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A demonstration of neuroimmune interactions between the candidate schizophrenia vulnerability gene Neuregulin 1 and peripheral and central cytokine levels: focus on IL-6 and GCSF

Peta Snikeris

University of Wollongong

UNIVERSITY OF WOLLONGONG

SCHOOL OF MEDICINE

A demonstration of neuroimmune interactions between the candidate schizophrenia vulnerability gene Neuregulin 1 and peripheral and central cytokine levels: focus on IL-6 and G-CSF

PETA SNIKERIS

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CERTIFICATION

I, Peta Snikeris, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Medicine, Faculty of Science, Medicine and Health, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. This manuscript has not been submitted for qualifications at any other academic institution.

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ABSTRACT

The development of schizophrenia is thought to involve an interaction of genetic susceptibility and environmental factors. In particular, immune insults have been suggested as relevant environmental impacts, with evidence of cytokine alterations in schizophrenia patients and an emerging role for cytokines in the brain. Neuregulin 1 (Nrg1) represents one potential candidate vulnerability gene and although murine and human studies indicate the potential for a Nrg1 mutation to influence immune function, the response to a direct immune stimulus has not been studied. The aim of the present study was to determine if a mutation in the Nrg1 gene altered sensitivity to an immune challenge by changing the peripheral cytokine response and corresponding cytokine levels in the brain.

The Nrg1 heterozygous transmembrane domain mutant (Nrg1 Het) mice and wild type-like (WT) littermates were subject to an immune stimulus in the form of B16F0 melanoma cells which established a chronic immune response over 9 days. Plasma levels of 32 peripheral cytokines were measured using a multiplexed flow cytometry assay. Additional measures of the peripheral immune response were analysed using flow cytometry while tumour makers for immune infiltration and angiogenesis were measured using qPCR and immuno-staining. Based on alterations in the peripheral immune system, interleukin 6 (IL-6) and granulocyte colony stimulating factor (G-CSF) were studied in the prefrontal cortex and the hippocampus, brain regions highly implicated in schizophrenia. Protein and mRNA expression levels for IL-6, IL-6Rα and gp130 receptor, as well as G-CSF and G-CSFR were measured using Western blot, flow cytometry and qPCR methods. In an additional exploratory study, qPCR was used to

measure mRNA expression of a number of pathway molecules involved in PI3K/Akt and Jak/Stat signalling, both candidates to integrate neuronal and peripheral immune function.

The immune challenged Nrg1 Het mice showed a threefold elevation in IL-6 while G-CSF was double when compared with immune challenged WT mice (chapter 2). WT mice subject to the immune stimulus showed an increase in IL-5 and KC demonstrating that an immune response was generated. There were no differences in circulating immune cells or tumour specific factors.

Following the confirmation of alteration in the periphery, the cytokines IL-6 and G-CSF, as well as their obligate signalling receptors were investigated in the brain (chapters 3 and 4). IL-6 was reduced in the prefrontal cortex of immune challenged Nrg1 mice compared with unchallenged Nrg1 mice. Additionally, IL-6Rα was increased in the same brain region when Nrg1 immune stimulated mice were compared to immune stimulated WT mice. Soluble gp130 was lower in the hippocampus of the Nrg1 Het compared to WT controls, and was even further reduced in both groups with an immune stimulus. The hippocampus also displayed alterations in G-CSF where WT mice demonstrated an increase in G-CSF compared to their unchallenged littermates. Further, Nrg1 mice revealed lower levels of G-CSF compared to the immune challenged WT animals.

In the final exploratory study, two pathways highly implicated in both neuronal function in schizophrenia as well as in the immune system, were considered: the PI3K/Akt pathway and the Jak/Stat pathway (chapter 5). The PFC showed increases in Akt1 and SOCS3 based on Nrg1 genotype, while ErbB2 and Pten were increased by the genotype

x immune interaction. The hippocampus, showed a decrease in Jak1 mRNA as a result of genotype plus immune insult. These alterations suggest a central role for PI3K/Akt signalling.

