the 4-day test period (one-way ANOVA:  $F_{(3,39)} = 3.2$ ,  $P = 0.031$ ; Figure 4.2A) were observed without any post hoc differences. Interestingly in the probe trial, that was used to measure spatial memory (Lower latency to the original escape box indicates effective memory consolidation, Figure 2B), a significant difference between strains was noted for latency to find the original position (one-way ANOVA:  $F_{(3,42)} = 3.67$ ,  $P = 0.019$ ). An analysis by Tukey's post hoc test showed TNF-R1<sup>-/-</sup> mice displayed significantly better memory than WT ( $P = 0.046$ ) and TNF-R2<sup>-/-</sup> mice ( $P = 0.019$ ), indicating that TNF-R1<sup>-/-</sup> mice had better memory than these other strains of mice.

### ***Decreased Depression-like Behaviour in TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> Mice***

Depression-like behaviour was measured using the FST (Figure 4.2C) and higher immobility time was considered a measure of greater despair, or depression-like behaviour. One-way ANOVA analysis showed a significant difference between strains ( $F_{(3,45)} = 16.35$ ,  $P < 0.0001$ ) and Tukey's multiple comparison tests indicated that TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice had significantly lower immobility than WT mice ( $P < 0.001$  for both strains). In addition, TNF<sup>-/-</sup> mice displayed significantly lower immobility than TNF-R1<sup>-/-</sup> mice ( $P = 0.011$ ). These results indicate that lack of TNF and TNF-R2 is associated with less depression-like behaviour as compared to WT mice in mid-adult mice.

**Figure 4.2: Cognition**



**Legend:** (A) Mean Latency to escape hole in Barnes Maze training phase (B) Latency to original position in the probe trial of the Barnes maze, (C) Time spent immobile during the last 4 min of the forced swim test. All data represent mean  $\pm$  SEM ( $n = 14$ /group for all strains). Data compared using 2-way ANOVA for Y maze and Bonferroni's post hoc test and one-way ANOVA for Barnes Maze analyses followed by Tukey's post hoc test (\*  $P < 0.05$ , \*\*\*  $P < 0.001$  compared to WT, ^  $P < 0.05$  compared to TNF<sup>-/-</sup> mice +  $P < 0.05$  compared to TNF-R1<sup>-/-</sup>).

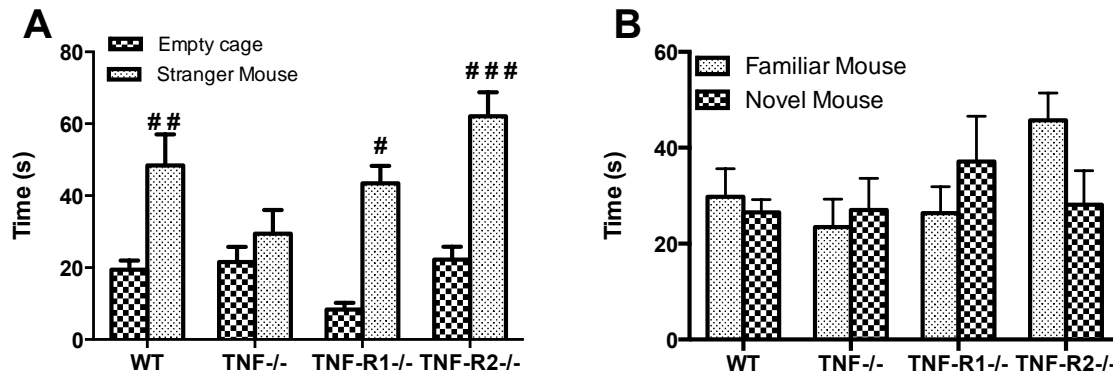
### **Social Behaviour in Mice**

Social behaviour was measured using the sociability test. The first phase of the test was used to measure normal social behaviour in mice, where more time spent by mice interacting with the stranger mouse over the empty cage indicated normal social behaviour (Figure 4.3A). Analysis by repeated measures two-way ANOVA showed that mice spent significantly more time interacting with the stranger mouse than they did with the empty cage (Interaction:  $F_{(3,44)} = 2.54$ ,  $P = 0.069$ , Strain:  $F_{(3,44)} = 4.77$ ,  $P = 0.0057$ , Stranger mouse/Empty Cage:  $F_{(1,44)} = 34.63$ ,  $P < 0.0001$ ). Bonferroni's post hoc test further showed that WT ( $P = 0.0053$ ), TNF-R1<sup>-/-</sup> ( $P = 0.021$ ) and TNF-R2<sup>-/-</sup> mice ( $P < 0.0001$ ) all spent significantly more time interacting with the stranger mouse over the empty cage, indicating normal social behaviour. TNF<sup>-/-</sup> mice however showed poor social behaviour as they did not spend

more time with the stranger mouse over the empty cage ( $P > 0.99$ ). A preference index was also calculated to verify these results. Analysis of the preference index showed that WT ( $0.67 \pm 0.04$ ), TNF-R1<sup>-/-</sup> ( $0.71 \pm 0.09$ ) and TNF-R2<sup>-/-</sup> mice ( $0.72 \pm 0.04$ ) had a preference index approaching 1, indicating preference for the stranger mouse, whereas TNF<sup>-/-</sup> mice had a preference index around 0.5 ( $0.51 \pm 0.08$ ) indicating no preference for the stranger mouse. Analysis however showed no significant difference between strains in preference for the stranger mouse over the empty cage (one-way ANOVA:  $F_{(3,42)} = 2.65$ ,  $P = 0.06$ ).

The second phase of the test was used to measure social novelty, wherein time spent interacting with the novel mouse over the familiar mouse indicated normal preference for social novelty behaviour. No significant difference across strains between time spent with familiar and novel mouse was noted (Interaction:  $F_{(3,44)} = 1.48$ ,  $P = 0.23$ , Strain:  $F_{(3,44)} = 1.90$ ,  $P = 0.14$ , Novel/Familiar mouse:  $F_{(3,44)} = 0.11$ ,  $P = 0.74$ , Subjects (matching)  $P = 0.91$ ; Figure 4.3B), indicating abnormal social recognition in all strains. Further analysis of preference index for time spent interacting with novel mouse over the familiar mouse showed that all strains had a preference index of 0.5 or below, indicating no significant preference for the novel stranger mouse, with no difference between strains seen (one-way ANOVA:  $F_{(3,43)} = 1.03$ ,  $P = 0.38$ ).

**Figure 4.3: Social Behaviour**



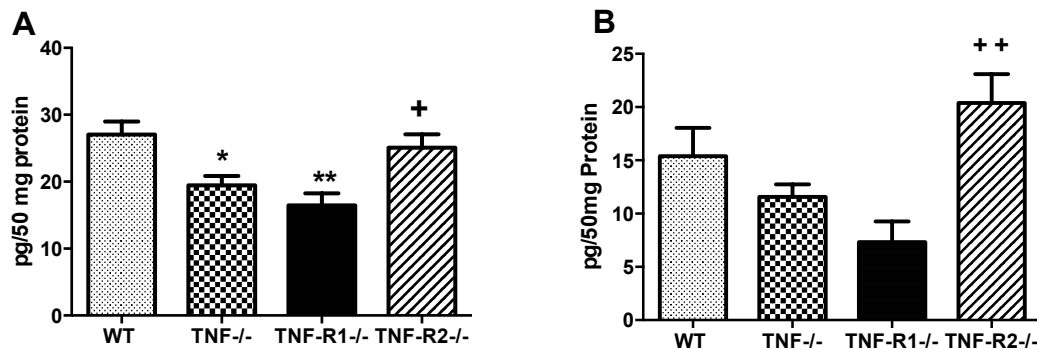
**Legend:** A) Time spent interacting with a stranger mouse compared to an empty cage in the Sociability stage, B) time spent interacting with a familiar and novel stranger mouse in the Preference for social novelty stage of the test. All data represent mean  $\pm$  SEM ( $n = 14/\text{group}$  for all strains). Data compared using 2-way ANOVA and Bonferroni's post hoc test (##  $P < 0.01$ , ###  $P < 0.001$ ).

### ***High Levels of BDNF and NGF in the Hippocampus of TNF-R2<sup>-/-</sup> Mice***

Hippocampal levels of BDNF and NGF were measured by ELISA. One-way ANOVA showed a significant difference between strains ( $F_{(3,25)} = 6.23$ ,  $P = 0.0026$ ; Figure 4.5A). Tukey's multiple comparison further showed that TNF<sup>-/-</sup> mice ( $P = 0.031$ ) and TNF-R1<sup>-/-</sup> mice ( $P = 0.0064$ ) had significantly lower levels than WT mice but no difference between TNF-R2<sup>-/-</sup> and WT mice was noted ( $P = 0.86$ ). TNF-R1<sup>-/-</sup> mice also had significantly lower BDNF levels than TNF-R2<sup>-/-</sup> mice ( $P = 0.022$ ).

Analysis of NGF in the hippocampus showed that a significant difference between strains (one-way ANOVA  $F_{(3,20)} = 5.11$ ,  $P = 0.002$ ; Figure 4.5B) and Tukey's multiple comparison showed TNF-R2<sup>-/-</sup> mice had significantly higher levels than TNF-R1<sup>-/-</sup> mice ( $P = 0.0066$ ).

Figure 4.4: Levels of BDNF and NGF



**Legend:** Levels of BDNF in the hippocampus (A) and levels of NGF in the hippocampus (B). All data represent mean  $\pm$  SEM ( $n = 8$ ). Data compared using One-way ANOVA with Tukey's post hoc comparison \*  $P < 0.05$  compared to WT, \*\*  $P < 0.01$  compared to WT, +  $P < 0.05$  compared to TNF-R1<sup>-/-</sup>, ++  $P < 0.01$  compared to TNF-R1<sup>-/-</sup>, ^  $P < 0.05$  compared to TNF<sup>-/-</sup>.

### Differences in Serum Cytokine Levels between Strains

To test whether there was any change in serum cytokine levels in the 6 month old adult mice, a CBA was performed on serum collected at the end of behavioural testing. TNF (total) levels were found to be significantly different between strains ( $F_{(3,20)} = 7.9$ ,  $P = 0.001$ ) and were very low in TNF<sup>-/-</sup> mice. This was reflected in Tukey's multiple comparison that showed significant differences between TNF<sup>-/-</sup> mice and other strains (Table 4.5), confirming the TNF knockout status of these mice. No differences between strains were observed for levels of IL-10, IL-6 and MCP-1. However all knockout mice displayed significantly lower levels of IFN- $\gamma$  compared to WT mice (One-way ANOVA  $F_{(3,19)} = 18.52$ ,  $P < 0.001$ ; TNF<sup>-/-</sup> compared to WT mice  $P = 0.0007$ , TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> compared to WT mice  $P < 0.0001$ ).



**Table 4.5: Serum cytokine analyses**

Cytokines	Strains			
	WT	TNF <sup>-/-</sup>	TNF-R1 <sup>-/-</sup>	TNF-R2 <sup>-/-</sup>
<b>TNF (total)</b>	5.02±0.67	0.13±0.13 <sup>2*</sup>	5.36±1.81	5.19±0.66
<b>IL-10</b>	7.42±0.07	4.99±1.36	3.51±3.04	6.02±3.08
<b>IL-12p70</b>	11.79±2.15	9.30 ±1.44	8.19±2.04	9.35±1.82
<b>MCP-1</b>	21.65±2.33	15.48± .28	30.14±12.48	26.68±2.47
<b>IL-6</b>	3.94±0.88	3.21±0.88	2.71±0.32	3.56±0.36
<b>IFN-<math>\gamma</math></b>	3.56±0.36	1.18±0.71 <sup>3*</sup>	0.22±1.22 <sup>3*</sup>	0.26± 0.08 <sup>3*</sup>

**Legend:** Representation of cytokine expression (across) measured by CBA in all strains of mice. All data represent mean  $\pm$  SEM (n = 14/strain). Data compared using One-way ANOVA with Tukey's post hoc comparison <sup>2\*</sup>  $P < 0.01$ , <sup>3\*</sup>  $P < 0.001$  compared to WT.

### ***Expression of Cytokines in the Hippocampus***

Levels of (mRNA) *Tnfa*, *Tnfr1*, and *Tnfr2*, *Il1b*, *Il10* in the hippocampus of mice were measured post behavioural testing (Table 4.6). This was done to measure the levels of TNF- $\alpha$  and its receptors in genetic knockout mice, and confirm mice are knockouts.

One-way ANOVA tests revealed no significant differences in the expression of *Tnfa* ( $F_{(2,9)} = 2.10$ ,  $P = 0.178$ ), *Tnfr1* ( $F_{(2,9)} = 2.76$ ,  $P = 0.11$ ) and *Tnfr2* ( $F_{(2,10)} = 1.68$ ,  $P = 1.16$ ) mRNA across the three knockout strains compared to WT controls. *Tnfa*, *Tnfr1* and *Tnfr2* mRNA levels were not detectable in the respective knockout strains as reported in Table 3 confirming the loss of gene function in these mouse strains and correlating with the results for TNF from CBA analysis.

A significant difference in mRNA levels of *Il10* ( $F_{(3,13)} = 3.77$ ,  $P = 0.039$ ) and *Il1b* ( $F_{(3,13)} = 4.76$ ,  $P = 0.029$ ) were noted. TNF<sup>-/-</sup> mice had significantly higher levels of *Il10* compared to TNF-R2<sup>-/-</sup> mice ( $P = 0.028$ ) whilst TNF-R2<sup>-/-</sup> mice had significantly lower levels of *Il1b* compared to WT mice ( $P = 0.031$ ).

**Table 4.6: Gene expression of cytokines measured in the hippocampus in TNF<sup>-/-</sup> and TNF- $\alpha$  receptor knockout mice**

Units: fold Difference (gene/ <i>Gapdh</i> )	Strains			
	WT	TNF <sup>-/-</sup>	TNF-R1 <sup>-/-</sup>	TNF-R2 <sup>-/-</sup>
<i>Tnfa</i>	0.90 $\pm$ 0.36	ND	0.75 $\pm$ 0.18	0.48 $\pm$ 0.54
<i>Tnfr1</i>	1.03 $\pm$ 0.15	1.61 $\pm$ 0.19	ND	1.60 $\pm$ 0.26
<i>Tnfr2</i>	1.07 $\pm$ 0.37	1.59 $\pm$ 0.23	1.49 $\pm$ 0.28	ND
<i>Il10</i>	0.89 $\pm$ 0.31	3.16 $\pm$ 0.63	1.36 $\pm$ 0.45	0.59 $\pm$ 0.37 ^
<i>Il1b</i>	0.81 $\pm$ 0.48	0.66 $\pm$ 0.72	0.46 $\pm$ 0.19	0.17 $\pm$ 0.08 *

**Legend:** Fold difference was calculated in relation to WT control control group. Results compared using One-way ANOVA with Tukey's multiple comparisons (delta Ct values used for analysis). ND indicates non-detectable limits. \* indicates differences compared to WT mice, ^ indicates significant difference compared to TNF<sup>-/-</sup> mice, \*/^  $p < 0.05$  ( $n = 4/\text{group}$ ).

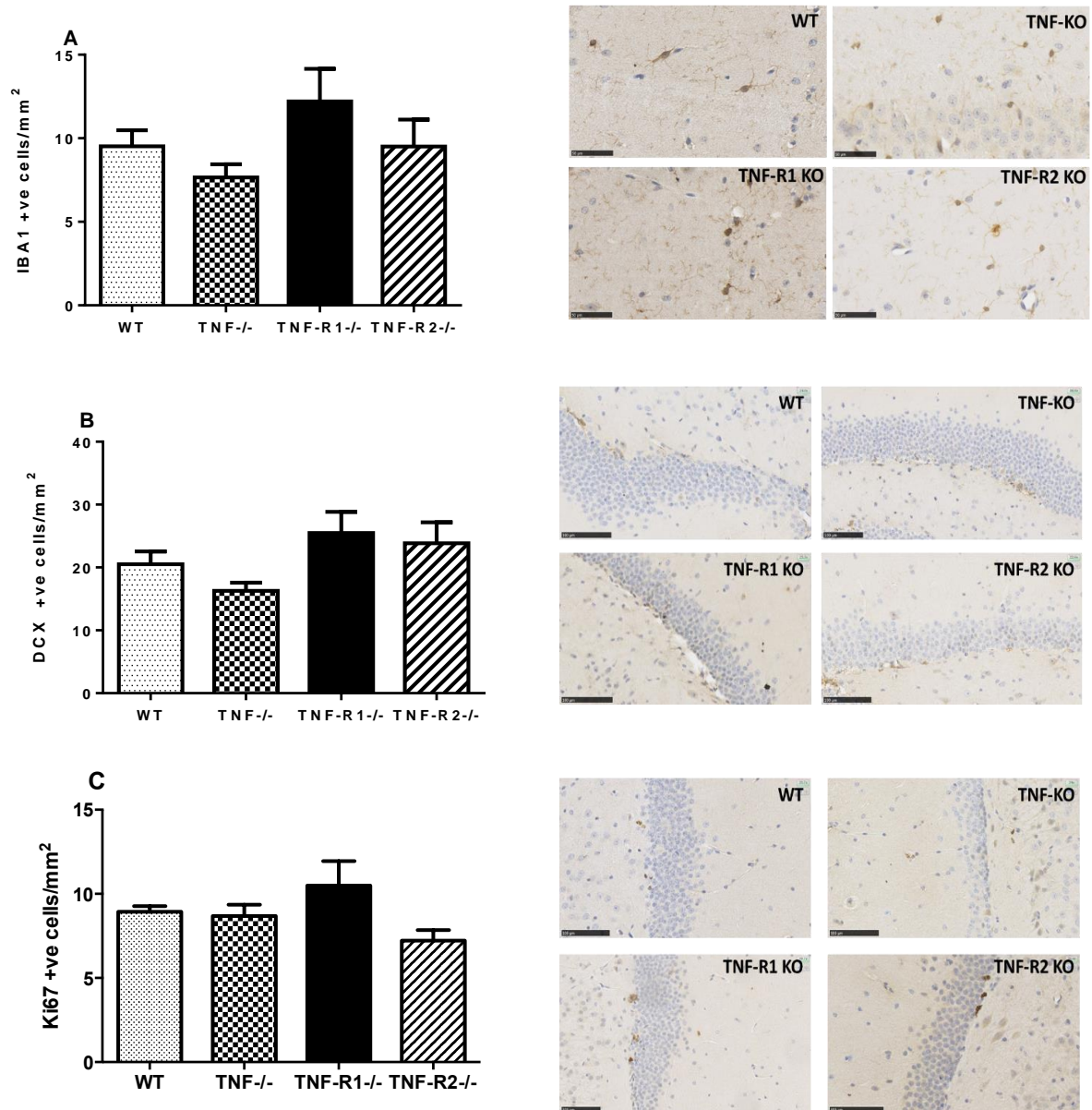
### **IHC Analysis**

Microglia in the dentate gyrus were measured using IHC using an IBA1 microglial marker. Due to the extensive interaction between microglia and TNF- $\alpha$  (Renno et al., 1995), we expected lower microglial numbers in knockout mice compared to WT due to absence of TNF- $\alpha$  and its receptor signalling. No difference between the strains was noted after one-way ANOVA analysis ( $F_{(3,11)} = 1.79$ ,  $P =$

0.20; Figure 4.5A), indicating that microglial numbers were not affected by lack of TNF- $\alpha$  and its receptors in mid-adult unchallenged mice.

Additional IHC analysis included number of proliferating cells (Ki67; Figure 4.5B) and immature neurons (DCX; Figure 4.5C) in the dentate gyrus to test whether lack of TNF- $\alpha$  and its receptor signalling would impair neurogenesis in 6 month old mice as no differences in 3 month old mice were observed (Camara et al., 2013). Analysis by one-way ANOVA showed no significant difference between strains for either of these markers ( $F_{(3,11)} = 2.51$ ,  $P = 0.11$  for Ki67;  $F_{(3,13)} = 2.78$ ,  $P = 0.082$  for DCX,).

**Figure 4.5: IHC Analysis**



**Legend:** Number of A) IBA1 positive cells in the dentate gyrus, B) DCX positive cells in the dentate gyrus and C) number of Ki67 positive cells in the dentate gyrus. All data represent mean  $\pm$  SEM (n = 8). Data compared using One-way ANOVA. No significant differences were observed between groups.

**Table 4.7: Mean  $\pm$  SEM values of all strains in all tests in this study.**

	WT mice	TNF <sup>-/-</sup> mice	TNF-R1 <sup>-/-</sup> mice	TNF-R2 <sup>-/-</sup> mice
Home Cage	9.69 $\pm$ 0.5	8.36 $\pm$ 0.2	7.53 $\pm$ 0.7	8.54 $\pm$ 0.7
OFT	13.44 $\pm$ 0.7	14.93 $\pm$ 1.5	13.60 $\pm$ 0.8	12.9 $\pm$ 1.1
EZM	51.96 $\pm$ 6.0	61.52 $\pm$ 6.9	51.29 $\pm$ 4.8	37.49 $\pm$ 5.5
BM Training	51.04 $\pm$ 7.6	66.68 $\pm$ 9.77	43.38 $\pm$ 6.6	74.81 $\pm$ 8.4
BM Probe Trial	20.15 $\pm$ 5.6	15.53 $\pm$ 3.7	3.7 $\pm$ 0.8	21.50 $\pm$ 4.6
HBE	50.50 $\pm$ 5.1	32.46 $\pm$ 4.1	28.50 $\pm$ 3.3	36.29 $\pm$ 3.14
FST	109.4 $\pm$ 11	31.3 $\pm$ 10.6	76.74 $\pm$ 10.6	47.50 $\pm$ 5.86
BDNF	27.05 $\pm$ 1.9	19.45 $\pm$ 1.4	16.46 $\pm$ 1.7	25.09 $\pm$ 1.9
NGF	15.38 $\pm$ 2.6	11.56 $\pm$ 1.1	7.33 $\pm$ 1.9	20.39 $\pm$ 2.7
<i>Tnfa</i> Expression	0.90 $\pm$ 0.36	ND	0.75 $\pm$ 0.18	0.48 $\pm$ 0.54
<i>Tnfr1</i> Expression	1.03 $\pm$ 0.15	1.61 $\pm$ 0.19	ND	1.60 $\pm$ 0.26
<i>Tnfr2</i> Expression	1.07 $\pm$ 0.37	1.59 $\pm$ 0.23	1.49 $\pm$ 0.28	ND
<i>Il10</i> Expression	0.89 $\pm$ 0.31	3.16 $\pm$ 0.63	1.36 $\pm$ 0.45	0.59 $\pm$ 0.37
<i>Il0b</i> Expression	0.81 $\pm$ 0.48	0.66 $\pm$ 0.72	0.46 $\pm$ 0.19	0.17 $\pm$ 0.08
Microglia	9.52 $\pm$ 0.9	7.65 $\pm$ 0.7	12.21 $\pm$ 1.9	9.50 $\pm$ 1.6
DCX	20.52 $\pm$ 2.0	16.28 $\pm$ 1.3	25.47 $\pm$ 3.3	23.85 $\pm$ 3.3
Ki67	11.77 $\pm$ 2.8	11.61 $\pm$ 1.2	13.20 $\pm$ 1.5	8.48 $\pm$ 0.7

**Legend:** The table shows the mean  $\pm$  SEM values of all strains of mice in behavioural and biological analyses. ND refers to non-detectable levels.