Together these data demonstrate strong cross talk between the immune system and the brain. The elevated IL-6 plasma level found in the Nrg1 Het mice was consistent with increased levels of IL-6 found in several studies on schizophrenia patients, as well as in humans carrying a Nrg1 mutation. While G-CSF has not been directly studied, a peripheral role is implicated in schizophrenia in relation to side effects caused by anti-psychotic medications. The implications of reduced IL-6 and GCSF as found in the brain are yet to be understood, however these results confirm a dysfunction of cytokines in the brain of Nrg1 Het mice. Although the detected alterations in the studied signalling pathways should be considered carefully due to study limitations; a potential dysregulation of the PI3K/Akt pathway is indicated. It remains to be determined whether these changes have neuromodulatory functions and can influence schizophrenia-relevant neurotransmission and behaviour. This study demonstrated, however, that peripheral cytokines and brain cytokines are perturbed when a Nrg1 mutation is combined with a direct peripheral immune stimulus, similar to previous reports in humans, in a plausibly schizophrenia-relevant manner.

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Saving the most important for last: To Mum and Shane: your unwavering belief in my abilities means more to me than you might know

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ABBREVIATIONS AND ACRONYMS

Akt	Protein kinase B (PKB): also known as Akt
Akt1	V-akt murine thymoma viral oncogene homolog 1
Akt2	V-akt murine thymoma viral oncogene homolog 2
Akt3	V-akt murine thymoma viral oncogene homolog 3
B16F0	Murine melanoma cell line
BBB	Blood brain barrier
CD	Cluster of differentiation
CNS	Central nervous system
Eotaxin	Eosinophil chemotactic protein
ErbB	Erythroblastic leukaemia viral oncogenes
ErbB2	V-erb-a erythroblastic leukaemia viral oncogene homolog 2
ErbB3	V-erb-a erythroblastic leukaemia viral oncogene homolog 3
ErbB4	V-erb-a erythroblastic leukaemia viral oncogene homolog 4
GABA	Gamma amino butyric acid
G-CSF	Granulocyte-colony stimulating factor
G-CSFR	G-CSF receptor
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp130	Glycoprotein 130
IFN	Interferon
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
IL-6Rα	IL-6 receptor alpha chain
IP-10	Interferon-gamma-inducible protein 10
Jak	Janus kinase
KC	Platelet-derived growth factor-inducible protein KC
LIF	Leukaemia inhibitory factor
LIX	Cytokine LIX, also known as C-X-C motif chemokine 5
LPS	Lipopolysaccharide
Ly6G	Lymphocyte antigen 6 complex, locus G
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage-colony stimulating factor

mgp130	Membrane bound gp130
MIG	Monokine induced by interferon-gamma
mIL-6Rα	Membrane bound IL-6Rα
MIP-1α	Macrophage inflammatory protein 1-alpha
MIP-1β	Macrophage inflammatory protein 1-beta
MIP-2	Macrophage inflammatory protein 2
NMDA-R	N-methyl-D-aspartate receptor
Nrg1	Neuregulin 1
Nrg1 Het	Neuregulin 1 heterozygous transmembrane domain mutant
pAkt	Phosphorylated Akt
PFC	Prefrontal Cortex
PI3K	Phosphatidylinositol-3 kinase
PKB	Protein kinase B also known as Akt
PolyIC	Polyinosinic-polycytidylic acid
Pten	Phosphatase and tensin homolog
RANTES	Regulated upon activation, normal T cell expressed and secreted
sgp130	Soluble gp130
sIL-2R	Soluble IL-2 receptor
sIL-6Rα	Soluble IL-6Rα
SOCS3	Suppressor of cytokine signalling 3
Stat	Signal transducers and activators of transcription
T_C	Cytotoxic T cell
TGF-β	Transforming growth factor-beta
T_H	T-helper cell
T_H1	T-helper cell type 1
T_H2	T-helper cell type 2
TNF	Tumour Necrosis Factor
VEGF	Vascular endothelial growth factor
WT	Wild type-like

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CHAPTER ONE

Introduction and literature review

Peta Snikeris
University of Wollongong

1.1 Introduction

Schizophrenia is a chronic and devastating psychiatric disorder. It is generally accepted that the illness is a neurodevelopmental disorder and that symptoms result from aberrant neurotransmission, with genetic vulnerability highly implicated in the disorder (Tandon et al., 2008a). While the symptoms of schizophrenia are well characterised, the genetic alterations, developmental underpinnings, symptom causation and possible coexistence with other diseases remain incompletely understood.

Presently, support is increasing for links between the aetiology of schizophrenia and a persistent malfunctioning of the immune system (Patterson, 2009). Epidemiological studies showed that schizophrenia patients have a lower incidence of a subset of cancers, rheumatoid arthritis and type II diabetes, diseases related to dysfunction of the immune system (Catts et al., 2008; Leucht et al., 2007). Accordingly, altered levels of several cytokines were shown in the blood and cerebrospinal fluid of schizophrenia patients (Miller et al., 2011; Potvin et al., 2008). Remarkably, the Schizophrenia Working Group of the Psychiatric Genomics Consortium (2014) demonstrated in their recent genome wide association study that several genes found to be associated with a schizophrenia diagnosis were enriched in immune loci. These results underline previous epidemiological findings linking schizophrenia and the immune system and add support for the hypothesis that combinations of genetic and immune-relevant environmental insults lead to the manifestation of schizophrenia (Tsuang, 2000).

Indeed, Ibi et al (2010) demonstrated that maternal immune activation, when combined with a Disrupted in Schizophrenia 1 (DISC1) genetic mutation, resulted in altered cytokine levels and a schizophrenia-like phenotype in adult murine offspring. While

Desbonnet et al (2012) showed that a genetic mutation in the schizophrenia susceptibility gene Nrg1 altered the immune response to stress in adult mice. In humans, Marballi et al (2010) demonstrated differing levels of cytokines in first degree relatives of schizophrenia patients who carried a schizophrenia relevant gene mutation compared to those relatives not carrying the mutation. However, whether a genetic vulnerability to schizophrenia would result in an altered response to a direct immune stimulus in early adulthood has not been studied. As a result, this thesis will examine if a mutation in a single schizophrenia candidate vulnerability gene can alter the response to a direct immune challenge in young adult mice.

1.2 Literature review

1.2.1 Schizophrenia

Schizophrenia is a chronic and devastating psychiatric disorder that affects around 1% of the population worldwide; it is associated with a great burden to patients, carers and health systems (Rossler et al., 2005). The disease is characterized by a number of symptoms that are classified as positive, negative and cognitive with the onset of symptoms generally during adolescence or early adulthood (Hafner, 1998; Miyamoto et al., 2003; Tandon et al., 2009; van der Werf et al., 2014). Studies investigating anatomical and physiological changes in brains of schizophrenia patients reveal altered cytoarchitecture, enlarged ventricles and reductions in cortical grey matter (Harrison, 2008; Keshavan et al., 2008; Tandon et al., 2008b). Although the underlying aetiology remains unknown, it is widely acknowledged that the symptoms of schizophrenia result from abnormalities in neurotransmission, particularly in the dopaminergic and glutamatergic, but also the gamma amino butyric acid (GABA)-ergic and serotonergic systems (Berretta et al., 2009; Harrison, 1999; Lang et al., 2007; Murray, 2002; Stone et

al., 2007). These changes do not appear to deteriorate following symptom onset and are therefore thought to result from abnormal development, resulting in the commonly accepted hypothesis of schizophrenia as a neurodevelopmental disorder (Harrison, 2008; Miyamoto et al., 2003; Tandon et al., 2008b). The disease, however, presents as a heterogeneous disorder with the underlying factors of its development, the causal factors of symptoms, and the possible coexistence with other diseases still to be resolved.

1.2.2 The genetic basis of schizophrenia

The most consistent risk factor for schizophrenia development is an inherited genetic predisposition (Tandon et al., 2008a). Adoption studies in particular have shown that a family history of schizophrenia is the most significant risk factor in developing the illness (Schultz et al., 2007). Monozygotic twins have a 40-50% concordance rate for schizophrenia and the risks of developing the disease are increased 10 fold in first degree relatives of schizophrenia patients (Weiser et al., 2005). In subsequent population studies, several susceptibility genes have been identified, including Neuregulin 1 (Nrg1), V-erb-a erythroblastic leukaemia viral oncogene homolog 4 (ErbB4), V-akt murine thymoma viral oncogene homolog 1 (Akt1), dystrobrevin-binding protein 1 (Dysbindin), Catechol-O-methyl transferase (COMT), and Disrupted-in-Schizophrenia 1 (DISC1) (Harrison and Weinberger, 2005; Norton et al., 2006; Singh et al., 2014; Stone et al., 2007). From this diverse array of candidate genes, the Nrg1 gene and its gene products were shown as attractive candidates for further study (Esper et al., 2006; Schmitt et al., 2008).