**Table 4.8: Comparison of knockout strains to WT mice**

	TNF <sup>-/-</sup>	TNF-R1 <sup>-/-</sup>	TNF-R2 <sup>-/-</sup>
HOME CAGE TEST	-	-	-
OPEN FIELD TEST	-	-	-
BARNES MAZE (TRAINING)	-	-	-
BARNES MAZE (PROBE TRIAL)	-	Improved Learning	-
ELEVATED ZERO MAZE	-	-	-
Y MAZE	-	-	-
HOLE BOARD EXPLORATION TEST	Decreased Exploration	Decreased Exploration	-
SOCIABILITY	Normal Social Behaviour	Normal Social Behaviour	Normal Social Behaviour
SOCIAL NOVELTY	Normal Social Behaviour	Normal Social Behaviour	Normal Social Behaviour
FORCED SWIM TEST	Decreased Depression	-	Decreased Depression
BDNF ELISA	Lower Expression	Lower Expression	-
NGF ELISA	-	-	Higher Expression
<i>Tnfa</i> EXPRESSION	Lower Levels	-	-
<i>Tnfr1</i> EXPRESSION	-	Lower Levels	-
<i>Tnfr2</i> EXPRESSION	-	-	Lower Levels
<i>Il10</i> EXPRESSION	-	-	-
<i>Il1b</i> EXPRESSION	-	-	Lower Levels
MICROGLIAL NUMBERS	-	-	-
DCX NUMBERS	-	-	-
Ki67 NUMBERS	-	-	-

**Legend:** Table summarising the differences between the strains compared to WT mice for Home Cage, OFT, BM, HBE, FST and ELISAs (—) represents no statistical significant difference compared to WT mice. Analysis of normal or impaired behaviour within a strain in reference to WT mice has been reported for Y-maze and sociability tests. ND refers to non-detectable levels.

**Table 4.9: Comparisons between TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice**

TEST	Difference between strains	TNF-R1 <sup>-/-</sup>	TNF-R2 <sup>-/-</sup>
Home Cage Test	No difference	7.53 $\pm$ 0.7	8.54 $\pm$ 0.7
Open Field Test	N difference	13.60 $\pm$ 0.8	12.9 $\pm$ 1.1
Elevated Zero Maze	No Difference	51.29 $\pm$ 4.8	37.49 $\pm$ 5.5
Barnes Maze (Training)	No difference	43.38 $\pm$ 6.6	74.81 $\pm$ 8.4
Barnes Maze (Probe Trial)	<b>Better memory in TNF-R1<sup>-/-</sup> mice</b>	3.7 $\pm$ 0.8	21.50 $\pm$ 4.6
Hole Board Exploration Test	No difference	28.50 $\pm$ 3.3	36.29 $\pm$ 3.14
Forced Swim Test	No difference	76.74 $\pm$ 10.6	47.50 $\pm$ 5.86
BDNF ELISA	<b>Higher Expression in TNF-R2<sup>-/-</sup> mice</b>	16.46 $\pm$ 1.7	25.09 $\pm$ 1.9
NGF ELISA	<b>Higher Expression in TNF-R2<sup>-/-</sup> mice</b>	7.33 $\pm$ 1.9	20.39 $\pm$ 2.7
Tnfa EXPRESSION	No difference	0.75 $\pm$ 0.18	0.48 $\pm$ 0.54
Tnfr1 EXPRESSION	<b>Higher Levels in TNF-R2<sup>-/-</sup> mice</b>	ND	1.60 $\pm$ 0.26
Tnfr2 EXPRESSION	<b>Higher Levels in TNF-R1<sup>-/-</sup> mice</b>	1.49 $\pm$ 0.28	ND
Il10 Expression	No difference	1.36 $\pm$ 0.45	0.59 $\pm$ 0.37
Il0b Expression	No difference	0.46 $\pm$ 0.19	0.17 $\pm$ 0.08
IBA1 Positive Cells	No difference	12.21 $\pm$ 1.9	9.50 $\pm$ 1.6
DCX Positive Cells	No difference	25.47 $\pm$ 3.3	23.85 $\pm$ 3.3
Ki67 Positive Cells	No difference	13.20 $\pm$ 1.5	8.48 $\pm$ 0.7

**Legend:** Representation of the differences between TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice. All data represent mean  $\pm$  SEM and P values (n = 14/group for TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice). ND refers to non-detectable levels.

## Discussion

At 6 months of age all mice displayed similar locomotor activity compared to WT mice. In the anxiety test however, TNF-R2<sup>-/-</sup> mice were significantly more anxious than TNF<sup>-/-</sup> mice but were no different to WT mice. Interestingly TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice showed poorer exploratory behaviour than WT mice in the HBE test. In the training phase of the Barnes maze no differences were detected between strains, however in the probe trial TNF-R1<sup>-/-</sup> mice displayed better memory than WT and TNF-R2<sup>-/-</sup> mice. WT, TNFR1<sup>-/-</sup> and TNFR2<sup>-/-</sup> mice demonstrated normal social behaviour while TNF<sup>-/-</sup> mice had significantly less preference for stranger mouse compared to empty cage than other strains. It is also interesting to note that both TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice exhibited lower immobility on the depression-like behaviour test, the FST, than WT mice. Additionally, TNF<sup>-/-</sup> mice expressed significantly lower levels of BDNF than WT mice in the hippocampus while TNF-R1<sup>-/-</sup> mice displayed significantly lower BDNF levels compared to both WT and TNF-R2<sup>-/-</sup> mice. TNF-R2<sup>-/-</sup> mice also displayed significantly higher levels of NGF compared to TNF-R1<sup>-/-</sup> mice. As expected, TNF<sup>-/-</sup> mice had undetectable levels of *Tnfa* in the hippocampus and very low levels of TNF (total) in serum compared to other strains. Similarly TNF-R1<sup>-/-</sup> mice and TNF-R2<sup>-/-</sup> mice had undetectable levels of *Tnfr1* and *Tnfr2* respectively in the hippocampus, compared to other strains. TNF<sup>-/-</sup> mice had significantly higher levels of *Il10* compared to TNF-R2<sup>-/-</sup> mice while TNF-R2<sup>-/-</sup> mice had significantly lower levels of *Il1b* compared to WT mice in the hippocampus, while serum IFN- $\gamma$  was decreased in all KO strains compared to WT.

TNF- $\alpha$  is an active participant in synaptic scaling, long term potentiation, and prevents neuronal excitotoxicity; a role that is mediated by signalling through TNF-



R1 despite TNF-R1 possessing neuro-inflammatory properties (Cheng et al., 1994, Beattie et al., 2002, Stellwagen et al., 2005, Baune et al., 2012b). To further develop on these findings, previous research within this group focussed on the behavioural changes mediated by TNF- $\alpha$  and its receptor signalling in knockout mouse strains at different stages of development. We previously demonstrated that while lack of TNF- $\alpha$  impaired cognitive ability in young adult TNF- $\alpha$  and receptor deficient mice (3 month old mice), lack of this cytokine improved cognition in aged mice (Baune et al., 2008, McAfoose et al., 2009, Camara et al., 2013), establishing a more complex role of TNF- $\alpha$  in mediating cognition. In order to build on the work that was previously conducted within this group we assessed the behavioural phenotypes of 6 month old mice. We have shown presently, for the first time that this transition from TNF- $\alpha$  being an essential component to a redundant factor in cognitive development begins in mid-adulthood. While the present study was not designed as a longitudinal assessment of TNF- $\alpha$  mediated behavioural phenotypes, comparisons and references to findings in the 3 month old mice can be made. In the present study we have shown that TNF<sup>-/-</sup> and receptor knockout mice at 6 months of age were no different to WT mice in the learning component of the Barnes maze. In contrast, 3 month old mice previously showed that TNF<sup>-/-</sup> mice displayed impaired learning in similar tasks compared to WT mice (Baune et al., 2008, Camara et al., 2013). These results lend support to studies conducted by McAfoose et al (2009), where a gradual improvement in cognition was seen from young adulthood (3 months) to old age (12 months) in TNF<sup>-/-</sup> mice, with a transition to better cognition beginning at mid-adulthood (6 months). In the present study we were able to extend these findings to TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice and showed that TNF-R1<sup>-/-</sup> mice had in fact better

memory than WT mice in the probe trial of the Barnes maze. TNF-R2<sup>-/-</sup> mice however were no different to WT mice in the probe trial, these results suggest that the 2 TNF- $\alpha$  receptors may have divergent functions in behaviour as opposed to being purely neurodegenerative or neuroprotective, as TNF-R1<sup>-/-</sup> mice were cognitively impaired at 3 months of age (Camara et al., 2013) but not presently at 6 months compared to WT mice. There also appears to be a gradual shift from a possible need for TNF- $\alpha$  in cognitive development to a beneficial effect of the lack of TNF- $\alpha$ , as animals' age, free from immune challenge.

This change in cognitive function from early developmental stages to adulthood is further evident in exploratory and other behaviours including depression and social behaviours in TNF- $\alpha$  and its receptor knockout mice. Here we showed that TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice had decreased exploratory behaviour in the HBE test, suggesting that these receptors are required for maintaining exploratory behaviours in mid-adulthood. This is in contrast to our previous study using 3 month old mice of the same strains where no differences were observed between knockout strains compared to WT mice in exploratory behaviour (Camara et al., 2013). It is unclear why this difference between the current study and our previous work has arisen, as tests were conducted under the same conditions, suggesting it is likely linked to the age of mice tested. Fiore et al (1998) showed decreased exploratory behaviour in TNF- $\alpha$  over-expressing mice that were only 45 days old.

In the present study, lack of TNF- $\alpha$  and TNF-R2 in mid-adult knockout mice prevented depression-like behaviour in the FST. The present findings differ from our previous work on the same strains aged to 3 months, where we observed no difference in depression-like behaviour between knockout strains compared to WT

mice (Camara et al., 2013). Despite the difference to our earlier results, the findings in the present study corroborate work done by Krugel et al (2013) who showed anti-depressant effects (tested in FST) in rats undergoing chronic mild stress after twice weekly IP etanercept treatment (over a period of 5 weeks). We know that TNF- $\alpha$  is associated with MDD in humans (Dowlati et al., 2010), and furthermore as ageing is a trigger for up-regulation of TNF- $\alpha$ , it can be expected that during normal ageing TNF- $\alpha$  related depression is a possibility. Studies on humans have shown that serum levels of both TNF- $\alpha$  receptors are associated with depression, in fact high serum TNF-R2 levels are associated with late life depression, while an up-regulation of TNF-R1 is seen in serum of patients undergoing anti-depressant treatment (Diniz et al., 2010, Tulner et al., 2011). Interestingly the present study substantiates these findings, as we observed decreased depression-like behaviour in mid-adult TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> but not TNF-R1<sup>-/-</sup> mice in the FST, suggesting that the 2 receptors of TNF- $\alpha$  may play differential roles in development of this behaviour. Additionally this study shows firstly, that the development of this depressive phenotype occurs from a stage closer to mid-adulthood and that secondly these findings corroborate studies on TNF- $\alpha$  antagonists that showed modulating TNF- $\alpha$  action may be beneficial to alleviate depressive symptoms (Kekow et al., 2011).

In this study, social behaviour was poor in TNF<sup>-/-</sup> mice, with these mice spending less time interacting with stranger and novel mice, in both phases of the sociability test. These results differ from our previous findings in young adult mice where we observed TNF- $\alpha$  and TNF- $\alpha$  receptor knockout mice had normal social behaviour compared to unchallenged 3 months old WT mice (Camara et al., 2013). Similarly in the present study we showed that lack of the TNF- $\alpha$  receptors did not

impair social behaviour and these mice exhibited similar social interactions to WT mice. It is unclear however, why TNF<sup>-/-</sup> mice displayed poorer social interactions, but more work will need to be done to better understand the role of TNF- $\alpha$  in social interactions in different age groups of mice.

As TNF- $\alpha$  up-regulation by ICV administration, is linked to activation of microglia resulting in a positive feedback loop of further activation (Nadeau and Rivest, 2000), it is unsurprising that in our study we noted no difference in measures of microglial activation in TNF- $\alpha$  and receptor knockout mice compared to WT mice, in either number or morphology of microglial cells. Microglia are believed to promote postnatal neurogenesis, however in inflammatory conditions it can hinder neurogenesis (Shigemoto-Mogami et al., 2014). TNF- $\alpha$  is shown to be a suppressor of adult neurogenesis (Iosif et al., 2006), therefore, we postulated that neurogenesis that occurs throughout life in the sub ventricular zone and the dentate gyrus (Biebl et al., 2000) would be higher in TNF<sup>-/-</sup> and TNF- $\alpha$  receptor knockout mice. However, we found no difference in markers of neurogenesis between strains compared to WT mice, indicating that lack of TNF- $\alpha$  and its receptors in mid-adult mice neither impairs nor stimulates neurogenesis at this stage of adulthood in immunologically unchallenged conditions. It may be worth exploring whether immune stimulation would impair neurogenesis in these strains of mice. Indeed we have demonstrated that LPS treatment systemically administered in WT mice was effective in increasing microglial numbers in the brain which were then brought back to baseline levels by administration of a TNF- $\alpha$  antagonist (Camara et al., 2014). It is also plausible that, as mice age, the effects of lack of TNF- $\alpha$  and its receptors on neurogenesis may become more pronounced and would be worth investigating for future studies.

BDNF levels in the present study were decreased in TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice compared to WT mice. It is unclear though why TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice had decreased BDNF levels in the present study as in our previous study on TNF<sup>-/-</sup> and TNF- $\alpha$  receptor knockout mice, we did not detect changes in BDNF levels in knockout mice compared to WT mice at 3 months of age, indicating that ageing may play a role in BDNF expression in these strains (Camara et al., 2013). There has been an interesting debate regarding the interactions between BDNF and TNF- $\alpha$ . Some studies have reported that high systemic levels of TNF- $\alpha$  are associated with decreased BDNF (Churchill et al., 2006) whereas others indicate no interaction between TNF- $\alpha$  and BDNF. For instance, a recent study showed that peripheral administration of LPS for 7 days increased TNF- $\alpha$  levels in the hippocampus and caused cognitive decline but did not affect BDNF levels in the hippocampus (Zhu et al., 2014a). On the other hand, in vitro studies have shown that astrocyte cultures treated with TNF- $\alpha$  were able to produce BDNF, specifically through NF $\kappa$ B pathways (Saha et al., 2006b). In the present study the effects of ageing in combination with the lack of TNF- $\alpha$  expression in TNF- $\alpha$  knockout mice may be responsible for the low levels of BDNF observed and further investigation into these mechanisms will help clarify this hypothesis.

Reports suggest that a decrease in NGF during ageing is associated with a reduction in long term potentiation in the dentate gyrus (Kelly et al., 2000). Under inflammatory conditions microglia can stimulate TNF- $\alpha$  to produce more NGF, creating a positive feedback loop (Takei and Laskey, 2008). As a result we expected to see a decrease in NGF levels in TNF<sup>-/-</sup> mice, as there would be no cross-talk between microglia and TNF- $\alpha$  to produce more NGF. However, in the present study,

we have shown that there were no differences between NGF levels in TNF<sup>-/-</sup>, TNF-R1<sup>-/-</sup> and WT mice, though TNF-R2<sup>-/-</sup> mice had significantly higher levels than TNF-R1<sup>-/-</sup> mice. While these results were observed in mid-adult and not aged mice, they may give an indication as to the direction in which neurotrophin levels in unchallenged TNF<sup>-/-</sup> and receptor deficient mice is headed with ageing. Especially since in 3 month old mice we found TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice had significantly lower NGF levels than WT mice (Camara et al., 2013). The increase in NGF levels seen presently can be linked to the lower depression-like behaviour we saw in the TNF-R2<sup>-/-</sup> mice, and provides a stronger link between TNF- $\alpha$  and NGF (Takei and Laskey, 2008, Takei and Laskey, 2011). The use of NGF knockout mice in conjunction with TNF- $\alpha$  agonists and antagonists may be useful in elucidating the exact mechanisms behind TNF- $\alpha$  -NGF interactions (Capsoni et al., 2000, Ruberti et al., 2000).

As expected, in the hippocampus, Tnfa levels in TNF<sup>-/-</sup> mice, Tnfr1 levels in TNF-R1<sup>-/-</sup> mice and Tnfr2 levels in TNF-R2<sup>-/-</sup> mice were too low to be detected confirming the knockout genotype of these strains. Serum TNF (total) was also significantly lower in TNF<sup>-/-</sup> mice, further confirming the status of these mice. Significantly higher levels of Il10 were seen in the hippocampus of TNF<sup>-/-</sup> mice compared to TNF-R2<sup>-/-</sup> mice. IL-10 is an anti-inflammatory cytokine that has been shown to suppress TNF- $\alpha$  production (Minter et al., 2000). It has also been shown to increase in production to a minor extent in contexts on low TNF- $\alpha$  expression (Ralph et al., 1992, Minter et al., 2000) and may explain why the TNF<sup>-/-</sup> mice had the lowest Il10 expression.

Studies have shown IL-1 $\beta$  can influence the regulation of TNF- $\alpha$  receptors, though the exact effects and mechanisms on TNF- $\alpha$  receptors are poorly understood

(Winzen et al., 1993, Hultner et al., 2000, Saperstein et al., 2009). A recent study showed that in mouse alveolar epithelial cells, treatment with IL-1 $\beta$  increased the expression of TNF-R2 (Saperstein et al., 2009). Similarly in the present study, TNF-R2<sup>-/-</sup> mice were found to have significantly lower levels of Il1b compared to WT mice and may be mediated by the strong association between IL-1 $\beta$  and TNF- $\alpha$  receptor regulation.

Additionally, serum levels of IFN- $\gamma$  were decreased in all KO strains compared to WT mice. Given the close association between TNF- $\alpha$  and IFN- $\gamma$  wherein IFN- $\gamma$  is known to stimulate the production of TNF- $\alpha$  (by interferon regulatory factors 1 and 8) (Celada and Maki, 1991, Vila-del Sol et al., 2008), the lack of TNF- $\alpha$  expression in knockout mice in this study may in turn deter the production of IFN- $\gamma$  but this mechanism is not yet clear.

It should be noted that In a TNF- $\alpha$  receptor knockout mice we would expect increased activity of the other TNF- $\alpha$  receptor, which would subsequently have different effects on behaviour. Thus a TNF- $\alpha$  receptor knockout mouse, would not be expected to behave exactly like a TNF-KO mouse, as was observed in the present findings. Furthermore, as TNF- $\alpha$  receptors can bind to lymphotoxin- $\alpha$  ligands in addition to TNF- $\alpha$ , It is plausible that some of the effects noted in the present studies in TNF receptor deficient mice may be due to interactions between lymphotoxin- $\alpha$  and TNF receptors (MacEwan, 2002). This would again result in different phenotypes in TNF receptor mice compared to TNF-KO mice. At this stage we are unable to verify these interactions due to limitations of the current studies but these

issues should be considerations for future work involving these strains of mice.

Overall these results show that in mid-adulthood, deficiency of TNF- $\alpha$  or its receptors can improve either cognition or depression-like behaviours, while decreasing exploratory behaviour compared to WT mice. Specifically, the results of the present study show that absence of TNF- $\alpha$  and TNF-R1 protect mice from depression-like behaviour. The findings of the present study also extend to BDNF and NGF levels in the hippocampus that appear to be regulated by TNF- $\alpha$  and its receptors even without the influence of external immune stimulation. These results also support conclusions on the changing role of TNF- $\alpha$  in mediating different behaviours in different age groups, with the mid-adult group representing a transition period of TNF- $\alpha$  function under unchallenged immune conditions. As with the results of the previous study, in a younger cohort of mice (Camara et al., 2013), it is plausible that in TNF- $\alpha$  receptor knockout mice, the behavioural and neurobiological effects observed are mediated by a deficiency in both sol and tm TNF- $\alpha$ . As this study did not differentiate between the two types of TNF- $\alpha$ , it is difficult to assign a particular mechanism of action to each type. To circumvent these shortcomings more thorough investigations into the mechanisms between the different forms of TNF- $\alpha$  will be undertaken in future studies.

Future studies should include conducting experiments following stress procedures in the mice to activate microglia, such as LPS administration, unpredictable chronic mild stress or early life stress protocols. Furthermore, the use



of Cre-loxed mice would greatly benefit the study of TNF- $\alpha$  and its receptors by specifically targeting these mice and overcome the limitations of developmental phenotype over actual knockout phenotypes. Additional studies would benefit from the use of randomised littermates as control mice to circumvent the epigenetic differences that may occur from the use of WT control mice from different colonies.

## **Chapter 5 : Centrally Administered Etanercept on Behaviour Following a Peripheral Immune Challenge**

## Effects of Centrally Administered Etanercept on Behaviour Following a Peripheral Immune Challenge

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## Effects of Centrally Administered Etanercept on Behavior, Microglia, and Astrocytes in Mice Following a Peripheral Immune Challenge

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Peripheral cytokines affect central nervous system (CNS) function, manifesting in symptoms of anxiety and cognitive decline. Although the peripheral blockade of tumor necrosis factor (TNF)- $\alpha$  has been effective in alleviating depression and rheumatoid arthritis, it is yet unknown whether central blockade of TNF- $\alpha$  is beneficial for immune-challenged CNS function. This study investigated the effects of central etanercept administration following a peripheral immune challenge on anxiety-like and cognition-like behaviors and microglia and astrocyte numbers. Twelve-week-old C57BL/6 mice ( $n=40$ ) were treated with either LPS or saline administered peripherally 24 h before being treated with either etanercept or artificial CSF (aCSF) by intracerebroventricular injection. Mice underwent behavioral analyses for locomotion, memory, and anxiety-like behavior 24 h post-etanercept/aCSF treatment, and tissue was collected to estimate the numbers of hippocampal microglia and astrocytes. Following peripheral immune challenge with LPS, mice showed increased anxiety-like behavior, which was significantly improved following treatment with etanercept (two-way ANOVA: Interaction:  $F_{(1,30)}=0.60$ ,  $P=0.44$ ; Saline/LPS challenge:  $F_{(1,30)}=23.92$ ,  $P<0.0001$ , etanercept vs aCSF:  $F_{(1,30)}=11.09$ ,  $P=0.0023$ ). For cognition, a significant interaction effect found by two-way ANOVA (Interaction:  $F_{(1,20)}=4.96$ ,  $P=0.037$ , Saline/LPS challenge:  $F_{(1,20)}=4.966$ ,  $P=0.31$ , aCSF/etanercept treatment:  $F_{(1,20)}=0.06$ ,  $P=0.80$ ) and post-hoc analysis revealed a significant decrease in cognition in LPS-aCSF compared with Sal-aCSF mice ( $P=0.038$ ), but no significant difference was noted between LPS-aCSF and LPS-Etan mice ( $P>0.9$ ). A significant reduction in the number of microglia within the hippocampus of these mice was noted (two-way ANOVA: Interaction:  $F_{(1,15)}=11.41$ ,  $P=0.0041$ ; Saline/LPS challenge:  $F_{(1,15)}=50.13$ ,  $P<0.0001$ , etanercept vs aCSF:  $F_{(1,15)}=3.36$ ,  $P=0.08$ ). Centrally administered etanercept improved anxiety-like behavior but not spatial memory under a peripheral immune challenge and was associated with a decrease in the hippocampal microglia numbers. This suggests that etanercept recovers anxiety-like behavior possibly mediated by a reduction of TNF- $\alpha$ -related central inflammation.

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## Overview

Peripheral cytokines affect central nervous system (CNS) function, manifesting in symptoms of anxiety and cognitive decline. Although the peripheral blockage of tumor necrosis factor (TNF- $\alpha$ ) has been effective in alleviating depression and rheumatoid arthritis, it is yet unknown whether central blockade of TNF- $\alpha$  is beneficial for immune challenged CNS function.

**AIM 3: To determine the behavioural and neurobiological effects of pharmacological blockade of TNF- $\alpha$  in the CNS of young adult immune activated mice.**

12 week old C57BL/6 mice (n = 40) were treated with either LPS or saline administered peripherally 24 hr before being treated with either etanercept or artificial CSF (aCSF) by intracerebroventricular injection. Mice underwent behavioural analyses for locomotion, memory and anxiety-like behaviour 24 hr post etanercept/aCSF treatment and tissue was collected to estimate numbers of hippocampal microglia and astrocytes.

Following peripheral immune challenge with LPS, mice showed increased anxiety-like behaviour, which was significantly improved following treatment with etanercept (2-way ANOVA: Interaction:  $F_{(1,30)} = 0.60$ ,  $P = 0.44$ ; Saline/LPS challenge:  $F_{(1,30)} = 23.92$ ,  $P < 0.0001$ , etanercept vs aCSF:  $F_{(1,30)} = 11.09$ ,  $P = 0.0023$ ). For cognition, a significant interaction effect found by 2-way ANOVA (Interaction:  $F_{(1,20)} = 4.96$ ,  $P = 0.037$ , Saline/LPS challenge:  $F_{(1,20)} = 4.966$ ,  $P = 0.31$ , aCSF/etanercept treatment:  $F_{(1,20)} = 0.06$ ,  $P = 0.80$ ) and post-hoc analysis revealed a significant decrease in cognition in LPS-aCSF compared to Sal-aCSF mice ( $P = 0.038$ ), but no significant difference was noted between LPS-aCSF and LPS-Etan mice ( $P > 0.9$ ). A significant reduction in the number of microglia within the hippocampus of these mice

was noted (2-way ANOVA: Interaction:  $F_{(1,15)} = 11.41$ ,  $P = 0.0041$ ; Saline/LPS challenge:  $F_{(1,15)} = 50.13$ ,  $P < 0.0001$ , etanercept vs aCSF:  $F_{(1,15)} = 3.36$ ,  $P = 0.08$ ,).

Centrally administered etanercept improved anxiety-like behaviour, but not spatial memory under a peripheral immune challenge and was associated with a decrease in hippocampal microglia numbers. This suggests that etanercept recovers anxiety-like behaviour possibly mediated by a reduction of TNF- $\alpha$  related central inflammation.

## Introduction

Tumour necrosis factor (TNF- $\alpha$ ) plays an important role in the development and progression of cognitive decline, as well as depressive and anxiety disorders (Baune et al., 2012b, Kaster et al., 2012, Bai et al., 2013, Camara et al., 2013). Indeed, peripheral inflammatory conditions like arthritis (RA) and psoriasis that show high levels of circulating TNF- $\alpha$  are associated with psychiatric symptoms like cognitive decline, anxiety and depression (Chandarana et al., 1987, Bassukas et al., 2008, Menter et al., 2010).

This association is likely related to extensive cross-talk occurring between immune cells within the central nervous system (CNS), like microglia, and peripheral TNF- $\alpha$  producing monocytes (Kerfoot et al., 2006). Peripheral LPS inflammation activates toll-like receptor 4 present on circumventricular organs and choroid plexus, resulting in NF $\kappa$ B activation and subsequent production of TNF- $\alpha$ . This activates microglia (Rivest, 2003), creating a positive feedback loop whereby activated microglia produce more TNF- $\alpha$  and other pro-inflammatory cytokines (Nadeau and Rivest, 2000). These pro-inflammatory cytokines activate neurons, causing an increase in  $\Delta$ FosB within regions like the limbic system that govern mood-like behaviours (Frenois et al., 2007). Indeed, infiltration of immune cells into the CNS can result in behavioural changes manifested by increased anxiety and depression (Dantzer et al., 2008).

One current method used to abate this inflammatory process is through use of TNF- $\alpha$  antagonists, like etanercept. It is a large (molecular weight of 150,000 Da) fusion protein that consists of extracellular region of human TNF-R2 fragment

coupled to an Fc region of human IgG that binds to transmembrane TNF- $\alpha$  and acts as a decoy receptor (Mohler et al., 1993). Etanercept can bind to both sTNF as well as tmTNF in addition to members of the lymphotoxin family (Thalayasingam and Isaacs, 2011). It is clinically used in the treatment of RA and psoriasis, via subcutaneous administration (Tyring et al., 2007, Hunt and Emery, 2013) and reduces co-morbidities of depression and cognitive impairment (Tyring et al., 2006, Kekow et al., 2011).

As this drug cannot cross the blood brain barrier (BBB) due to its large size (Boado et al., 2010), it is unable to directly interact with TNF- $\alpha$  present within the CNS, and exerts its effects peripherally. However etanercept indirectly reduces CNS inflammation by decreasing peripheral inflammation and preventing cross-talk (Kerfoot et al., 2006). This principal was evident in a 12 week treatment trial in psoriasis patients, where improved depressive symptoms were observed following subcutaneous etanercept injections (Tyring et al., 2006).

However, it is uncertain whether central blockade of TNF- $\alpha$  is efficacious in preventing CNS manifestations of peripheral inflammatory diseases. Furthermore, although research into the effects of centrally and peripherally administered TNF- $\alpha$  blockade on cognition and depressive symptoms has improved our understanding of cytokine actions in the CNS (Tobinick and Gross, 2008, Couch et al., 2013), little work has been undertaken to enhance our knowledge on the effects of TNF- $\alpha$  blockade on anxiety behaviours. This is surprising since peripheral inflammatory conditions like RA and psoriasis, involving TNF- $\alpha$  up-regulation are commonly associated with anxiety (Salim et al., 2011, Chen et al., 2013), although the exact mechanisms whereby inflammation induces psychiatric symptoms are unclear

(Chandarana et al., 1987, Couch et al., 2013). It is suggested that peripheral inflammation can cause up-regulation of TNF- $\alpha$ , which in turn activates macrophages peripherally and microglia centrally (Qin et al., 2007) and is further linked to the development of anxiety-like behaviour (Wohleb et al., 2011). Recently a study has shown that chronic peripheral administration of etanercept in rats reduced both depression-like and anxiety-like behaviour in the absence of immune stimulation (Bayramgurler et al., 2013a). While the study showed that chronic blockade of TNF- $\alpha$  action under physiologically normal conditions may be beneficial against anxiety-like behaviour (Bayramgurler et al., 2013a), it is unclear whether this mechanism will translate to peripheral inflammatory conditions. Astrocytes are known to be activated by LPS stimulation to produce cytokines like TNF- $\alpha$ , though the involvement in the development of behavioural disruptions is unknown (Zhang et al., 2014).

The present study sought to first determine the effects of peripheral immune challenge on anxiety-like and cognition-like behaviours by treating mice with an acute dose of LPS. Due to the link between peripheral cytokines and immune cells of the CNS (Kerfoot et al., 2006), we sought also to understand whether a single peripheral immune challenge would be sufficient to activate CNS immune cells. Our next aim was to investigate whether central blockade of TNF- $\alpha$  by etanercept would reverse the behavioural and neurobiological effects seen after LPS challenge. We hypothesized that centrally administered etanercept reduces anxiety-like behaviours, improves cognition-like behaviour and reduces CNS inflammation in mice that have received a peripheral immune challenge.



## **Methods**

### ***Mice***

Male C57BL/6 mice (Jackson stock number: 000664), aged 12 weeks, were purchased from the University of Adelaide breeding facility and used in this study (n = 40). Male mice were chosen for this study to avoid the possibility of confounding factors arising out of sexually dimorphic behaviour (Brown et al., 2000). All mice were housed in groups of 5 per cage during the experimental period, with food and water available ad libitum. Ambient temperature of the housing and testing rooms was  $22 \pm 1^{\circ}\text{C}$ . Mice were housed under a 12 hr light-dark cycle, lights on at 0700hr, and all behavioural testing was conducted between 0800 hr and 1600 hr. Animal procedures were approved by the University of Adelaide Animal Ethics committee.

### ***Drugs***

#### **Lipopolysaccharide (LPS)**

To induce a systemic inflammatory reaction for experimental procedures, LPS from *Escherichia coli* (Sigma Chemical Co., St. Louis, MO; 0111:B4) was diluted in saline and injected via intraperitoneal injection (IP) at a dose of 1mg/kg at 1400h. Control mice were injected with saline only by IP injection. Pilot studies in our lab using the same dose of LPS showed inflammation 24 hours post LPS in the form of increased serum levels of the cytokines TNF- $\alpha$ , IL-6, and MCP-1, as well as hippocampal and PFC gene expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-10. This led us to choose this dose of LPS and time point for administering etanercept, in the hope it would decrease TNF- $\alpha$  and other signs of inflammation, including glia.

### ***Etanercept***

Etanercept (Enbrel, Wyeth-Ayerst laboratories, 25 mg/kg) was diluted 1:10 in artificial cerebrospinal fluid (aCSF, composition detailed in Table 5.1) and administered intracerebroventricularly (ICV). A total volume of 2 µl was injected as previously established by Nilsberth et al (2009). 18hr post LPS/saline administration, mice were anaesthetised with isoflurane (1%), mounted in a stereotaxic frame, and kept at 37°C through a feedback controlled heating pad. A 0.3 mm burrhole was made at the point relative to Bregma: 1 mm to the right and 0.5mm posterior to inject into the lateral ventricle. A 33 gauge needle connected to a 5 µl Hamilton syringe was then lowered 2.5mm and either etanercept or aCSF (2 µl) injected at a rate of 0.5 µl/min. The needle was then left in place for 2 min, before being removed to suture the skin. Mice were then placed on a heat pad to recover. Once mice had regained normal mobility they were returned to their home cage with unlimited access to food and water and checked regularly for 24 hr to ensure there were no adverse effects from surgery.

**Table 5.1: Composition of artificial cerebrospinal fluid**

	Working Conc (mM)	1X 1Liter	10X 1liter
NaCl	125	7.305	73.05
KCl	2.5	0.186	1.86
MgCl <sub>2</sub> ·6H <sub>2</sub> O	1	0.2033	2.033
NaH <sub>2</sub> PO <sub>4</sub>	1.25	0.15	1.5
CaCl <sub>2</sub> ·2H <sub>2</sub> O	2	0.294	2.94
NaHCO <sub>3</sub>	25	2.1025	21.025
Glucose	25	4.505	45.05
pH with NaOH to 7.3			

**Legend:** The table details the composition of aCSF used in chapter 5

Once mice had regained normal mobility they were then returned to their home cage with unlimited access to food and water and checked regularly for 24 hr to ensure there were no adverse effects from surgery. Due to the quick turnover of the cerebrospinal fluid (Saunders et al., 1999), behavioural testing commenced 24 hr post-surgery and mice were given at least 1 hr of rest between each test to prevent interference. Mice were sacrificed immediately post behavioural testing.

### ***Design and Treatment Groups***

Mice were divided into 4 treatment groups (n = 40). Two groups were challenged with an acute dose of LPS (n = 20; IP administration) while 2 groups received saline (n = 20; IP administration). Of the LPS groups, one group received etanercept via ICV administration (n = 10; LPS-Etan: LPS challenge IP with etanercept administered ICV) and a control group received aCSF also via ICV administration (n = 10; LPS-aCSF: LPS challenge IP with aCSF administered ICV). Similarly with the saline treated mice, one group (n = 10) received etanercept through ICV injection (Sal-Etan: Saline treatment IP with etanercept administered ICV) while the other group (n = 10) received aCSF also through ICV injection (Sal-aCSF: Saline treatment IP with aCSF administered ICV).

### ***Behavioural Analysis***

A behavioural battery was carried out to assess locomotor activity, anxiety-like behaviour and cognition-like behaviour. Mice were given an hour between tests due to short half-life of etanercept and to gain the maximum results of the drug effects. A single test was not repeated in the same mouse. Tests were conducted 24 hr post ICV injections, as detailed in Table 5.3. Equipment for all tests was purchased from Stoelting Co. (USA) and all movements were tracked using ANY-MAZE imaging software Stoelting Co. (USA).

#### **Locomotor Activity: Open Field Test (OFT)**

Basal locomotor activity was measured in the OFT. Mice were placed at the centre of a brightly lit plexi-glass box (40 x 40 cm) and movements were tracked over a 5 min period as previously published (Baune et al., 2008, Camara et al., 2013). Distance travelled and time spent in the outer and inner zones of the box were measured.

#### **Anxiety-like Behaviour: Elevated Zero Maze (EZM)**

The EZM is used as a measure of anxiety-like behaviour wherein anxiety is measured as the time spent in the open arm of the maze. The apparatus consists of a grey Perspex circular platform of 50 cm diameter and width of 5 cm, located at a height of 40 cm above ground. The platform is divided into 4 equal quadrants (arms), two closed quadrants with grey Perspex walls (27 cm high) on the inside and outside, and two open quadrants without walls. This setup differs from the elevated plus maze in that the use of the zero maze over the elevated plus maze (EPM)

addresses and eliminates the issue of ambiguity with time spent in the central square of the EPM. This seems to make the zero maze a more robust apparatus over the plus maze (Shepherd et al., 1994).

Mice were placed at the centre of either one of the open arms and allowed to explore the apparatus for 5 min. Movement and time spent in open and closed arms was recorded. Time spent in open arms was used as a measure of anxiety-like behaviour, where more anxious mice spend less time in the open arms. Head dipping behaviour and time spent in stretch/attend posture from the closed arm were also recorded as measures of anxiety-like behaviour wherein increased time spent in these behaviour was taken as a measure of low anxiety-like response.

### **Spatial Recognition Memory: Y Maze**

The Y maze is used to assess hippocampal spatial recognition memory. It consists of a three-armed chamber, with the arms at a 120° angle from each other. Each arm is 35 cm long, 5.0 cm wide and 10 cm high (Choy et al., 2008).

**Phase 1:** One arm of the maze was closed off. Mice were placed at the bottom of the “start” arm and allowed to explore the two arms for 10 min.

**Phase 2:** 45 min after phase one, mice were tested with all three arms left open. Mice were again placed at the bottom of the start arm and allowed to explore the three arms for 5 min. Mice have a preference for exploring novel environments (Dulawa et al., 1999). Thus normal mice should spend more time exploring the novel arm, indicating normal spatial recognition memory. This was determined by calculating a preference for the novel arm for each mouse, using the following equation:  $\text{Time in novel arm} / (\text{Time in novel arm} + \text{time in familiar arm}) * 100$ . Mice

that did not enter either the familiar or novel arms during Phase 2 were excluded from analysis. As all mice started in the same arm (start arm) time spent in this arm was not analysed.

### ***Gene Expression Analysis (RT-qPCR)***

A subset of mice was selected for qPCR analysis to measure the expression of *Tnfa* post LPS and etanercept treatment (n = 5/treatment group). Mice were sacrificed via a lethal dose of pentobarbital via IP injection and hippocampal and prefrontal cortex (PFC) tissue from mice was collected and stored in RNAlater (Ambion, Life Technologies) at -20°C until required for RNA extraction. The hippocampus was analysed as it plays a role in spatial cognition (Sweatt, 2004) as well as some emotional responses (Sweatt, 2004), while the PFC was chosen for analysis as it plays a role cognitive control (Miller and Cohen, 2001) and goal oriented emotional processing (Davidson, 2002).

Brain tissue was lysed with TissueLyserLT (Qiagen, Australia) with 5mm stainless steel lysis bead (Qiagen, Australia). RNA was then extracted from the lysed tissue using PureLink® RNA mini extraction kit (Ambion) as per manufacturer's instructions. RNA was then quantified using Nanodrop Lite (Thermo Fischer Scientific). Gene expression analysis for *Tnfa* was performed by RT-qPCR in two steps. First, complementary DNA (cDNA) was synthesised from the total RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen) in a thermal cycler (MJ research) following manufacturer's instructions. The products were then diluted to attain uniform concentration of 10 ng/µl of cDNA for qPCR.

In the next step qPCR was performed on the cDNA using the Applied Biosystems 7500 Fast real-time PCR machine (ABI, Life technologies). Expression of *Tnfa* was quantified with *Gapdh* used as the endogenous reference gene using the primers listed below;

*Tnfa*-forward: CCACCACGCTCTTCTGTCTA

*Tnfa*-reverse: AGGGTCTGGGCCATAGAACT

*Gapdh*-forward: TGTTCTACCCCCAATGTGT

*Gapdh*-reverse: CCTGCTTCACCACCTTCTTG

Samples and negative controls (without cDNA) for each gene were run in triplicates on a 96 well MicroAmp Fast optical-qPCR plate (ABI, Life technologies). Briefly, 4µl of cDNA was added to a master mix consisting of 10 µl of 2X *Power* SYBR Green PCR master mix (ABI, Life technologies), 0.14 µl each of forward and reverse primer along with 5.72 µl of double distilled water to make a total reaction volume of 20 µl. The raw data consisting of the Ct (Cycle threshold) values for individual reactions were exported to excel files and analysis was conducted manually using the delta-delta CT method (Livak and Schmittgen, 2001).



**Table 5.2: Dosing and testing schedule of experiments**

Day 1	Day 2	Day 3
LPS/ Saline Dosing	Etanercept /aCSF Treatment by ICV injection	OFT (9-10am)
		EZM (11-12 am)
		Y maze (1-3pm)
		Tissue Collection

**Legend: LPS: Lipopolysaccharide, aCSF: Artificial Cerebrospinal Fluid, OFT: Open Field Test, EZM: Elevated Zero Maze**

### ***Immunohistochemistry (IHC)***

Following behavioural testing, animals were injected IP with pentobarbital and perfused via transcardiac injection with 10% neutral buffered formalin. Brains were then processed and embedded in paraffin wax. Serial 5 µm sections spaced 150 µm apart were taken of the entire length of the hippocampus. Sections were then assessed for levels of neuroinflammation (IBA1, GFAP) via immunohistochemistry. Briefly, slides were de-waxed, dehydrated and endogenous peroxidase activity was blocked, before undergoing antigen retrievably heating slides at boiling point for 10 min in citrate solution. Non-specific binding was then specific binding blocked with normal horse serum, before application of the appropriate primary antibody with incubation overnight (Abcam, IBA1 1:1000, Santa Cruz, GFAP 1:40,000, Dako). The next day slides were incubated with the appropriate species of secondary antibody (Abacus, 1:250) (Table 5.4) followed by

streptavidin peroxidase. The antigen-antibody complex was then detected with Diaminobenzadine (DAB) (Sigma) followed by counter staining with haematoxylin prior to dehydration and mounting with DePex mounting media.

Slides were digitally scanned using the Nanozoomer (Hamamatsu) and viewed with the associated software (NDP view). Serial images of the CA region of the hippocampus and the dentate gyrus of each section were captured as jpegs. These images were then exported to Image J (NIH) and the number of positive cells manually counted.

**Table 5.3: Antibodies employed within this study**

Cells/Proteins of Interest	Primary Antibody	Dilution		
		(In Normal Horse Serum)	Secondary Antibody	Retrieval Buffer
<b>Microglia</b>	IBA1	1:1000	Anti-Goat	Citrate
<b>Astrocytes</b>	GFAP	1:40,000	Anti-Rabbit	Citrate

**Legend:** Table shows the list of antibodies that were used in this study for IHC analysis.

### ***Statistical Analysis***

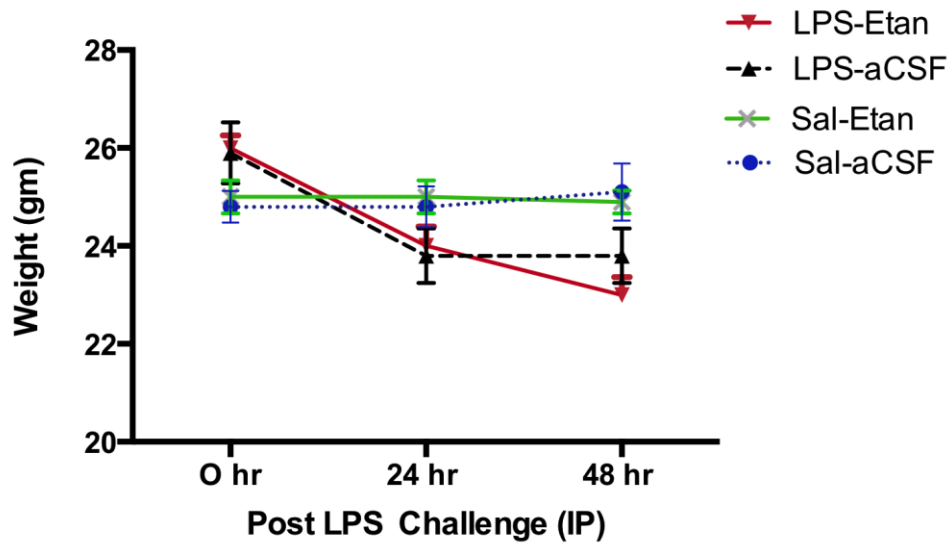
Data analyses were carried out using GraphPad Prism software (version 6.01). All data are presented as mean  $\pm$  SEM. Comparisons between groups were carried out using 2-way ANOVA with Bonferroni's multiple comparisons post-hoc test where appropriate. Weight of mice was analysed using a 2 x 2 x 3 ANOVA. A significance level of  $p < 0.05$  was regarded as significant in all tests.

## Results

### *Age and Weight of Animals*

All male mice that were used in this study were tested and killed at 13 weeks of age. Weight was measured at baseline and 24 hr following LPS challenge as well as 24 hr following etanercept treatment (48 hr after LPS). Change in weight over time was analysed using a 2 x 2 x 3 ANOVA. Tests of within subjects' measures showed a significant effect of time ( $F_{(2,36)} = 42.2$ ,  $P < 0.001$ ), indicating a decrease in weights over the 48 hr period of immune challenge and etanercept treatment. In addition, a significant effect of time\*LPS ( $F_{(2,36)} = 23.6$ ,  $P < 0.001$ ) was observed, indicating LPS significantly reduced weights in mice over time. However an effect of time\*etanercept was not seen ( $F_{(2,36)} = 2.34$ ,  $P = 0.10$ ), suggesting etanercept had no effect on weight, and was not able to reverse the decrease in weight caused by LPS treatment. No significant 3-way interaction between time, LPS, and etanercept ( $F_{(2,36)} = 0.99$ ,  $P = 0.37$ ) was observed (Figure 5.1).

Figure 5.1: Weight of mice before and after LPS dosing

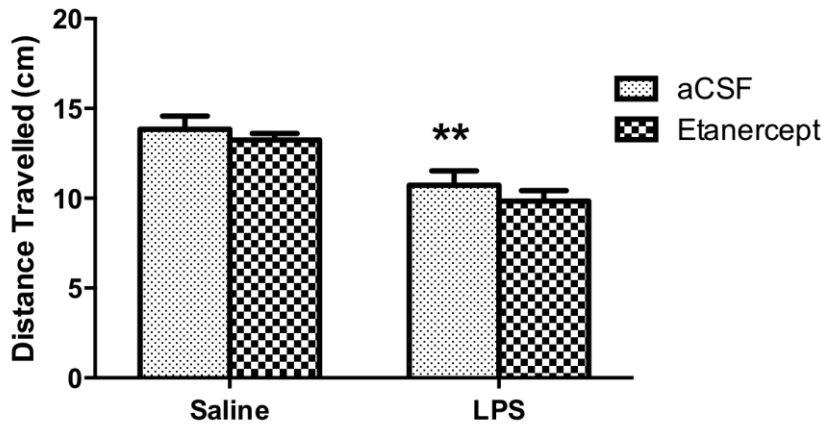


Legend: The figure depicts the weight of mice over the 48hr experimental period. All data represent mean  $\pm$  SEM (n=10/group). (*Sal-aCSF*: Saline treatment IP with aCSF administered ICV, *Sal-Etan*: Saline treatment IP with etanercept administered ICV, *LPS-aCSF*: LPS challenge IP with aCSF administered ICV, *LPS-Etan*: LPS challenge IP with etanercept administered ICV). Data compared using 2 X 2 X 3-way ANOVA with Bonferroni's multiple comparison test.

### Locomotor Activity

Locomotor activity was measured under stressful conditions using the open field test. Analysis by 2-way ANOVA found no interaction effect or effect of etanercept treatment on locomotor activity but a significant effect of LPS challenge was observed (Figure 5.2: 2-way ANOVA: Interaction:  $F_{(1,36)} = 0.049$ ,  $P = 0.8254$ , Saline/LPS challenge:  $F_{(1,36)} = 25.84$ ,  $P < 0.0001$ , aCSF/etanercept treatment:  $F_{(1,36)} = 1.35$ ,  $P = 0.25$ ). Bonferroni's multiple comparison showed that LPS-aCSF treated mice were less active than Sal-aCSF treated mice ( $P = 0.009$ ). These results suggest that while treatment with LPS impaired locomotor activity, etanercept was ineffective in reversing this impairment.

Figure 5.2: Locomotor Activity (OFT)



**Legend:** Distance travelled in OFT, as a measure of baseline locomotor activity All data represent mean  $\pm$  SEM (n=10/group). (*Sal-aCSF*: Saline treatment IP with aCSF administered ICV, *Sal-Etan*: Saline treatment IP with etanercept administered ICV, *LPS-aCSF*: LPS challenge IP with aCSF administered ICV, *LPS-Etan*: LPS challenge IP with etanercept administered ICV). Data compared using 2-way ANOVA, (Interaction:  $P = 0.8254$ , Saline/LPS challenge:  $P < 0.001$ , aCSF/etanercept treatment:  $P = 0.25$ ). \*\*  $P < 0.01$  LPS-aCSF compared to Sal-aCSF treated mice, analysed by Bonferroni's multiple comparison

### Anxiety-like Behaviour

Anxiety-like behaviour was measured using the EZM (Figure 5.3). Mice were allowed to move freely between open and closed arms. Time spent in the open arm is regarded as an indicator of lower levels of anxiety-like behaviour, as are time spent in head-dipping and stretched posture (Shepherd et al., 1994). No significant interaction effect was seen between groups for time in open arms, but a significant effect for aCSF/etanercept treatment was noted (2-way ANOVA: Interaction:  $F_{(1,31)} = 0.64$ ,  $P = 0.42$ , Saline/LPS challenge:  $F_{(1,31)} = 1.18$ ,  $P = 0.28$ , aCSF/etanercept treatment:  $F_{(1,31)} = 4.58$ ,  $P = 0.04$ ).

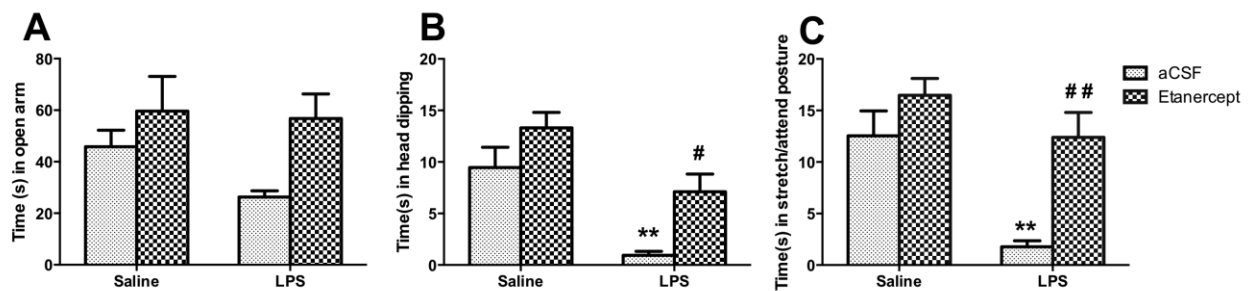
No interaction effect for time spent in head dipping behaviour was observed, however an effect of LPS challenge and etanercept treatment was seen (2-way ANOVA: Interaction:  $F_{(1,30)} = 0.60$ ,  $P = 0.44$ , Saline/LPS challenge:  $F_{(1,30)} = 23.92$ ,  $P < 0.001$ , aCSF/etanercept treatment:  $F_{(1,30)} = 11.09$ ,  $P = 0.0023$ ; Figure 5.3B). Bonferroni's multiple comparison of LPS effect showed that LPS-aCSF treated mice spent less time in head dipping behaviour than Sal-aCSF mice ( $P = 0.0031$ ), suggesting LPS increases anxiety-like behaviour. Bonferroni's multiple comparison for etanercept effect showed that LPS-Etan treated mice spent more time in head dipping behaviour than LPS-aCSF mice ( $P = 0.025$ ), indicating that etanercept was able to reverse the effects on LPS on head dipping behaviour.

Similar results were also seen for stretch-attend posture (2-way ANOVA: Interaction:  $F_{(1,31)} = 2.98$ ,  $P = 0.096$ ; Saline/LPS challenge:  $F_{(1,31)} = 14.55$ ,  $P = 0.0006$ , , aCSF/etanercept treatment:  $F_{(1,31)} = 13.93$ ,  $P = 0.0008$ ; Figure 5.3C). Bonferroni's multiple comparison of LPS effect showed that LSP-aCSF treated mice spent less time in stretch attend posture compared to Sal-aCSF mice ( $P = 0.0032$ ), indicative of LPS induced anxiety-like behaviour. Again, Bonferroni's multiple comparison for etanercept effects showed that LPS-Etan treated mice spent more time in stretch attend posture than LPS-aCSF mice ( $P = 0.002$ ), indicating that etanercept can reduce anxiety behaviours in LPS treated mice.

Distance travelled in the open arm was also measured between groups and no significant differences were detected (2-way ANOVA: Interaction:  $F_{(1,34)} = 1.17$ ,  $P = 0.28$ ; Saline/LPS challenge:  $F_{(1,34)} = 0.15$ ,  $P = 0.69$ ; aCSF/etanercept treatment:  $F_{(1,34)} = 1.20$ ,  $P = 0.81$ ; Mean  $\pm$  SEM: Sal-aCSF:  $10.4 \pm 1.05$ , LPS-aCSF  $8.189 \pm 1.9$ , Sal-Etan:  $8.45 \pm 1.06$ , LPS-Etan:  $9.45 \pm 1.83$ ). This indicates that though there were

significant changes observed in the OFT between LPS and Saline treated mice, this difference did not extend to the EZM and the differences observed in this test were reflections of anxiety-like behaviour and not locomotor deficits.

**Figure 5.3: Anxiety-like Behaviour (EZM)**



**Legend:** A) Time spent in open arm B) Time spent in head dipping behaviour, C) Time spent in stretch/attend posture. All data represent mean  $\pm$  SEM (n=10/group). (*Sal-aCSF*: Saline treatment IP with aCSF administered ICV, *Sal-Etan*: Saline treatment IP with etanercept administered ICV, *LPS-aCSF*: LPS challenge IP with aCSF administered ICV, *LPS-Etan*: LPS challenge IP with etanercept administered ICV). Data compared using 2-way ANOVA (A: Interaction:  $P = 0.42$ , Saline/LPS challenge:  $P = 0.28$ , aCSF/etanercept treatment:  $P = 0.04$ ; B: Time in head dipping behaviour: Interaction:  $P = 0.44$ , Saline/LPS challenge:  $P < 0.001$ , aCSF/etanercept treatment:  $P = 0.0023$ ; C: Stretch-attend posture: Interaction:  $P = 0.096$ , Saline/LPS challenge:  $P = 0.0006$ , aCSF/etanercept treatment:  $P = 0.0008$ ). \*\*  $P < 0.01$  LPS-aCSF compared to Sal-aCSF mice, #  $P < 0.05$ , ##  $P < 0.01$  LPS-Etan compared to LPS-aCSF mice, analysed by Bonferroni's multiple comparisons.

### Cognition-like Behaviour

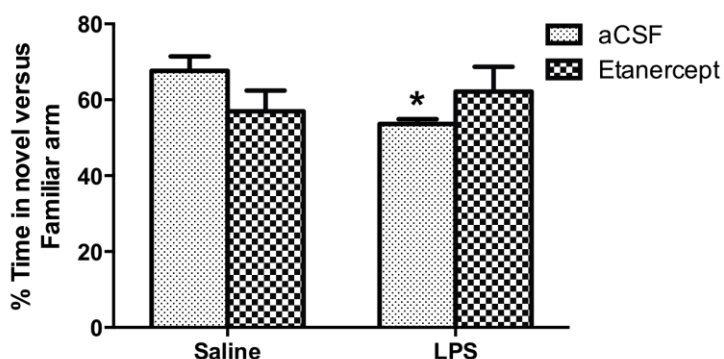
The Y maze was used as a measure of spatial recognition memory. . As all mice started in the same arm (start arm) time spent in this arm was not analysed. Analysis however showed that all mice spent similar amount of time in start arm.



A preference index for the novel arm was calculated and a significant interaction effect was found by 2-way ANOVA (Interaction:  $F_{(1,20)} = 4.96$ ,  $P = 0.037$ , Saline/LPS challenge:  $F_{(1,20)} = 1.04$ ,  $P = 0.31$ , aCSF/etanercept treatment:  $F_{(1,20)} = 0.06$ ,  $P = 0.80$ ). Bonferroni's post-hoc analysis revealed a significant decrease in spatial recognition memory in LPS-aCSF compared to Sal-aCSF mice ( $P = 0.038$ ), but no significant difference was noted between LPS-aCSF and LPS-Etan mice ( $P > 0.9$ ) suggesting this impairment was not rescued by etanercept.

Distance travelled in the Y maze was also measured between groups and no significant differences were detected (2-way ANOVA: Interaction:  $F_{(1,36)} = 0.96$ ,  $P = 0.33$ ; Saline/LPS challenge:  $F_{(1,36)} = 0.91$ ,  $P = 0.34$ ; aCSF/etanercept treatment:  $F_{(1,34)} = 1.81$ ,  $P = 0.18$ ; Mean  $\pm$  SEM: Sal-aCSF:  $6.39 \pm 1.28$ , LPS-aCSF:  $10.87 \pm 3.78$ , Sal-Etan:  $5.54 \pm 0.63$ , LPS-Etan:  $5.47 \pm 2.24$ ).

**Figure 5.4: Cognition-like Behaviour (Y maze)**



**Legend:** A) Percentage of time spent in the novel arm vs. the familiar arm of the retention phase of Y maze, All data represent mean  $\pm$  SEM (n=5-7/group). (*Sal-aCSF*: Saline treatment IP with aCSF administered ICV, *Sal-Etan*: Saline treatment IP with etanercept administered ICV, *LPS-aCSF*: LPS challenge IP with aCSF administered ICV, *LPS-Etan*: LPS challenge IP with etanercept administered ICV). Data compared using 2-way ANOVA (Interaction:  $P = 0.037$ , Saline/LPS challenge:  $P = 0.31$ ,

aCSF/etanercept treatment:  $P = 0.80$ ). \*  $P < 0.05$  LPS-aCSF compared to Sal-aCSF treated mice, analysed by Bonferroni's multiple comparison.

### ***Expression of (mRNA) *Tnfa* in the Hippocampus and PFC***

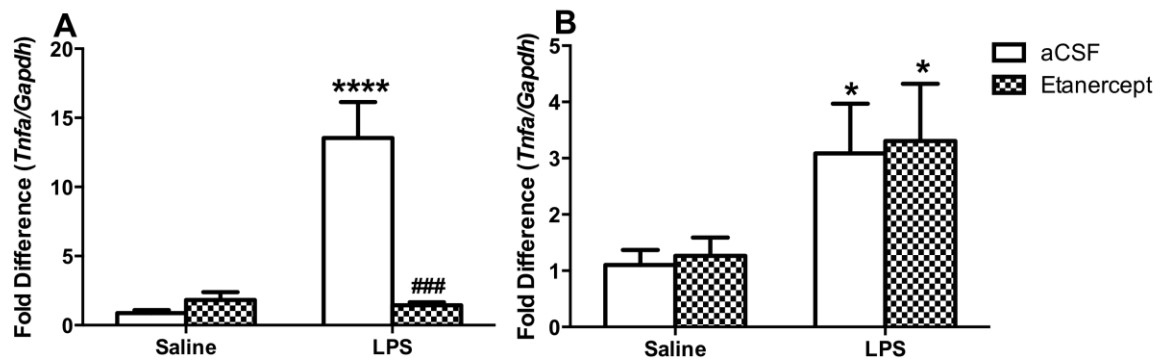
Levels of (mRNA) *Tnfa* in the hippocampus and the PFC of mice were measured 48 hr after LPS challenge. This was done firstly, to test whether LPS would increase *Tnfa* levels in these CNS areas, and then to test whether etanercept would be effective in reversing the up-regulation of *Tnfa*.

In the hippocampus (Fig 5.5A), we found significant difference in mRNA levels of *Tnfa* between groups (Interaction:  $F_{(1,13)} = 28.04$ ,  $P = 0.0001$ , Saline/LPS challenge:  $F_{(1,13)} = 24.52$ ,  $P = 0.0003$ , aCSF/etanercept treatment:  $F_{(1,13)} = 6.84$ ,  $P = 0.021$ ). Post hoc analysis showed that indeed LPS-aCSF mice had significantly higher levels of *Tnfa* compared to Sal-aCSF mice ( $P < 0.001$ ) indicating that peripheral LPS was effective in increasing CNS *Tnfa* levels. Furthermore etanercept treatment was successful in significantly reducing *Tnfa* levels in LPS-Etan mice compared to LPS-aCSF mice ( $P = 0.001$ ).

In the PFC (Fig 5.5B) the difference between treatment groups was not as robust as in the hippocampus. 2-way ANOVA comparing the different treatment groups found a significant positive effect of LPS in increasing *Tnfa* expression (Interaction:  $F_{(1,15)} = 0.09$ ,  $P = 0.76$ , Saline/LPS challenge:  $F_{(1,15)} = 14.25$ ,  $P = 0.018$ , aCSF/etanercept treatment:  $F_{(1,15)} = 0.07$ ,  $P = 0.79$ ) but no significant interaction effects, suggesting LPS was effective in increasing *Tnfa* but etanercept was ineffective in reversing this effect. Post hoc analysis of LPS effect confirmed that LPS was effective in increasing *Tnfa* levels in LPS challenged mice (LPS-aCSF)

compared to Sal-aCSF mice ( $P = 0.026$ ). LPS-Etan mice also had significantly higher *Tnfa* levels than Sal-Etan mice ( $P = 0.045$ ).

Figure 5.5: Expression levels of (mRNA) *Tnfa* in the PFC and Hippocampus



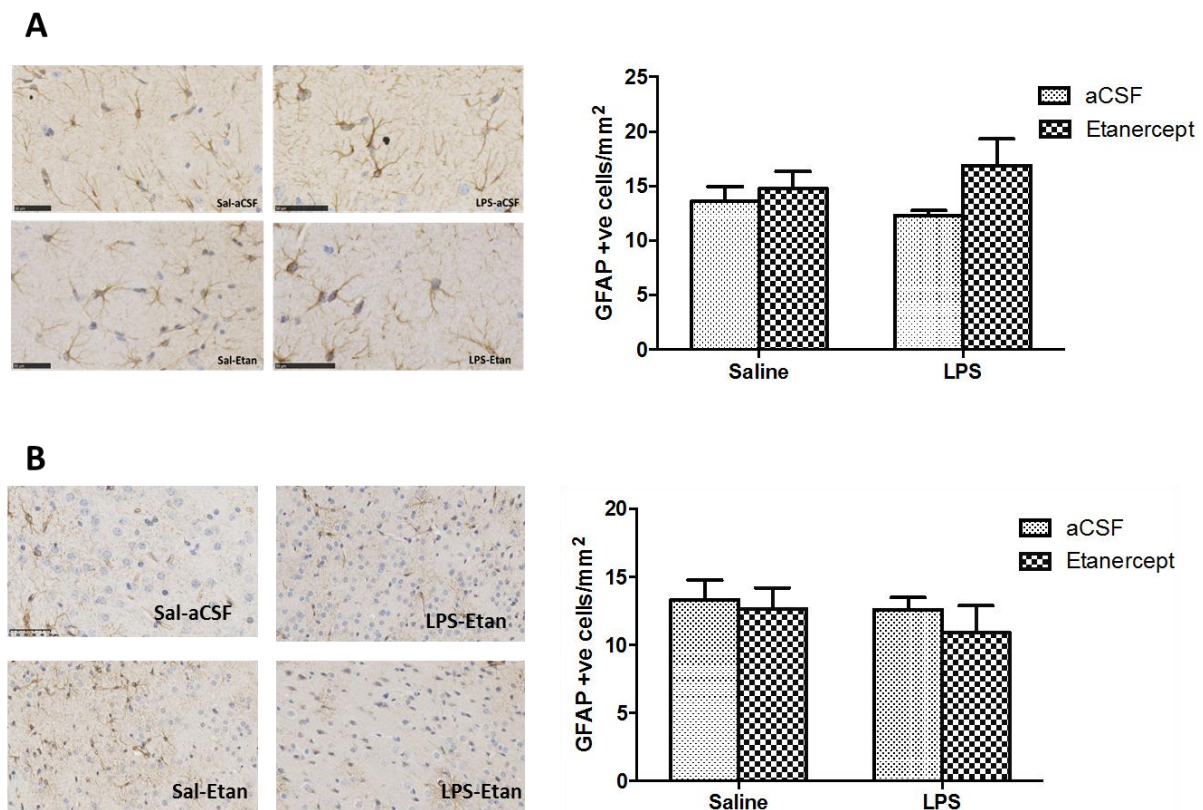
Legend: A) Fold difference of *Tnfa* expression between treatment groups in the hippocampus, B) Fold difference of *Tnfa* expression between treatment groups in the PFC, All statistical analysis were done using the delta Ct values (expression of *Tnfa* normalised to *Gapdh*) and the data represent mean  $\pm$  SEM ( $n = 5/\text{group}$ ). (Sal-aCSF: Saline treatment IP with aCSF administered ICV, Sal-Etan: Saline treatment IP with etanercept administered ICV, LPS-aCSF: LPS challenge IP with aCSF administered ICV, LPS-Etan: LPS challenge IP with etanercept administered ICV). Data compared using 2-way ANOVA (A: Interaction:  $P = 0.0001$ , Saline/LPS challenge:  $P = 0.0003$ , aCSF/etanercept treatment:  $P = 0.021$ ; B: Interaction:  $P = 0.76$ , Saline/LPS challenge:  $P = 0.018$ , aCSF/etanercept treatment:  $P = 0.79$ ). \*  $P < 0.05$ , \*\*\*\*  $P < 0.0001$  compared to Sal-aCSF mice, ###  $P < 0.001$  LPS-Etan compared to Sal-Etan, analysed with Bonferroni's post hoc test

### Examination of Microglial and Astrocyte Numbers following LPS treatment

To study the effects of LPS and etanercept on resident immune cells in the brain, microglial (IBA1) and astrocyte (GFAP) numbers were examined by staining hippocampal slices using IHC, and counting the number of positive cells in the dentate gyrus and the PFC. Immune challenge with LPS had no effect on astrocyte

number within the dentate gyrus (2-way ANOVA: Interaction:  $F_{(1,15)} = 1.24$ ,  $P = 0.28$ , Saline/LPS challenge:  $F_{(1,15)} = 0.077$ ,  $P = 0.78$ , aCSF/etanercept treatment:  $F_{(1,15)} = 3.58$ ,  $P = 0.07$ , Fig 5.6A), or in the PFC (2-way ANOVA: Interaction:  $F_{(1,12)} = 0.11$ ,  $P = 0.73$ , Saline/LPS challenge:  $F_{(1,12)} = 0.69$ ,  $P = 0.42$  aCSF/etanercept treatment:  $F_{(1,12)} = 0.63$ ,  $P = 0.44$ , Fig 5.6B).

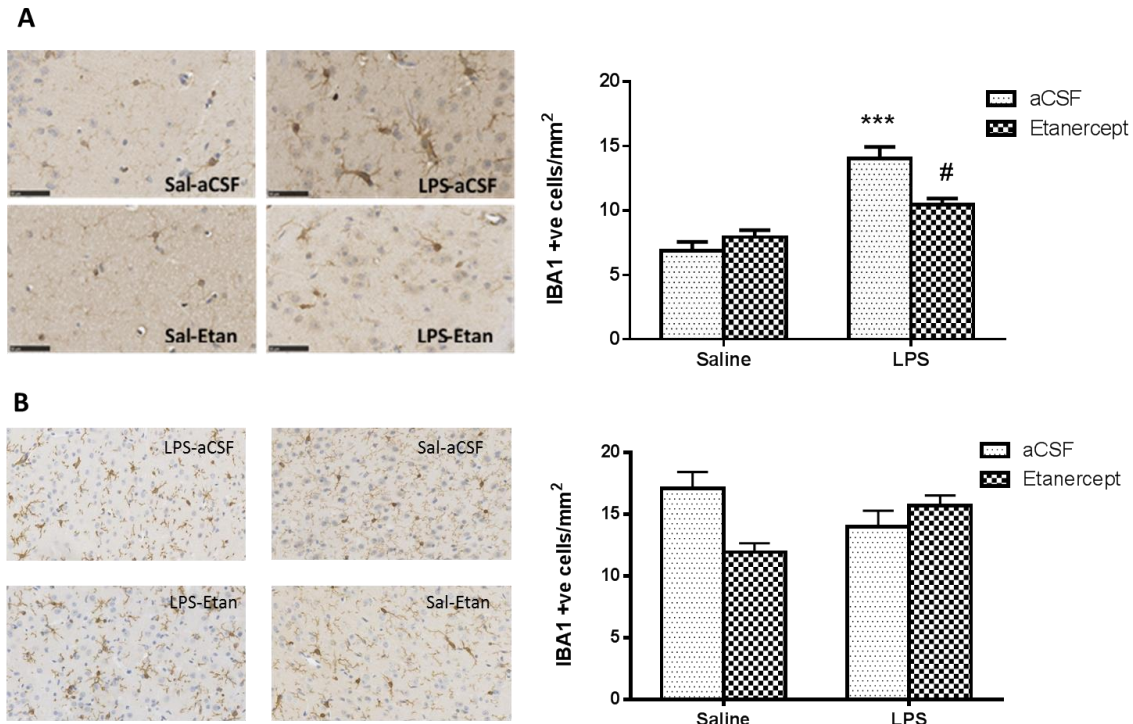
**Figure 5.6: Astrocyte counts across experimental conditions**



**Legend:** A) the figure shows IHC images of astrocytes in the dentate gyrus while the graph represents the number of GFAP positive cells in the dentate gyrus. B) The figure shows IHC images of astrocytes in the PFC while the graph represents the number of GFAP positive cells in the PFC. All data represent mean  $\pm$  SEM ( $n = 5$ /group, *Sal-aCSF*: Saline treatment IP with aCSF administered ICV, *Sal-Etan*: Saline treatment IP with etanercept administered ICV, *LPS-aCSF*: LPS challenge IP with aCSF administered ICV, *LPS-Etan*: LPS challenge IP with etanercept administered ICV). Data compared using 2-way ANOVA.

LPS however did lead to a significant increase in microglial numbers in the hippocampus, with a significant interaction effect between LPS challenge and etanercept treatment (2-way ANOVA: Interaction:  $F_{(1,15)} = 11.41$ ,  $P = 0.0041$ , Saline/LPS challenge:  $F_{(1,15)}=50.13$ ,  $P<0.001$ , aCSF/etanercept:  $F_{(1,15)} = 3.36$ ,  $P = 0.08$ , Figure 5.7A). Bonferroni's multiple comparison of LPS effect on microglial numbers showed that LPS-aCSF treated mice had higher number of IBA1 positive cells in the dentate gyrus compared to Sal-aCSF mice ( $P < 0.001$ ). Bonferroni's multiple comparison of etanercept effects showed that LPS-Etan mice had lower number of BA1 positive cells within the dentate gyrus compared to LPS-aCSF ( $P = 0.016$ ). In the PFC, however, we found that while there was a significant interaction effect between treatment groups, there was no significant effect of LPS or etanercept (2-way ANOVA: Interaction:  $F_{(1,9)} = 6.81$ ,  $P = 0.02$ , Saline/LPS challenge:  $F_{(1,9)} = 0.05$ ,  $P = 0.81$ , aCSF/etanercept treatment:  $F_{(1,15)} = 1.7$ ,  $P = 0.22$ , figure 5.7B). These results indicate that LPS increased microglial numbers only in the hippocampus and not PFC, and this increase was prevented with etanercept treatment.

**Figure 5.7: Microglial counts across experimental conditions**



**Legend:** A) the figure shows IHC images of the microglia in the dentate gyrus while the graph represents the number of IBA1 positive cells in the dentate gyrus. B) The figure shows IHC images of the microglia in the PFC while the graph represents the number of IBA1 positive cells in the PFC. All data represent mean  $\pm$  SEM ( $n = 5/\text{group}$ , *Sal-aCSF*: Saline treatment IP with aCSF administered ICV, *Sal-Etan*: Saline treatment IP with etanercept administered ICV, *LPS-aCSF*: LPS challenge IP with aCSF administered ICV, *LPS-Etan*: LPS challenge IP with etanercept administered ICV). Data compared using 2-way ANOVA (A: Interaction:  $P = 0.0041$ , Saline/LPS challenge:  $P < 0.001$ , aCSF/etanercept:  $P = 0.08$ ; B: Interaction:  $P = 0.02$ , Saline/LPS challenge:  $P = 0.81$ , aCSF/etanercept treatment:  $P = 0.22$ ). \*\*\*\*  $P < 0.001$  LPS-aCSF compared to Sal-aCSF, #  $P < 0.05$  LPS-Etan compared to LPS-aCSF analysed by Bonferroni's multiple comparisons.

## Discussion

In the present study we have shown that acute systemic immune challenge with LPS resulted in sickness behaviour in mice, evident by a significant decrease in weight at 24 hr, and impaired locomotor activity in the OFT. Furthermore, LPS significantly increased some aspects of anxiety-like behaviour in the EZM and significantly reduced preference for the novel arm in the Y Maze. Etanercept treatment post-LPS was able to prevent the effects of LPS on anxiety-like behaviours in the EZM, but we were unable to observe a significant effect on cognition-like behaviour (Y Maze), weight loss or locomotor activity. LPS challenge increased (mRNA) *Tnfa* expression in the hippocampus and PFC of treated mice, while etanercept treatment was significantly effective in reversing these effects in the hippocampus only. The effects of etanercept were also associated with a significant reduction in microglial numbers within the hippocampus of these mice following LPS administration.

LPS is a potent and robust model of acute sickness behaviour, triggering activation of toll-like receptors and up-regulation of chemokines and cytokines like TNF- $\alpha$ , IL-1 $\beta$  and CXCL1 (Ortega et al., 2011). This leads to acute changes in food intake, fever, hypo-activity, lethargy, and changes in mood in mice (Dantzer et al., 2008), akin to clinical features of depression and anxiety-like behaviour (Yirmiya et al., 2001, Dantzer et al., 2008). These features were replicated in this study, with LPS causing weight loss, reduction in locomotor activity as well as cognitive impairment and increased anxiety-like behaviour 48 hr post administration. This corresponds with previous studies that showed that while the peak of peripheral LPS activity, marked by increased cytokine expression, occurs 4-8 hr post administration,



its central effects are delayed. Indeed, it is reported that LPS related effects on cognition-like behaviour manifest as early as 4 hr and are present up to 24 hr post administration, whilst depression-like behaviours are evident only from 6 hr, up to 24 hr post administration (Frenois et al., 2007, Noble et al., 2007). In our study we extended these findings, by determining that changes in cognition and anxiety-like behaviour were still present 48 hr after LPS administration, although the peak effects of LPS induced behavioural changes may have been missed due to the delay between LPS treatment and behavioural analyses. The exact mechanisms whereby inflammation induces neurological symptoms are not yet clearly understood (Chandarana et al., 1987, Cavailles et al., 2013, Couch et al., 2013) and in the present study the anxiety-like effects of LPS appear to be partially dependent on TNF- $\alpha$ , as anxiety-like behaviour on the EZM could be reduced by central administration of etanercept. While these effects were significant for head dip and stretch attend measures only, time spent in open arms showed the same trend across groups, suggesting the effects of LPS and etanercept are consistent across measures. Higher number of mice may have led to all three measures showing significant differences between groups. Additionally no differences between groups were observed for distance travelled in this test, indicating that the behaviour observed was representative of anxiety-like responses and not merely locomotor differences. A role for TNF- $\alpha$  in anxiety-like behaviour is supported by studies demonstrating that chronic peripheral administration of TNF- $\alpha$  antagonists reduce levels of depression and anxiety in conditions including chronic pain (Chen et al., 2013) and RA (Uguz et al., 2009), whilst genetic knockout of TNF- $\alpha$  lowers anxiety-like behaviour in young mice (Camara et al., 2013). The role of TNF- $\alpha$  in regulating



emotional responses within the CNS is yet to be determined with suggestions that TNF- $\alpha$  may be involved in the regulation of the serotonin transporter pathway, as well as stimulation of the hypothalamic-pituitary axis, which are involved in generating emotional responses (Himmerich et al., 2006, Zhu et al., 2006, Bayramgurler et al., 2013a).

Supporting the present findings, that a single challenge with LPS systemically can increase central TNF- $\alpha$  expression, Bossu *et al* (2012), showed that a single dose of LPS (5 mg/kg) was effective in increasing TNF- $\alpha$  expression in the hippocampus and PFC from 7 days after LPS challenge, lasting up to 10 months after challenge and these changes were associated with cognitive impairments. These results indicate that TNF- $\alpha$ , through systemic LPS can have lasting neuroinflammatory effects in the brain and may lead to cognitive decline. High doses of LPS have also been shown to cause loss of neuronal loss in the frontal cortex along with cognitive impairment (Semmler et al., 2007). LPS challenge has also been linked to decreased parvalbumin reactivity (required for inhibitory neurons) in neurons of the hippocampus without any corresponding change in the frontal regions of rodents (Jenkins et al., 2009). Furthermore extremely high doses of LPS (10 mg/kg and 5 mg/kg) were shown to reduce cholinergic innervations (Semmler et al., 2007), cause degeneration of dopaminergic neurons (Qin et al., 2007). High LPS (5mg/kg) also increased TNF- $\alpha$  levels in the brain, serum and liver (Qin et al., 2007). These studies support the current findings of increased (mRNA) *Tnfa* in the hippocampus and PFC 48 hr after systemic LPS challenge of a relatively lower dose.

In the present study, TNF- $\alpha$  appears to play an important role in mediating the anxiety-like effects induced by LPS as etanercept reduced anxiety-like behaviours.

This role of TNF- $\alpha$  is supported by previous studies where genetic knockout of TNF- $\alpha$  reduced anxiety-like behaviour in young mice (Camara et al., 2013), while central blockade reduced experimental autoimmune encephalitis (EAE) induced anxiety in mice (Haji et al., 2012). Additionally TNF- $\alpha$  antagonists reduced anxiety in chronic pain models in mice after peripheral administration (Chen et al., 2013) and this was supported by decreased anxiety observed in RA patients after anti-TNF- $\alpha$  treatment (Uguz et al., 2009). Haji *et al* (2012) showed that the release of TNF- $\alpha$  from activated microglia altered excitatory transmission within the striatum promotes the development of anxiety-like behaviour while etanercept decreased decay time and duration of mini excitatory post-synaptic currents (mEPSC), indicating a reduction in glutamatergic signalling. Other potential mechanisms whereby TNF- $\alpha$  may induce the development of anxiety-like behaviour include the stimulation of microglia to release glutamate causing neurotoxicity by cell death (Takeuchi et al., 2006) and by silencing cell survival signals (Tamminga, 1998, Venters et al., 2000). Moreover, dysfunction in glutamate signalling has been related to cognitive decline and development of psychotic symptoms (Francis, 2009). Furthermore, it has been suggested that TNF- $\alpha$  modifies the serotonin transporter uptake through p38 mitogen activated protein kinases and thereby altering emotional behaviour, which has been related to anxiety, depression and other mood disturbances like aggression (Zhu et al., 2006).

Although the present study demonstrated a significant effect of LPS challenge on cognition-like behaviours, independent of changes in locomotor activity in this test, etanercept failed to significantly reverse these deficits. Despite the LPS model not being directly translatable to RA, continuous peripheral TNF- $\alpha$  antagonism in RA

patients improved cognition (Chen et al., 2010), suggesting that chronic rather than a single dose administration is needed to achieve such effects. However, it should be noted that Tobinick *et al* (2006, 2008) observed a rapid improvement in cognition in a small cohort of patients with Alzheimer's disease (AD) following a single dose of perispinal etanercept and was supported by animal research on AD models where antagonism of TNF- $\alpha$  by ICV injection had immediate effects on cognition (Medeiros et al., 2007). Additionally, these findings suggest that centrally administered etanercept is more effective in attenuating cognitive impairment in CNS disease models like AD, which are associated with more substantial cognitive deficits, than in our peripheral inflammatory model induced with a single dose of LPS. The lack of significant effects of etanercept in cognition-like behaviour in our study may be linked to the time delay between LPS administration and etanercept treatment. Since major effects of LPS on cognition have previously only been reported up to 24 hr post LPS (Noble et al., 2007), the present study may have missed the peak of cognitive dysfunction, although significant deficits were still noted in the LPS treated mice. Thus, measuring the efficacy of etanercept at an earlier time-point post LPS administration may be necessary to observe significant treatment effects on cognition-like behaviour. Another alternative for the non-significant effect of etanercept on cognition is the high variation within groups in this test compared to others. Using increased numbers, or a more sensitive test of cognition could have helped us see a significant effect of etanercept treatment. Future studies should take this into account as well as timing of drug treatments.

Interestingly, our study failed to observe effects of LPS on astrocytic as opposed to microglia numbers, suggesting differential activation of the two glial cell

types, whereby microglia may be more sensitive to peripheral administration of LPS than astrocytes. This may relate to the dose of LPS, as chronic administration (daily for 7 days) generated an astrocyte response in another study, whereas in our study only a single LPS administration was employed (Bian et al., 2013). Another possibility is that there is a temporal difference between the reactivity of the two cell types, with microglia responding more quickly to this insult than astrocytes (Kuhlmann and Guilarte, 2000, Ni et al., 2010). Kuhlmann *et al* (2000) also reported that while microglia responded within 48 hr to an insult generated by administration of neurotoxicant trimethyltin, astrocytic response was delayed, seen at 14 days, but not 48hr following injury. This may explain the lack of findings of peripheral LPS administration on astrocytic number in the current study and future work would need to look at delayed effects of LPS on astrocytes.

The behavioural deficits seen in this study were associated with increased microglia numbers within the dentate gyrus in LPS treated mice, 48 hr post-administration, but not in the PFC. It is known that peripherally stimulated monocytes affect the CNS via the BBB and the blood-CSF barrier and express cytokines like TNF- $\alpha$  (Ubogu et al., 2006). Indeed, this peripheral-central immune cross-talk is further supported by a study that showed how a single high dose of LPS (5 mg/kg) given IP was effective in increasing cytokine expression, particularly TNF- $\alpha$ , in the hippocampus and prefrontal cortex even 7 days after administration (Bossu et al., 2012). Thus, activation of peripheral inflammation can promote central activation and proliferation of microglia and subsequent production of cytokines within the brain itself (Qin et al., 2007, Bossu et al., 2012), providing a potential mechanism to explain the increase in microglia numbers noted within this study only in the

hippocampus but not PFC. The differences in microglia findings in the hippocampus and the PFC between the present study and previous results by Bossu *et al* (2012) may be due to the relatively low dose of LPS used in this study. It may be plausible that a higher dose of LPS is required to illicit a change in microglial numbers in the PFC, but the hippocampus is more susceptible to immune activation, however these speculations will need to be clarified by further studies.

Chronic and acute stressors are known to prime microglia, leading to up-regulation of pro-inflammatory cytokines like TNF- $\alpha$ . Microglia are also believed to be activated by psychological and peripheral immune factors, which can result in behavioural changes (Li *et al.*, 2014). Zhu *et al* (2014b) proposed that, as activated microglia that are known to produce pro-inflammatory cytokines, this may cause neuronal toxicity through free radicals, cytokines and decreased neurogenesis leading to behavioural impairments, particularly social impairment and prepulse inhibition. Additionally chronic stress has been shown to increase microglial activation leading to increased uptake of peripheral monocytes perpetuating cognitive and emotional impairment possibly by impairing synaptic connections (Wohleb *et al.*, 2011, Hinwood *et al.*, 2012, Wohleb *et al.*, 2013, Li *et al.*, 2014). Furthermore High levels of TNF- $\alpha$  promote microglia activation inducing a positive feedback loop causing more activation and proliferation of microglia and TNF- $\alpha$  (Takeuchi *et al.*, 2006). Thereby our study supports that blocking TNF- $\alpha$  action in the brain would be expected to reduce microglia proliferation by stopping this feedback loop. Indeed studies on traumatic brain injury (TBI) in rats showed that etanercept administration is effective in reducing microglia numbers within the CNS following injury (Chio *et al.*, 2013).

Overall, this study shows that following LPS induced behavioural changes and increased microglia in the dentate gyrus, a single dose of etanercept can significantly reduce anxiety-like behaviour and microglia numbers 48hr post peripheral immune challenge. Future work should explore whether a single dose of etanercept administered at the peak of LPS activity (4-8 hr) will be more effective in reducing inflammation as well as in improving cognition and mood-like behaviours compared to a later point of administration. Furthermore it would be worth investigating whether prolonged etanercept administration would be more effective than simply increasing the dosage concentration.

## **Chapter 6 : Behavioural Characterization of a TNF- $\alpha$ Over-Expressing Mouse Strain**

## Overview

In addition to the studies detailed in chapters 3.4 and 5, I performed a supplementary study on a TNF- $\alpha$  over-expressing strain. The study was conducted to validate a currently available model of CNS specific TNF- $\alpha$  over-expression, a GFAP-TNF<sup>+/+</sup> mouse model (Stalder et al., 1998). As TNF- $\alpha$  is a vital biomarker and contributor to a range of inflammatory conditions leading to CNS pathology, there is a need to understand the effects of not only the lack of TNF- $\alpha$  signalling but also the behavioural and neurobiological implications of TNF- $\alpha$  over-expression in the CNS.

**AIM 4:** To determine the effects of over-expression of TNF- $\alpha$  in a mouse model of CNS specific TNF- $\alpha$  up-regulation, on the behaviour and neurobiology of mice.

Transgenic mice over-expressing TNF- $\alpha$  under the control of glial fibrillary acidic protein (GFAP) promoter (astrocyte specific) were compared to age and sex matched control wild-type C57BL/6 mice (WT). This study was conducted in 2 parts: In the first part of the study, behavioural analysis was conducted on 12 week old mice (n = 14 for GFAP-TNF<sup>+/+</sup> mice, n = 21 for WT mice) whereas in the second part of this study a second group of mice were aged to 28 weeks of age and tested in a similar battery as the first part of the study (n = 14/group). Behaviour of mice was tested in battery measuring locomotor activity, cognition-like behaviour, anxiety-like behaviour, exploratory behaviour and depression-like behaviour. Additionally mRNA levels of TNF- $\alpha$  were measured using qPCR and hippocampal levels of NGF and BDNF by ELISA.

The present study failed to note any significant differences between mice, at 3 months and 7 months of age compared to WT counterparts. There was no difference



in TNF- $\alpha$  expression between GFAP-TNF<sup>+/+</sup> and WT mice, both at 3 months of age and also at 7 months of age. These strains of mice also did not differ in locomotor activity, cognition-like behaviour, exploratory behaviour or depression-like behaviour at both age groups. While anxiety-like behaviour between strains was not different at 3 months of age, older GFAP-TNF<sup>+/+</sup> mice spent significantly less time than WT mice in the open arm of the maze, indicating higher anxiety-like behaviour. No differences between strains in neurotrophin expression were noted for both age groups.

Overall the present study failed to establish the GFAP-TNF<sup>+/+</sup> model as an effective model of neurodegeneration by TNF- $\alpha$  up-regulation. The lack of significant findings that differ from the original analysis of this strain may be due to epigenetic changes that may have occurred within the strain during breeding but a thorough biological analysis is required to verify this. Immune stimulation by LPS may be an avenue for further research to validate the GFAP-TNF<sup>+/+</sup> model.

## Introduction

TNF- $\alpha$  is a major biomarker in a range of pathologies that manifest in behavioural defects (Baune et al., 2012b). It is up-regulated in major depression and Alzheimer's disease to name a few (Tobinick and Gross, 2008, Dowlati et al., 2010). Apart from this, treatment of inflammatory conditions like rheumatoid arthritis and Psoriasis with anti-TNF- $\alpha$  drug, etanercept has shown promising results in improving not only disease symptoms but also decreasing pain, depression and improving cognitive decline associated with these pathologies (Tyring et al., 2006, Uguz et al., 2009, Chen et al., 2010).

These factors make the necessity for finding a model of TNF- $\alpha$  over-expression vital not only to understand how behaviour is affected by TNF- $\alpha$  but also the consequences of TNF- $\alpha$  over-expression on neurobiology. This study was therefore conducted to characterize the behavioural changes in a currently available TNF- $\alpha$  over-expressing mouse model in young and mid-adult mice.

Stalder et al (1998) developed a TNF- $\alpha$  over-expression model that was specific to astrocytes. The model appeared to be a valuable tool in understanding TNF- $\alpha$  mediated CNS pathology. As the initial assessment of the mouse strain showed that the over-expressing mice developed severe motor deficits as well as CNS pathology resulting in lesions and up-regulation of TNF- $\alpha$  in the brain, it appeared to be the ideal model for behavioural characterisation of TNF- $\alpha$  specific pathology (Stalder et al., 1998, Carrasco et al., 2000). Therefore this study was carried out to characterize the behavioural changes in this model, both, before the

onset of disease (3 month old mice) and after the commencement of TNF- $\alpha$  over-expression (from 7 months of age).

## **Methods**

### ***Mice***

Transgenic mice over-expressing TNF- $\alpha$  under the control of Glial Fibrillary Acidic Protein (GFAP) promoter (Astrocyte Specific) were used and obtained from Iain Campbell's lab (GFAP-TNF<sup>+/+</sup>). The generation and subsequent early phenotypic characterisation of this strain has been extensively described by Stalder et al (1998). These test mice were then compared to age and sex matched control wild-type C57BL/6 mice (WT). In the first part of the study, behavioural analysis was conducted on 12 week old mice (n = 14 for GFAP-TNF<sup>+/+</sup> mice, n = 21 for WT mice). In the second part of this study a second group of mice were aged to 28 weeks of age and tested in a similar battery as the first part of the study (n = 14/group).

### ***Gene Expression Analysis (qPCR)***

A subset of mice was selected for qPCR analysis to measure the expression of *Tnfa*, (n = 4/ group). Mice were sacrificed after a lethal dose of pentobarbital via IP injection and hippocampal tissue from mice was collected and stored in RNAlater (Ambion, Life Technologies) at -20°C until required for RNA extraction. Brain tissues were lysed with TissueLyserLT (Qiagen, Australia) with 5 mm stainless steel lysis bead (Qiagen, Australia). RNA was extracted from the lysed tissue using Ambion

PureLink RNA mini extraction as per manufacturer's instructions. RNA was then quantified using Nanodrop Lite (Thermo Fischer Scientific).

Gene expression analysis for *Tnf* was performed by RT-qPCR in two steps. First, complementary DNA (cDNA) was synthesised from the total RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen) in a thermal cycler (MJ research) following manufacturer's instructions. The products were then diluted to attain a uniform concentration of 10 ng/ $\mu$ l of cDNA for qPCR.

In the next step qPCR was performed on the cDNA using the Applied Biosystems 7500 Fast real-time PCR machine (ABI, Life technologies). Expression of *Tnfa* was quantified with *Gapdh* used as the endogenous reference gene. Samples and negative controls for each gene (without cDNA) were run in triplicates on a 96 well MicroAmp Fast optical-qPCR plate (ABI, Life technologies). Briefly, 4 $\mu$ l of cDNA was added to a master mix consisting of 10  $\mu$ l of 2X Power SYBR Green PCR master mix (ABI, Life technologies), 0.14  $\mu$ l each of forward and reverse primer along with 5.72  $\mu$ l of double distilled water to make a total reaction volume of 20  $\mu$ l. The raw data consisting of the Ct (Cycle threshold) values for individual reactions were exported to excel files and relative quantification of expression was conducted manually using the delta-delta Ct method (Livak and Schmittgen, 2001). Statistical tests were conducted on the delta Ct values (expression normalised to endogenous reference) of each strain

### ***Behavioural Analysis***

A comprehensive behavioural battery was carried out incorporating several different behavioural types. Tests were conducted in the following order: (1) Home Cage Locomotor Activity, (2) Open Field Test (OFT), (3) Hole-Board Exploration (HBE), (3) Elevated Zero Maze (EZM), (4) Barnes Maze (BM) and (5) Forced Swim Test (FST) and took 4 weeks to complete. Mice were given at least one day off between tests and tests were conducted in order of least to most stressful, to minimise effects of previous testing (Lad et al., 2010). A single test was not repeated on the same mouse. Equipment for all tests was purchased from Stoelting Co. (USA) and all movements were tracked using ANY-MAZE imaging software Stoelting Co. (USA).

#### **Locomotor Activity: Home Cage Locomotor Activity**

Mice were individually tested for general locomotor activity in home cages with 2 day old bedding under basal non-stressful conditions according to previous published results (Baune et al., 2008). Total distance covered over a 5 min period was measured.

#### **Open Field Test (OFT)**

Under more stressful conditions basal locomotor activity was measured. Mice were placed at the centre of a brightly lit plexiglass box (40 X 40 cm) and movements were tracked over a 5 min period as previously published (Baune et al., 2008). Distance travelled and time spent in the outer and inner zones of the box was calculated.

### **Cognition-Like Behaviour: Spatial Memory and Learning: Barnes Maze (BM)**

The Barnes maze consists of a bright, circular white platform (91 cm) with 20 holes, which contain either false boxes, or one hidden escape box. The false boxes remove visual cues that might be observed through an open hole. BM procedures were carried out over a six day period according to published protocols with the time taken to find the escape box recorded (Baune et al., 2008).

**Pre-training (Day 1):** Mice were pre-trained to enter the escape box, first by placement of the mouse into the escape box for 2 min, then by guidance to the escape box before being left for 2 min in the escape box, and finally placement outside the escape box within a glass chamber for up to 3 min at the end of which the mice were again placed into the escape box for 2 min.

**Training (Days 2-5):** Mice were briefly placed in the centre of the maze under a removable chamber and given 3 min to locate the escape box. Mice that failed to enter the escape box within 3 min were guided to the box and placed there for 2 min prior to returning to their home cage. Each mouse was subjected to 4 trials per day, separated by 15 min, for 4 days. The average of the time taken to find the escape box across the 4 day period was then calculated. Mice that failed to enter the escape box during the trial period were given a test time of 3min. Lower escape latencies representing good spatial memory.

**Probe trial (Day 6):** Each mouse was given a probe trial of 3 min duration each, with the escape box rotated 90°. Latency to the old and new escape locations was recorded. Retention memory for the (known) old escape box was considered as the tendency of the mice to explore the old escape box in the probe trial instead of exploring the new (unknown) box location. Thus, short latencies to locate the old

escape location were considered to indicate spatial memory retention for the original location.

### **Exploratory Activity: Hole Board Exploration Test (HBE)**

The hole-board exploration test is set up in an apparatus similar to the open field test, and utilises a hole-board insert, which has 16 holes of 1.5 cm in diameter, spaced 6.0 cm apart. Mice are placed into the apparatus and allowed to explore for 5 min. Head dipping behaviour is manually recorded and is an indicator of exploration (Hart et al., 2010).

### **Anxiety-Like Behaviour: Elevated Zero Maze (EZM)**

The elevated zero maze is used as a measure of anxiety-like behaviour wherein anxiety is measured as the time spent in the open arm of the maze, as well as in head dipping and stretch/attend behaviour (Cryns et al., 2007). The apparatus consists of a grey Perspex circular platform of 50 cm diameter and width of 5 cm, located at a height of 40 cm above ground. The platform is divided into 4 equal quadrants, two closed quadrants with grey Perspex walls (27 cm high) on the inside and outside, and two open quadrants without walls. Mice were placed at the centre of either one of the open arms and allowed to explore the apparatus for 5 min. Movement and time spent in open and closed quadrants was recorded.

### **Depression-Like Behaviour: Forced Swim Test (FST)**

The forced swim test is a measure of despair and depression-like behaviour, and was performed according to previously published results (Petit-Demouliere et al., 2005). The apparatus consists of a 4 L cylinder (20 cm diameter) filled up to 20 cm with water (23-24°). Mice were placed in water and movement was tracked over a period of 6 min. Immobility was considered a measure of depression-like behaviour, and was defined as lack of motion beyond which was required for the mouse to keep its head above water. For analysis, the first 2 min were excluded, since mice are mobile during that time and familiarizing themselves with the environment and any immobility during this time therefore cannot be attributed to despair.

### ***Protein Analyses: ELISA***

In order to evaluate whether there were any underlying changes in neurotrophin levels within the TNF- $\alpha$  transgenic mice, a subset of mice (n = 7 mice), were sacrificed by Pentobarbital overdose and the brain rapidly removed. The hippocampus was isolated and stored fresh frozen at -80°C until analysed. Tissue was homogenised in a solution containing 50 mL TRIS, one protease inhibitor tablet (Roche) and 50  $\mu$ L of Triton-X. Homogenates were then centrifuged at 8500 rpm (Davidson, 2002) for 15 min at 4°C, and the supernatant transferred to 1.5 mL eppendorf tubes. All samples were stored at -80°C until analysis. Amount of protein was quantified using the Bradford method and samples then diluted to contain 50 ng of protein. NGF and BDNF ELISAs were then performed using the E<sub>max</sub> Immuno



Assay system (Promega) according to the manufacturer's instructions and as previously published (Sei et al., 2000, Gilmore et al., 2003).

### ***Statistical Analysis***

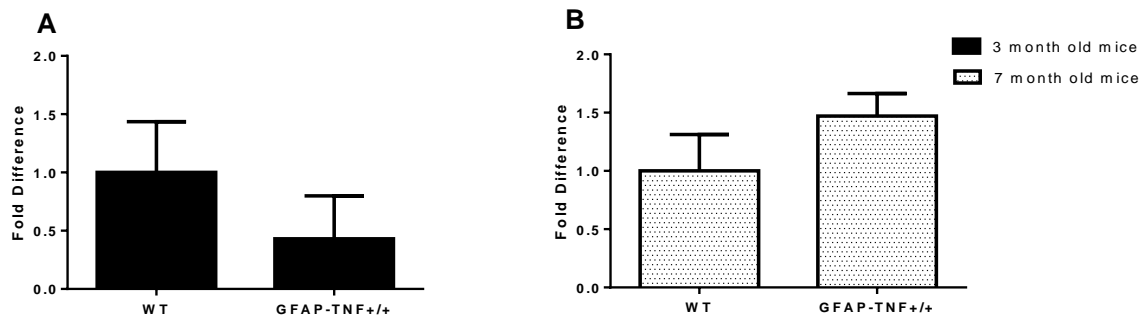
Data analyses were carried out using GraphPad Prism software (version 6.01). All data are presented as mean  $\pm$  SEM. Comparisons between groups were carried out using a t test for comparison of 2 groups.  $P < 0.05$  was the level of significance for all tests.

## Results

### **No Difference in *Tnfa* Expression in Mouse Brains**

Levels of (mRNA) *Tnfa* in the hippocampus of mice were measured post behavioural testing from both WT and GFAP-TNF<sup>+/+</sup> mice at 3 and 7 months of age (Figure 6.1). Fold difference of *Tnfa* was calculated against reference gene expression, *Gapdh*, but no significant difference was observed between strains for either age group. At 3 months of age, GFAP-TNF<sup>+/+</sup> mice had a 0.5 fold decrease in *Tnfa* expression compared to WT mice, while at 7 months of age there was a 0.5 fold increase in *Tnfa* expression in GFAP-TNF<sup>+/+</sup> mice compared to WT mice.

**Figure 6.1: RNA expression of TNF- $\alpha$  in the hippocampus**

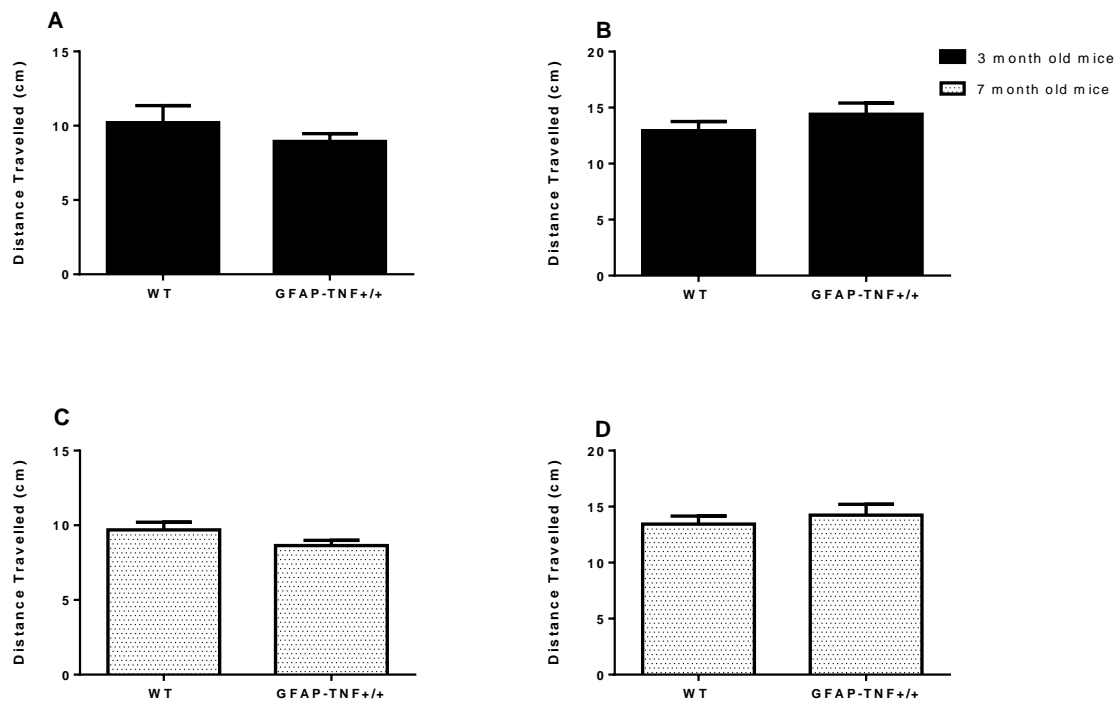


**Legend:** The figure shows the expression of TNF- $\alpha$  in the hippocampus of mice at (A) 3 month age and (B) 7 month age. N = 21 for WT mice and n =14 for GFAP-TNF<sup>+/+</sup> at 3 months of age; n = 4/strain/age group. All data represent mean  $\pm$  SEM. No significant differences were observed between groups.

### Normal Locomotor Activity in 3 and 7 month old mice

Locomotor activity was measured in both strains of mice at 3 months and at 7 months (Figure 6.2). In the home cage and open field test there were no differences between strains at 3 months (Figure 6.2A:  $P = 0.40$  HC, Figure 6.2B:  $P = 6.26$  OFT) or 7 months of age (Figure 6.2C:  $P = 0.09$  HC, Figure 6.2D:  $P = 0.51$ , OFT), as indicated by t-test. These results indicate that locomotor activity was not affected by either genetic manipulation or ageing and that variances observed in any of the subsequent tests were a result of behavioural and not locomotor differences.

Figure 6.2: Baseline locomotor activity



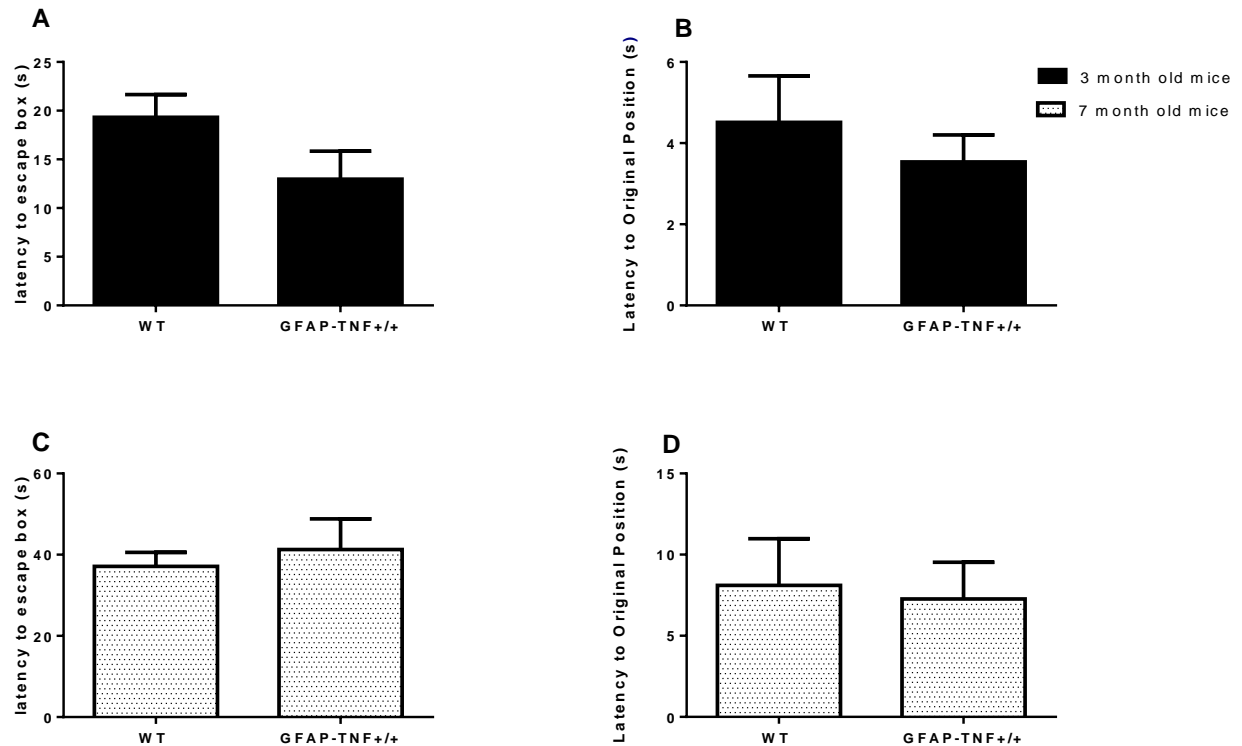
Legend: The figure shows the Home cage activity in (A) 3 month old mice and (C) 7 month old mice. Open field test in (B) 3 month old mice and (D) 7 month old mice.  $n = 21$  for WT mice and  $n = 14$  for GFAP-TNF<sup>+/+</sup> at 3 months of age;  $n = 14$  for both strains at 7 months of age. All data represent mean  $\pm$  SEM. Data compared using T test. No significant differences were observed between groups.

### ***No Difference in Learning and Memory on the Barnes maze***

Spatial learning and memory in GFAP-TNF<sup>+/+</sup> mice and WT mice was measured using the Barnes maze (Figure 6.3). At 3 months of age no difference between strains in latency to the original escape hole in training (Figure 6.3A:  $P = 0.09$ ) or probe trial phase (Figure 6.3B:  $P = 0.51$ ) was detected.

Interestingly, though higher latencies to the original position in the 7 month old GFAP-TNF<sup>+/+</sup> mice were expected, no difference in both, training (Figure 6.3C:  $P = 0.62$ ) and probe trial (Figure 6.3D:  $P = 0.81$ ) phase of the test were observed.

Figure 6.3: Learning and memory in the Barnes maze



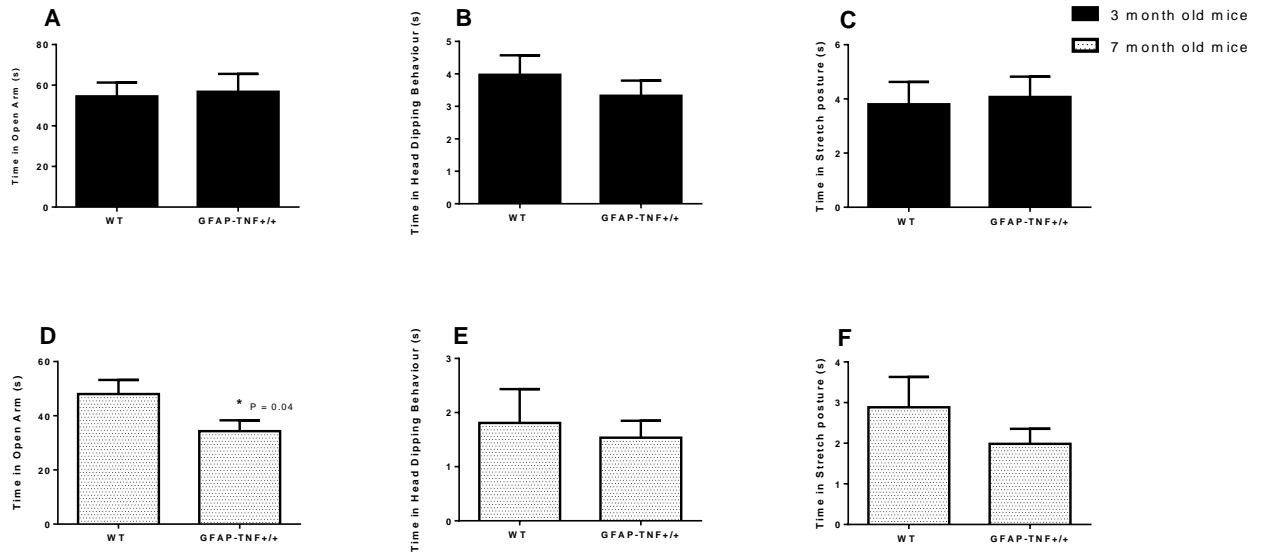
### ***Anxiety-Like Behaviour***

Anxiety-like behaviour in this study was measured using the elevated zero maze. To measure anxiety, time in the open arm as well as time spent in head dipping and stretch attend posture was recorded (Figure 6.4)

As expected there was no difference in any of the three parameters in the 3 month old mice (Figure 6.4A:  $P = 0.87$  time in open arm, Figure 6.4B:  $P = 0.12$  time in head dipping behaviour, Figure 6.4C:  $P = 0.82$  time in stretch/attend posture).

Unsurprisingly GFAP-TNF<sup>+/+</sup> mice displayed more anxiety-like behaviour than WT mice at 7 months of age by spending less time in the open arm than WT mice (Figure 6.4D:  $P = 0.04$ ). However this result did not extend to head dipping behaviour and time in stretch/attend behaviour with no difference in strains observed for these limits (Figure 6.4E:  $P = 0.15$  time in head dipping behaviour, Figure 6.4F:  $P = 0.12$  time in stretch/attend posture).

**Figure 6.4: Anxiety-like behaviour in the elevated zero maze**



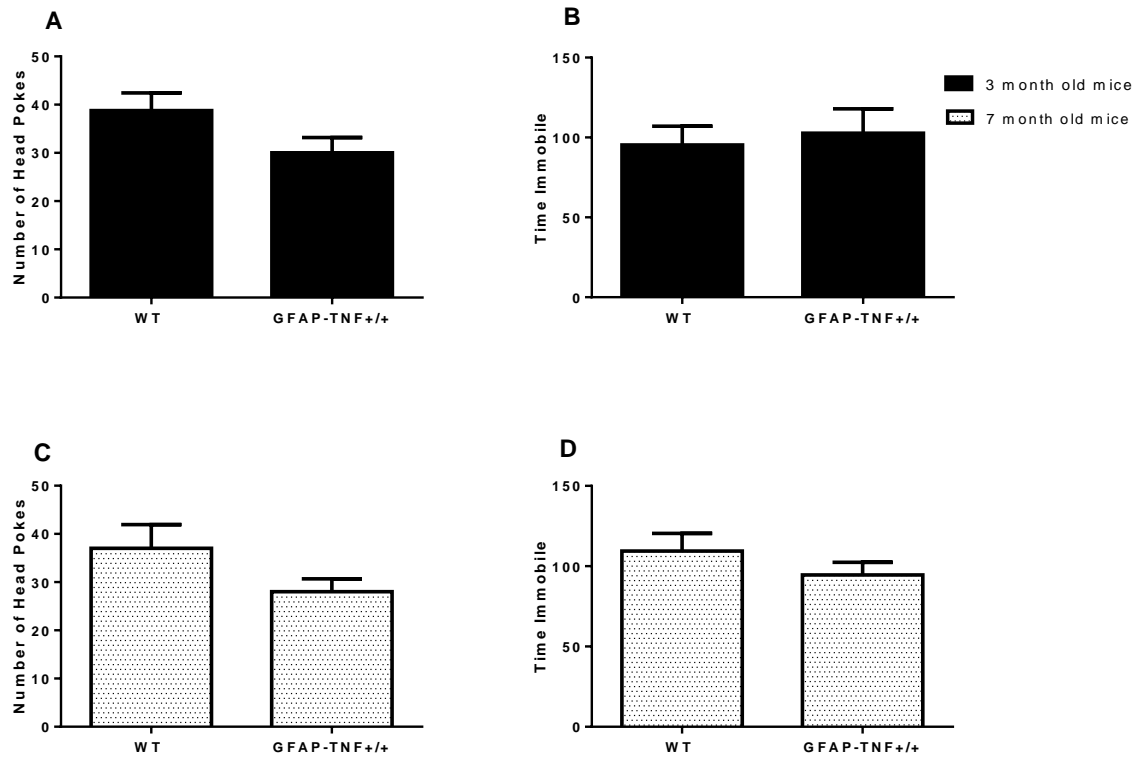
**Legend:** Time spent in open arm at 3 months (A) and 7 months (D). Time in head dipping behaviour at 3 months (B) and 7 months of age (E). Time in stretch/attend behaviour at 3 months (C) and 7 months of age (F).  $n = 21$  for WT mice and  $n = 14$  for GFAP-TNF<sup>+/+</sup> at 3 months of age;  $n = 14$  for both strains at 7 months of age. All data represent mean  $\pm$  SEM. Data compared using T test. \*  $P < 0.05$  compared to WT mice.

### ***Exploratory and Depression-like Behaviour***

To measure exploration in this study, the hole board exploration test was used but no difference between strains for number of head pokes at both 3 months (Figure 6.5A:  $P = 0.10$ ) and 7 months (Figure 6.5C:  $P = 0.09$ ) of age were seen.

These findings extended to depression-like behaviour that was measured on the forced swim test. No change in immobility time was detected between strains at either 3 months (Figure 6.5B:  $P = 0.70$ ) or 7 months (Figure 6.5D:  $P = 0.28$ ) of age. These results are unexpected and against the hypothesis of higher depression-like behaviour in GFAP-TNF<sup>+/+</sup> mice.

**Figure 6.5: Exploratory and depression-like behaviour**



**Legend:** The figure shows the number of head pokes in (A) 3 month old mice and (C) 7 month old mice, the time immobile in the forced swim test at (B) 3 months of age (D) and 7 months of age.  $n = 21$  for WT mice and  $n = 14$  for GFAP-TNF<sup>+/+</sup> at 3 months of age;  $n = 14$  for both strains at 7 months of age. All data represent mean  $\pm$  SEM. Data compared using T test. No significant differences were observed between groups.

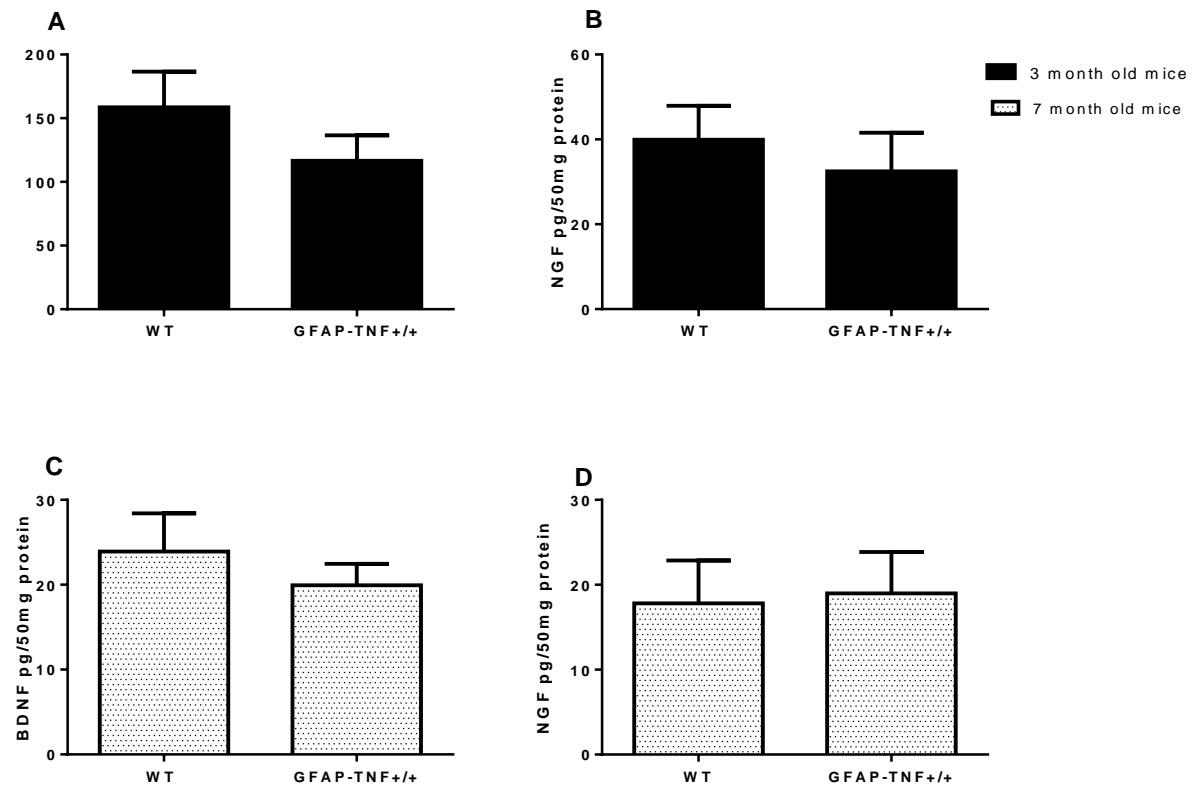


### ***NGF and BDNF Levels in the Hippocampus***

To test whether changes in neurotrophin levels occurred between GFAP-TNF<sup>+/+</sup> and WT mice, levels of NGF and BDNF in the hippocampus of these mice at both 3 and 7 months of age (Figure 6.6) were measured.

At the 3 months of age there was no difference in strains for BDNF (Figure 6.6A:  $P = 0.25$ ) and NGF (Figure 6.6B:  $P = 0.55$ ) expression. This result was replicated in the older 7 month cohort of mice and no difference was seen between strains for both, BDNF (Figure 6.6C:  $P = 0.45$ ) and NGF (Figure 6.6D:  $P = 0.39$ ) expression.

**Figure 6.6: Levels of BDNF and NGF in the hippocampus**



**Legend:** Expression of BDNF in (A) 3 month old and (C) 7 month old mice, Expression of NGF in (B) 3 month old mice and (D) 7 month old mice. n = 21 for WT mice and n =14 for GFAP-TNF<sup>+/+</sup> at 3 months of age; n = 14 for both strains at 7 months of age. All data represent mean  $\pm$  SEM. Data compared using T test. No significant differences were observed between groups.

## Discussion

This study failed to note any differences between mice, at 3 months and 7 months of age compared to WT counterparts. There was no difference in TNF- $\alpha$  expression in the brains of GFAP-TNF<sup>+/+</sup> mice compared to WT mice, both at 3 months of age and also at 7 months of age. No differences between strains in locomotor activity were noticed at both age groups, in either the home cage or the open field test. In the Barnes maze, there was no difference between GFAP-TNF<sup>+/+</sup> and WT mice at 3 months of age. At 7 months old, while no differences between strains was observed in the Barnes maze, GFAP-TNF<sup>+/+</sup> mice surprisingly showed a trend of lower latency to the escape hole in the probe trials, indicating a tendency towards better memory. In the EZM, anxiety-like behaviour between strains was not different, however in older mice, the GFAP-TNF<sup>+/+</sup> mice spent significantly less time that WT mice in the open arm of the maze, indicating higher anxiety-like behaviour. Unfortunately these results did not extend to the time spent in head dipping and stretch behaviour in the EZM. In case of exploratory and depression-like behaviour, there were no differences between either strains at 3 and 7 months of age, for both tests. No differences between strains in neurotrophin expression were noted for both age groups.

The lack of significant findings in the present study is disappointing. While it was previously reported that the GFAP-TNF<sup>+/+</sup> strain was liable to have motor impairment from 6 months of age (Stalder et al., 1998), the present study failed to notice any difference in locomotor activity. This lack of significant findings may be attributed to the absence of TNF- $\alpha$  over-expression seen in the present study. The initial analyses of this strain by Stadler et al (1998), showed a significant over-

expression of TNF- $\alpha$  in the brains of these mice compared to WT mice, which the present study failed to detect. At 7 months of age, there was only a 1.5 fold increase in TNF- $\alpha$  expression in the brains of the current mice as opposed to the high fold increase previously reported (Stalder et al., 1998).

Anxiety in 7 month old mice however was higher in GFAP-TNF<sup>+/+</sup> mice. This was an expected result, especially since TNF- $\alpha$  is associated with the development of anxiety behaviour in a range of disorders like RA and psoriasis that have an up-regulation of TNF- $\alpha$  (Uguz et al., 2009, Chio et al., 2013). Surprisingly these results did not extend to depression-like behaviour or the expression of neurotrophins.

The absence of changes in this study may be due to epigenetic changes that occurred with breeding the mice. The lack of penetrance of the genes or epigenetic changes, may have occurred by either breeding or housing conditions of mice between the initial strain developed and the one tested in the present study (Miko, 2008). Alternatively an external stimulant like LPS or physical trauma may be required to activate the over-expression of TNF- $\alpha$  in the GFAP-TNF<sup>+/+</sup> mice, as observed. This may cause more profound and symptomatic changes than the ones currently observed. Another possible explanation for the want of significant changes may be the age of mice tested. However it is unlikely that ageing mice to more than 7 months will provide similar results to the initial findings as these mice appear to be healthy, similar to WT mice (not shown).

There still is a need for understanding the effects of over-expression of TNF- $\alpha$  in mice, so as to develop treatments to treat inflammatory disorders that have an up-regulation of TNF- $\alpha$ . Pharmacological treatment of WT mice with either TNF- $\alpha$

antibodies or inflammatory triggers like LPS may help provide more insight into the workings of TNF- $\alpha$  over-expression on behaviour and neurobiology.

## **Chapter 7 : Conclusions and Future Directions**

## Summary of Principal Findings

This body of work was conducted to study the role of TNF- $\alpha$  on behavioural phenotypes in mouse models. Aside from the known roles of TNF- $\alpha$  in immunity, TNF- $\alpha$  is an interesting player in the CNS. Various studies have shown its involvement in a range of CNS activities, from synaptic scaling to neurotrophin expression (Aloe et al., 1999, Beattie et al., 2002). As it is produced by the resident immune cells of the CNS, microglia and astrocytes, as well as by certain neuronal populations (Lieberman et al., 1989, McCoy and Tansey, 2008), it is an active participant in many physiological and homeostatic functions occurring in the CNS (Baune et al., 2008, Baune et al., 2012b). However when the delicate balance of homeostasis is disrupted, be it by trauma, peripheral or central inflammation or even by psychological stress, an up-regulation of TNF- $\alpha$  can result, which disrupts the normal physiological function of TNF- $\alpha$  and can result in harmful consequences for the CNS (Baune et al., 2012b). This is evident from studies on a large number of disorders such as AD, RA and MDD, to name a few, which have presented with not only central TNF- $\alpha$  dysregulation but also an imbalance in peripheral cytokine levels (Alvarez et al., 1996, Korner and Sedgwick, 1996, Dowlati et al., 2010). These changes at the protein and molecular level are met with changes in behaviour – increased anxiety, depression and cognitive impairment (Chen et al., 2010).

As TNF- $\alpha$  is such an active biomarker in neuroinflammation, an important aim of this body of work was to determine the consequence of effective and reliable blockade of TNF- $\alpha$  and/or its receptor action. This led to the use of TNF- $\alpha$  and TNF- $\alpha$  receptor knockout mice (chapters 3 and 4) and TNF- $\alpha$  antagonists (chapter 5).

### ***Behavioural and Neurobiological Implications of Genetic Deletion of TNF- $\alpha$ and its Receptors on Young and Mid-Adult Mice***

To understand whether targeting TNF- $\alpha$  signalling as well as individual receptor signalling would be beneficial or harmful not only to behaviour, but also to neural development, the first study was conducted in young mice (3 month old mice) (Camara et al., 2013). The main findings from this study were

- TNF<sup>-/-</sup> mice had impaired learning and memory but TNF-R2 deficient mice, though slow learners, had effective memory consolidation.
- Decreased anxiety in TNF<sup>-/-</sup> as well as TNF-R2<sup>-/-</sup> mice was observed.
- There appeared to be no apparent effect of lack of TNF- $\alpha$  and its receptor signalling on exploratory or depression-like behaviour in any of the knockout strains.
- All mice appeared to have normal social behaviour.
- Low levels of NGF were seen in the TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice.

These initial findings were very interesting and showed that uninterrupted signalling of TNF- $\alpha$  and its receptors are required for normal cognitive function. This study also showed that a lack of TNF- $\alpha$  in young adult mice might be beneficial in preventing anxiety-like behaviour. Furthermore as these behavioural changes were linked to low levels of NGF, it indicates that this cytokine might be required for normal expression of NGF and that this may be an explanation for the poor cognition-like behaviour observed in these mice.

Based on these initial findings, the next step was to test whether these phenotypes would change over the course of the animal's life. To test this, mice of



the same strains, that is,  $TNF^{-/-}$ ,  $TNF-R1^{-/-}$  and  $TNF-R2^{-/-}$  mice and C57BL/6 WT mice, were aged to 6 months. These mice then underwent a similar behavioural and biological testing protocol. In this study on mid-adult mice it was found that:

- $TNF^{-/-}$  and  $TNF-\alpha$  receptor knockout mice displayed similar learning compared to WT mice,
- $TNF-R1^{-/-}$  mice displayed better memory than WT as well as other strains of mice.
- Impaired social behaviour was observed in  $TNF^{-/-}$  mice.
- Exploratory behaviour in  $TNF^{-/-}$  and  $TNF-R1^{-/-}$  mice was decreased compared to WT mice.
- $TNF^{-/-}$  and  $TNF-R2^{-/-}$  mice exhibited lower depression-like behaviour than WT mice.
- $TNF^{-/-}$  mice expressed significantly lower levels of BDNF than WT mice in the hippocampus
- $TNF-R1^{-/-}$  mice displayed significantly lower BDNF levels compared to both WT and  $TNF-R2^{-/-}$  mice.
- $TNF-R2^{-/-}$  mice displayed significantly higher levels of NGF compared to  $TNF-R1^{-/-}$ , WT and  $TNF^{-/-}$  mice.

These results were an exciting contrast to those observed at 3 months of age. Table 6.1 summarizes the results from chapters 3 and 4 and shows at a glance, the difference between the 2 age groups of mice.

Table 7.1: Summary and comparison of results from chapters 3 and 4

3 MONTH AGE GROUP				Comparison Against WT Mice	6 MONTH AGE GROUP		
STRAINS	TNF <sup>-/-</sup>	TNF-R1 <sup>-/-</sup>	TNF-R2 <sup>-/-</sup>		TNF <sup>-/-</sup>	TNF-R1 <sup>-/-</sup>	TNF-R2 <sup>-/-</sup>
Learning (BM)	-	Impaired Learning	Impaired Learning		-	-	-
Memory (BM - probe trial)	Impaired Memory	-	-		-	Better Memory	-
Exploration (HBE)	-	-	-		Decreased Exploration	Decreased Exploration	Trend for Decreased Exploration
Anxiety-like behaviour (EZM)	Decreased Anxiety	-	-		-	-	-
Social behaviour	-	-	-		Decreased Social Behaviour	-	-
Depression-like behaviour (FST)	-	-	-		Decreased Depression-like behaviour	Trend for Decreased Depression-like behaviour	Decreased Depression - behaviour

<b>BDNF ELISA</b>	-	<b>Trend for Lower Expression</b>	<b>Trend for Lower Expression</b>		<b>Lower Expression</b>	<b>Lower Expression</b>	-
<b>NGF ELISA</b>	<b>Lower Expression</b>	-	<b>Lower Expression</b>		-	<b>Trend for Lower Expression</b>	<b>Higher Expression</b>
<b>DCX positive cells</b>	-	-	-		-	-	-
<b>Ki67 Positive Cells</b>	-	-	-		-	-	-

**Legend:** The table summarises the findings in chapters 3 and 4. BM: Barnes Maze, HBE: Hole Board Exploration, EZM: Elevated Zero Maze, FST: Forced Swim Test, BDNF: Brain Derived Neurotrophic Factor, NGF: Nerve Growth Factor. ' – ' Indicates no difference to WT mice.

In the older cohort of mice it was interesting to note that cognition-like behaviour, which was impaired at 3 months of age, had normalised by 6 months, and that TNF-R1<sup>-/-</sup> mice even exhibited improved memory on the Barnes maze compared to WT mice. This indicates that while normal signalling of TNF- $\alpha$  and its receptors is required in early stages of growth, this is not the case as the onset of mid-adulthood occurs. The role of TNF- $\alpha$  is in developing a normal cognitive phenotype not maintaining it. Indeed these findings are in keeping with previous work within this group where similar results were seen in TNF<sup>-/-</sup> mice, with poor cognition at 3 months of age and improved cognition at 6 and 12 months of age (McAfoose et al., 2009).

In the case of exploratory behaviour, while at 3 months of age there was no difference between knockout strains and WT mice, at 6 months of age, TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice both had poor exploration. This indicates that normal signalling of TNF- $\alpha$  and its receptors is required to maintain a normal exploratory phenotype.

Where anxiety-like behaviour was concerned, at 3 months of age only TNF<sup>-/-</sup> mice displayed low anxiety-like behaviour and by 6 months of age there were no differences in strains compared to WT mice. This is unexpected given that depression-like behaviour was decreased in the 6 month age group (TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice) but not in the 3 month old mice.

Biological changes in neurotrophin expression were also seen between the two age groups. BDNF levels that at 3 months of age only showed trends of lower expression (in TNF-R1<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice), were significantly lower than WT mice at 6 months of age (in TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice). While NGF expression was found to be lowered in TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> mice at 3 months of age and increased at 6 months of age in the TNF-R2<sup>-/-</sup> mice.

The findings from these two studies demonstrate that although a lack of TNF- $\alpha$  signalling can improve cognitive function and decrease depression-like behaviour in mid-adult mice (i.e. is beneficial), normal functioning of TNF- $\alpha$  and its receptors is still required to maintain other behavioural phenotypes like exploration and social behaviour.

The outcomes of these studies led us to explore the role of TNF- $\alpha$  in the CNS further. Now the focus of these investigations turned to exploring the effects of pharmacological blockade of TNF- $\alpha$  in young adult mice, after a peripheral immune challenge. The aim of this was to see if the positive effects of TNF- $\alpha$  alteration could be temporarily applied to the CNS, whilst avoiding the negative impact of long-term alterations to TNF- $\alpha$  activity.

***Behavioural Implications of Pharmacological Blockade of TNF- $\alpha$  in the CNS***

The study detailed in chapter 5 was carried out to determine the effects of peripheral immune challenge on anxiety-like and cognition-like behaviour by treating mice with an acute dose of LPS. As there is extensive cross-talk between monocytes and peripheral cytokines and CNS immune cells (Kerfoot et al., 2006), the aim of this study was to test whether a peripheral immune challenge would be sufficient to activate CNS immune cells, and if so what the effect of blocking TNF- $\alpha$  activity within the CNS would be. Would central blockage of TNF- $\alpha$  by etanercept be able to reverse the behavioural and neurobiological effects of an immune challenge? The main findings from this study are

- Peripheral administration of LPS reduced locomotor activity, impaired cognition-like behaviour and increased anxiety-like behaviour.
- This was associated with an increase in microglia numbers in the dentate gyrus.
- Centrally administered etanercept significantly reduced LPS induced anxiety-like behaviour and LPS induced microglia numbers.
- LPS however had no significant effect on cognition-like behaviour and astrocyte numbers.

This study showed LPS to be effective in inducing behavioural changes in the mice studied and also to increase the numbers of microglia present in the dentate gyrus. Furthermore the study showed that a single dose of etanercept was able to significantly reduce anxiety-like behaviour and microglia numbers 48 hr post peripheral immune challenge.

## Conclusions and Discussion

The results of the studies presented are intriguing and demonstrate a complex role for TNF- $\alpha$  in governing behaviour, due to its ability to exert biological changes within the CNS.

### ***Benefits TNF- $\alpha$ Deficiency on Cognition-Like Behaviour***

This study has shown that TNF- $\alpha$  is an important candidate in the development of behavioural phenotypes. The observation of poor cognition-like behaviour in young 3 month old mice indicates an important role for this cytokine in establishing normal cognitive functioning. These findings provide support to studies showing TNF- $\alpha$  to be an essential component in mediating AMPAR trafficking to neuronal surfaces and decreasing GABA receptors (Beattie et al., 2002, Stellwagen et al., 2005). Interestingly, however, when knockout mice were aged to 6 months (mid-adulthood) no difference in learning between these strains compared to WT mice were observed. In fact TNF-R1<sup>-/-</sup> mice had improved memory compared to WT mice, suggesting that an absence of this receptor may actually be beneficial to cognition.

This result indirectly supports and builds on an earlier study that demonstrated high levels of TNF- $\alpha$  inhibited long term potentiation (LTP) in the hippocampus (Butler et al., 2004). Given this previous finding an absence of TNF- $\alpha$ , or its receptors, in older mice would be expected to show improved cognitive functioning when compared to WT animals. This would be because TNF- $\alpha$  activities within the

CNS would be reduced and this would prevent any harmful effects of TNF- $\alpha$  expression on LTP and improve cognitive function. The results of this study certainly supported this finding in part: cognitive functioning in the TNF- $\alpha$  deficient mice improved from 3 to 6 months of age. However, a further study looking at these effects on adult mice aged beyond 6 months would be needed to fully support this.

As TNF- $\alpha$  has been shown to be required for normal development of cognitive function (Camara et al., 2013) yet potentially harmful to cognitive activity if levels are increased in the CNS (Baune et al., 2012b). The next stage was to investigate the effects of temporary blockade of the activities of TNF- $\alpha$  in the CNS. Unfortunately, this study yielded mixed results. While a LPS protocol was effective at inducing impaired cognition, etanercept was ineffective in completely reversing this effect. It should be pointed out however that a large number of studies in both animal and human work have shown antagonism/blockade of TNF- $\alpha$  to be effective in alleviating cognitive decline (Tobinick and Davoodifar, 2004, Tobinick et al., 2006, Tobinick and Gross, 2008, Chen et al., 2010) and the lack of significant findings in this body of work may be due to limitations of the present study.

A disadvantage of the methodology used in chapter 5 is the time delay between administering LPS and observation of the effects of etanercept on cognition-like behaviour (something that is an intrinsic design flaw of this type of study), and this will need to be investigated further and in more detail in future studies.

Despite this methodological limitation, the results of these studies on genetically deficient TNF- $\alpha$  mice, show that the cytokine is an integral part of early



cognitive development but that its role can change to a less beneficial, almost deleterious one depending upon the developmental stage and inflammatory condition of the individual. While these studies were set in the context of ‘normal ageing’ (chapters 3 and 4), the question still remains as to whether these findings can be translated to inflammatory conditions where TNF- $\alpha$  activity has been increased/amplified (chapter 5).

### ***Impact of TNF- $\alpha$ Deficiency on Exploratory and Social Behaviour***

The effects of lack of TNF- $\alpha$  on social and exploratory behaviour were less profound than that on cognition-like behaviour. At 3 months there were no observable differences between knockout mice and WT mice in either of these behavioural phenotypes, suggesting a lack of involvement of TNF- $\alpha$  in the development of these behaviour (Camara et al., 2013). Surprisingly this appeared to not be the case as in older, mid-adult mice where had decreased social behaviour in TNF<sup>-/-</sup> mice, which was demonstrated by the poor exploration behaviour recorded in both TNF<sup>-/-</sup> and TNF-R1<sup>-/-</sup> mice. The results showed improved cognition-like behaviour in 6 month old knockout mice alongside poorer social and exploratory behaviour. This finding adds another complex dimension to the role of TNF- $\alpha$  in governing a range of behaviour.

These findings suggest that a continued blockade of the action of TNF- $\alpha$  may not necessarily benefit individuals in all facets of neurobiology and that selective modulation of the activities of TNF- $\alpha$  may be the best course of action. There appears to be a very complex role for TNF- $\alpha$  in defining various behavioural

phenotypes at different stages of development and this could be linked to either the receptor TNF- $\alpha$  is signalling through or the role that TNF- $\alpha$  plays in modulating synaptic transmission (Butler et al., 2004, Stellwagen et al., 2005, Camara et al., 2013).

### ***Benefits of TNF- $\alpha$ Deficiency on Emotion-Like Behaviour***

As with social and exploratory behaviour, there appeared to be a minimal effect of deletion of TNF- $\alpha$  on emotional behaviour in young mice. While decreased anxiety-like behaviour in young TNF<sup>-/-</sup> mice was observed, there was no effect on depression-like behaviour in these same young mice (Camara et al., 2013). These results were intriguing as they support the immune-endocrine hypothesis that showed TNF- $\alpha$  involvement in anxiety and depression-like behaviour (Bernardini et al., 1990). This hypothesis argues that TNF- $\alpha$  can cause hypothalamic pituitary adrenal (HPA) axis activation, promoting depression and anxiety through corticosterone release, whereas blockade of TNF- $\alpha$  has the opposite effect (Bernardini et al., 1990, Turnbull et al., 1997). TNF- $\alpha$  involvement has also been extensively studied in major depressive disorders and is a key player in the cytokine hypothesis of depression (Dean et al., 2010, Dowlati et al., 2010). These past studies were supported by the results from the 6 month old mice, in which significantly lower depression-like behaviour was observed in TNF<sup>-/-</sup> and TNF-R2<sup>-/-</sup> strains. Suggesting that this cytokine does indeed promote depressive behaviour and that targeting TNF- $\alpha$  action (modulation rather than complete blockade) may prove beneficial in the treatment of depressive and mood disorders.

In addition, this study supports the growing body of evidence of TNF- $\alpha$  involvement in mood behaviour, by demonstrating that an inflammatory response within the CNS resulting in anxiety-like behaviour could be alleviated by the TNF- $\alpha$  antagonist etanercept, which had the effect of reducing anxiety-like behaviour but not cognition-like behaviour in LPS-etanercept treated mice. These results tie in well with work showing decreased anxiety and depressive symptoms in psoriasis and rheumatoid arthritis patients treated with etanercept (Uguz et al., 2009, Chen et al., 2013). This study has also provided supporting evidence to the literature demonstrating a role for TNF- $\alpha$  as an active biomarker, if not a cause, for depressive and anxious symptoms.

### ***Neurotrophins, Neurogenesis, Glial Cells and TNF- $\alpha$***

In this study there were clear consequences as a result of genetic deletion or pharmacological blockade of TNF- $\alpha$  on the observed behaviour of the mice. However, the results of these studies did not provide information supporting a role for TNF- $\alpha$  in neurogenesis. There appeared to be no effect on the number of proliferating cells and young neuronal cells at either 3 or 6 months of age following alteration to TNF- $\alpha$  activity. Likewise, microglial cells were unaffected by deletion of TNF- $\alpha$  and its receptors in mid-adult mice.

In an inflammatory setting (peripheral inflammation by LPS), there was a significant increase in microglial numbers compared to mice that didn't receive LPS. This is expected since there is extensive cross-talk between peripheral and central immune systems leading to up-regulation of cytokines in both periphery and CNS,

even if there has been no specific trauma or injury to the CNS. Furthermore, TNF- $\alpha$  is able to exert an effect on microglia allowing for a positive feedback loop to become established where microglia produce TNF- $\alpha$  which further activates microglia and promotes TNF- $\alpha$  production. The blockade of this activity through the inhibition/antagonism of TNF- $\alpha$  action would be expected to decrease microglial numbers within the CNS, as observed and detailed in chapter 5.

Despite the absence of evidence of a role of TNF- $\alpha$  specifically in neurogenesis, a complex relationship between TNF- $\alpha$  and the neurotrophins, NGF and BDNF emerges from the results of this study (Camara et al., 2013). The findings contained within this thesis suggest a role for TNF- $\alpha$  and its receptors that adapts and alters neurotrophins in different ways over the course of an animal's life (Baune et al., 2012b).

Both NGF and BDNF are required for development and are regulators of neuronal survival and plasticity (Lewin and Barde, 1996, Huang and Reichardt, 2001, Baune et al., 2012b) and NGF is required specifically for hippocampal development (Golan et al., 2004). TNF- $\alpha$  and NGF are both produced by and cause activation of microglia under inflammatory conditions to produce more TNF- $\alpha$  and NGF resulting in a feedback loop (Takei and Laskey, 2008, Takei and Laskey, 2011). Similarly TNF- $\alpha$  deprivation in the hippocampus has been linked to low levels of BDNF suggesting a close association between the two. Additionally both NGF and BDNF are postulated to be biomarkers in the development of depressive and cognitive impairments (Peng et al., 2005, Castren et al., 2007).

While some of the behavioural changes seen in the TNF- $\alpha$  and TNF- $\alpha$  receptor knockout mice can be attributed to an influence over the activity of neurotrophins such as NGF and BDNF, the exact mechanisms for by which this occurs are yet unclear. More work will need to be done to specifically look at the effect TNF- $\alpha$  exerts over these neurotrophins and exactly how this links to observed behaviour.

## Considerations and Future Work

In this study, the main aim was to test the behavioural consequences of genetic deletion of TNF- $\alpha$  and its receptors on mice at different stages of development. This was done by testing mice in early adulthood (3 month old mice) and at mid-adulthood (6 months of age) and through the use of a range of genetically adapted mouse species. Initially, there were plans to further extend on these studies and to test the same mice strains at 12 months of age and older. However, it was not logistically feasible and out of the scope of this PhD project, due to the time constraints within the PhD and the extensive time requirements needed to breed mice to the required age before testing.

Mice improved in cognition-like behaviour and decreased depression-like behaviour compared to WT mice at 6 months of age and as opposed to the poor cognitive performance seen in the 3 months mice (Camara et al., 2013) (chapters 3 and 4). However, a further analysis of mice at 12 and even 15 months of age will be able to provide a more comprehensive picture of how a deficiency of TNF- $\alpha$  and its receptors alters cognitive function and emotional behaviour throughout development and into older age. I believe more work in studying older cohorts of the TNF- $\alpha$  and TNF- $\alpha$  receptor knockout mice strains would significantly add to the literature in the field and should be a consideration for future research.

Moreover, as both Tm and sol TNF- $\alpha$  have different functions and different affinities for the different receptors, wherein TNF-R1 has a higher affinity for solTNF- $\alpha$ , while TNF-R2 has a higher affinity for tmTNF, as has been mentioned previously in this thesis. In the present body of work, the knockout

mice were generated to lack total TNF- $\alpha$ . However it is very likely that in the TNF- $\alpha$  receptor knockout mice, the behavioural and neurobiological effects observed were due to a deficiency in both sol and tm TNF- $\alpha$ . As this study did not differentiate between the two types of TNF- $\alpha$ , it is difficult to assign a particular mechanism of action to each type. To circumvent these shortcomings more thorough investigations into the mechanisms between the different forms of TNF- $\alpha$  will be undertaken in future studies.

It would also be interesting to repeat the first two studies (chapters 3 and 4) of this study using a longitudinal design/methodology so that the results could be compared to those presented here. In the present study, two different groups of mice, of the same strain (chapter 3 and 4) were used rather than a single cohort of mice. This was as a result of the need to sacrifice the mice following behavioural testing in order to conduct biological and molecular analysis of the CNS tissue. Using one group of mice and testing them, first, at 3 months and then later at 6 months of age, could achieve a direct comparison of the behavioural analyses. Despite this limitation, however, there was still room in the current studies, to make speculative comparisons between the 2 age groups of mice. In addition, the use of WT mice in both studies allowed for a direct comparison of the effects of genetic deletion compared to 'normal' behavioural development.

For future work, using the same mouse cohorts for age-specific testing will enable analysis of whether cognition-like and depression-like behaviour actually improve with ageing in knockout mice or whether the improvement is only relative to a phenotypically 'normal' mouse (as was seen in chapter 4). Though this analysis

was not possible in the present study, the results still provide valuable insight on the behavioural effects of TNF- $\alpha$  deletion. It would also be beneficial to use randomised littermate controls in behavioural studies to minimize epigenetic differences colonies. Furthermore, the use of Cre-loxed mice would greatly benefit the study of TNF- $\alpha$  and its receptors by specifically targeting these mice and overcome the limitations of developmental phenotype over actual knockout phenotypes.

In addition to looking at the genetic deletion of TNF- $\alpha$  and its receptors, this study also sought to test a TNF- $\alpha$  over-expression strain (GFAP-TNF<sup>+/+</sup>, detailed in *appendix*). This strain was tested for behaviour at 3 months and then later at 7 months of age. This strain of mouse, as detailed by a previous study (Stalder et al., 1998) was known to suffer from locomotor deficits leading to ataxia and muscle spasms commencing from 6 months of age and resulting in death of the animal by 12 months of age, due to an over-expression of TNF- $\alpha$  by astrocytes. It was also associated with severe CNS pathology leading to high expression of TNF- $\alpha$  in the brain compared to WT mice, with lesions in areas like the cerebellum and spinal cord (Stalder et al., 1998, Carrasco et al., 2000).

However studies failed to show either up-regulation of TNF- $\alpha$  or any such phenotypic changes (i.e. locomotor deficits) in these mice. The results were unfortunately not what was expected of the transgenic model used and it is speculated that epigenetic changes occurred in these mice over the course of breeding, altering the phenotype (Miko, 2008).

As TNF- $\alpha$  has been postulated to be an important biomarker and is up-regulated in a range of disorders like MDD and AD (Baune et al., 2012b), the need



for understanding the neurobiological effects of TNF- $\alpha$  over-expression is critical. Though the model of over-expression employed in this study failed to provide us with the necessary answers, future work should look at either the development of a more robust TNF- $\alpha$  over-expression models or look towards pharmacological methods of TNF- $\alpha$  up-regulation, in order to mimic disease symptoms and provide insight into how this occurs.

Despite the lack of success in the GFAP-TNF<sup>+/+</sup> model (TNF- $\alpha$  over-expression strain), certain aspects of TNF- $\alpha$  up-regulation in the CNS as a part of this study could still be examined. In the experiments detailed in chapter 5, peripheral LPS administration to initiate an immune response within the CNS was successfully used. This experimental design produced an up-regulation of TNF- $\alpha$  activity within in the CNS and thus mimicked, to some extent, an overexpression model of TNF- $\alpha$ . The use of a TNF- $\alpha$  specific antagonist, etanercept, administered through ICV was effective in reducing the anxiety-like behaviour exhibited in this model and decreased the microglial numbers in the CNS. This data supports the hypothesis that the behavioural changes observed occurred as a consequence of TNF- $\alpha$  activation, and lends weight to the theory that immune activation within the CNS plays a pivotal role in the development of depressive and anxiety-like behaviour.

Whilst the results of the immune challenge study positively demonstrate and reinforce key findings regarding the role of TNF- $\alpha$  in the aetiology of mood disorders, there are some aspects of the study design that could have been improved upon. For instance, in this study mice were tested with an acute dose of peripheral LPS

and then treated with etanercept 24 hr after initial stimulation. However, it would be preferable to test the effects of etanercept on immune activation at a time-point earlier than 24 hr as this lies outside the peak of LPS activity (Frenois et al., 2007). Analysis at 4 hr and 8 hr post LPS would provide a more comprehensive picture of the effects of LPS, and the effects of etanercept administration, during the peak of LPS activity. This would certainly be a recommendation for future and further work using an LPS method of immune activation.

Lastly, in the studies presented in this thesis, the analysis of neurotrophin expression (NGF and BDNF) was limited to the use of ELISA, though a more robust approach like qPCR may have provided more definite results. ELISA methodology was chosen as this analytical technique is well established within the research group and provides more consistency with previous work undertaken using these transgenic strains (Camara et al., 2013). However the results presented here do provide an accurate representation of the protein levels of these neurotrophins within the hippocampus, especially since the protocols used for these analyses have been validated for brain tissue (Gilmore et al., 2003).

Overall, this study provides a comprehensive investigation into the behavioural consequences of targeting CNS TNF- $\alpha$  activity at 2 different stages of development. The results demonstrate conclusively that TNF- $\alpha$  is a key player at all stages of development and normal function. However, there are a number of aspects of the study that can be investigated further to more clearly determine the neurobiological and behavioural role of TNF- $\alpha$  and its receptors within the CNS as a

part of the normal development and functioning of the CNS and in terms of driving cognitive behaviour during inflammatory conditions.

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## **Appendix: Published Papers**

# Tumour Necrosis Factor-alpha Mediated Mechanisms of Cognitive Dysfunction

Review Article • DOI: 10.2478/s13380-012-0027-8 • Translational Neuroscience • 3(3) • 2012

Translational Neuroscience



## TUMOUR NECROSIS FACTOR ALPHA MEDIATED MECHANISMS OF COGNITIVE DYSFUNCTION

### Abstract

**Background:** Tumour necrosis factor - alpha (TNF- $\alpha$ ) is a pro-inflammatory cytokine that combines a plethora of activities in the early stages of an immune response. TNF- $\alpha$  has gained increasing importance given TNF- $\alpha$  up-regulation in multiple brain pathologies like neuropsychiatric conditions such as depression, schizophrenia, as well as neuroinflammatory disorder like multiple sclerosis (MS).

**Aim:** The aim of this review is to critically analyse neurobiological, immunological and molecular mechanisms through which TNF- $\alpha$  influences the development of cognitive dysfunction.

**Principal findings/results:** The review presents several lines of original research showing that the immunological properties of TNF- $\alpha$  exacerbate inflammatory responses in the central nervous system such as microglial and endothelial activation, lymphocytic and monocytic infiltration and the expression of downstream pro-inflammatory cytokines and apoptotic factors. Depression, schizophrenia, and MS all manifest symptoms of activated immune response along with cognitive dysfunction, with TNF- $\alpha$  overexpression as a central clinical feature common to these disorders. Furthermore, TNF- $\alpha$  acts negatively on neuroplasticity and the molecular mechanisms of memory and learning (i.e., long-term potentiation and long-term depression). TNF- $\alpha$  also exerts influence over the production of neurotrophins (i.e., nerve growth factor and brain-derived neurotrophic factor, neurogenesis, and dendritic branching). **Conclusions/significance:** This review outlines that TNF- $\alpha$  and its receptors have a substantial yet underappreciated influence on the development and progression of neuropsychiatric symptoms across several disease entities. An improved understanding of these underlying mechanisms may help develop novel therapeutic targets in the form of drugs specifically targeting downstream products of TNF- $\alpha$  activation within the central nervous system.

### Keywords

• Tumour necrosis factor alpha • Cytokines • Neuropsychiatry • Neuroinflammation • Depression • Neuroplasticity • Neurotrophins

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# TNF- $\alpha$ and its Receptors Modulate Complex Behaviours and Neurotrophins in Transgenic Mice

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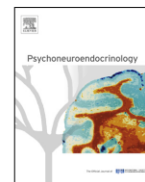


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## TNF- $\alpha$ and its receptors modulate complex behaviours and neurotrophins in transgenic mice



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### KEYWORDS

TNF- $\alpha$ ;  
TNF-R1;  
TNF-R2;  
Cognition;  
Depression;  
Anxiety;  
Sociability;  
Neurotrophins;  
Neurogenesis

**Summary** Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) plays an important role not only in immunity but also in the normal functioning of the central nervous system (CNS). At physiological levels, studies have shown TNF- $\alpha$  is essential to maintain synaptic scaling and thus influence learning and memory formation while also playing a role in modulating pathological states of anxiety and depression. TNF- $\alpha$  signals mainly through its two receptors, TNF-R1 and TNF-R2, however the exact role that these receptors play in TNF- $\alpha$  mediated behavioural phenotypes is yet to be determined.

**Methods:** We have assessed TNF- $^{-/-}$ , TNF-R1- $^{-/-}$  and TNF-R2- $^{-/-}$  mice against C57BL/6 wild-type (WT) mice from 12 weeks of age in order to evaluate measures of spatial memory and learning in the Barnes maze (BM) and Y-maze, as well as other behaviours such as exploration, social interaction, anxiety and depression-like behaviour in a battery of tests. We have also measured hippocampal and prefrontal cortex levels of the neurotrophins nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) as well as used immunohistochemical analyses to measure number of proliferating cells (Ki67) and immature neurons (DCX) within the dentate gyrus.

**Results:** We have shown that young adult TNF- $^{-/-}$  and TNF-R1- $^{-/-}$  mice displayed impairments in learning and memory in the BM and Y-maze, while TNF-R2- $^{-/-}$  mice showed good memory but slow learning in these tests. TNF- $^{-/-}$  and TNF-R2- $^{-/-}$  mice also demonstrated a decrease in anxiety like

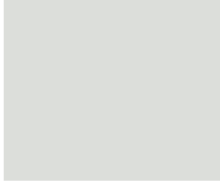
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behaviour compared to WT mice. ELISA analyses showed TNF  $\alpha$  and TNF-R2  $\alpha$  mice had lower levels of NGF compared to WT mice.

*Conclusion:* These results indicate that while lack of TNF- $\alpha$  can decrease anxiety-like behaviour in mice, certain basal levels of TNF- $\alpha$  are required for the development of normal cognition. Furthermore our results suggest that both TNF-R1 and TNF-R2 signalling play a role in normal CNS function, with knockout of either receptor impairing cognition on the Barnes maze.

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# Effects of Centrally Administered Etanercept on Behaviour, Microglia, and Astrocytes in Mice Following a Peripheral Immune Challenge

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## Effects of Centrally Administered Etanercept on Behavior, Microglia, and Astrocytes in Mice Following a Peripheral Immune Challenge

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Peripheral cytokines affect central nervous system (CNS) function, manifesting in symptoms of anxiety and cognitive decline. Although the peripheral blockade of tumor necrosis factor (TNF)- $\alpha$  has been effective in alleviating depression and rheumatoid arthritis, it is yet unknown whether central blockade of TNF- $\alpha$  is beneficial for immune-challenged CNS function. This study investigated the effects of central etanercept administration following a peripheral immune challenge on anxiety-like and cognition-like behaviors and microglia and astrocyte numbers. Twelve-week-old C57BL/6 mice ( $n=40$ ) were treated with either LPS or saline administered peripherally 24 h before being treated with either etanercept or artificial CSF (aCSF) by intracerebroventricular injection. Mice underwent behavioral analyses for locomotion, memory, and anxiety-like behavior 24 h post-etanercept/aCSF treatment, and tissue was collected to estimate the numbers of hippocampal microglia and astrocytes. Following peripheral immune challenge with LPS, mice showed increased anxiety-like behavior, which was significantly improved following treatment with etanercept (two-way ANOVA: Interaction:  $F_{(1,30)}=0.60$ ,  $P=0.44$ ; Saline/LPS challenge:  $F_{(1,30)}=23.92$ ,  $P<0.0001$ , etanercept vs aCSF:  $F_{(1,30)}=11.09$ ,  $P=0.0023$ ). For cognition, a significant interaction effect found by two-way ANOVA (Interaction:  $F_{(1,20)}=4.96$ ,  $P=0.037$ , Saline/LPS challenge:  $F_{(1,20)}=4.966$ ,  $P=0.31$ , aCSF/etanercept treatment:  $F_{(1,20)}=0.06$ ,  $P=0.80$ ) and post-hoc analysis revealed a significant decrease in cognition in LPS-aCSF compared with Sal-aCSF mice ( $P=0.038$ ), but no significant difference was noted between LPS-aCSF and LPS-Etan mice ( $P>0.9$ ). A significant reduction in the number of microglia within the hippocampus of these mice was noted (two-way ANOVA: Interaction:  $F_{(1,15)}=11.41$ ,  $P=0.0041$ ; Saline/LPS challenge:  $F_{(1,15)}=50.13$ ,  $P<0.0001$ , etanercept vs aCSF:  $F_{(1,15)}=3.36$ ,  $P=0.08$ ). Centrally administered etanercept improved anxiety-like behavior but not spatial memory under a peripheral immune challenge and was associated with a decrease in the hippocampal microglia numbers. This suggests that etanercept recovers anxiety-like behavior possibly mediated by a reduction of TNF- $\alpha$ -related central inflammation.

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