1.2.3 Neuregulin 1

Nrg1 belongs to a family of growth factors with four individually identified genes (Nrg1-4). The Nrg1 gene encodes six types of proteins with different signalling properties (Mei and Xiong, 2008). All six Nrg1 type proteins (I-VI) contain an intracellular cytoplasmic tail or intracellular domain, a transmembrane domain and an extracellular epidermal growth factor like domain. These six Nrg1 type proteins can be further processed into at least 31 known isoforms. Most of the Nrg1 isoforms are synthesized as a pro-neuregulin transmembrane precursor that undergoes further proteolytic processing, principally at the cell surface (Kalinowski et al., 2010). Nrg1 has been identified in range of different tissue types, including nervous, cardiac, lung and normal as well as cancerous breast tissues (Finigan et al., 2013; Holmes et al., 1992; Lemmens et al., 2007; Mei and Xiong, 2008). The full functional implications of mutations on the Nrg1 gene continue to be investigated.

Nrg1 signalling occurs by stimulation of the family of erythroblastic leukaemia viral oncogenes (ErbB) tyrosine kinase receptors, ErbB2, -3 and -4. The ErbB4 receptor is the only one of these receptors that can interact with the Nrg1 ligand and produce signalling when in the homodimeric conformation. ErbB3 is able to bind the Nrg1 ligand, but does not transmit a signal unless it has formed a heterodimer, preferably with the ErbB2 receptor. ErbB2 on the other hand has no intrinsic ability to bind Nrg1, but when acting in concert with ErbB3 or ErbB4, it can be activated by ligand binding to its heterodimeric partner. Here, the ErbB4 homodimers were found to signal with weaker potency, than if a heterodimer is formed with ErbB2 (Yarden and Sliwkowski, 2001a). Nrg1 and its signalling through the ErbB receptors have been shown essential

for both neuronal and cardiac development, as mice deficient in Nrg1, ErbB2, -3 or -4 are not viable (Yarden and Sliwkowski, 2001a).

In addition to the canonical signalling pathway, a lesser studied non-canonical, or backward signalling pathway has been demonstrated for Nrg1. This back-signalling pathway occurs when pro-Nrg1, acting as a receptor, binds ErbB4 which stimulates gamma-secretase cleavage of the Nrg1 intracellular domain (Dejaegere et al., 2008; Talmage, 2008). The intracellular domain translocates to the nucleus where it has been shown to effect a number of functions including: inhibition of NMDA toxicity-induced apoptosis, the regulation of PSD-95 transcription and an increase in surface expression of nAChR-alpha7, where the intracellular domain of Nrg1 type III is cleaved (Bao et al., 2003, 2004; Buonanno, 2010; Zhang et al., 2008). Although further study is required to fully elucidate the mechanisms for Nrg1 back-signalling and the functional consequences of this pathway, it is possible that this back-signalling could play a role in the Nrg1 induced changes in the brain and the peripheral immune system.

a) Neuregulin 1 in the brain

As a growth factor in the central nervous system (CNS), Nrg1 has been shown to be critically involved in neurogenesis, neuronal survival, neuronal migration and synaptic plasticity (Mei and Xiong, 2008). Upon binding to its ErbB receptors in the brain, forward signalling triggers various intracellular signalling pathways, including the phosphatidylinositol-3 kinase-protein kinase B (PI3K/PKB, also known as PI3K/Akt) and Janus kinase/signal transducers and activators of transcription (Jak/Stat) signalling (Banerjee et al., 2010; Liu and Kern, 2002). Nrg1 signalling was additionally shown to

directly influence GABAergic, glutamatergic and dopaminergic neurotransmission (Buonanno, 2010; Kwon et al., 2008). It is proposed that this direct interaction occurs because the location of the primary Nrg1 receptor, ErbB4 is on GABAergic interneurons and is in close proximity to glutamatergic N-methyl-D-aspartate receptors (NMDA-R) (Neddens and Buonanno, 2010; Neddens et al., 2011). These functions of Nrg1 represent a potential link between Nrg1 dysfunction and the major neurotransmitter theories of schizophrenia (Buonanno, 2010; Collier and Li, 2003; Harrison and Weinberger, 2005; Neddens et al., 2009; Stone et al., 2007).

b) Neuregulin 1 in schizophrenia

Stefansson et al (2002) first identified Nrg1 as a promising schizophrenia candidate gene in a genome wide association study in an Icelandic population. Subsequent genetic and mRNA expression studies have confirmed that alterations of the Nrg1 system are associated with this illness in a range of populations, (Alaerts et al., 2009; Law et al., 2006; Stefansson et al., 2004), as well as in a meta-analysis (Munafò et al., 2006) and systematic review (Agim et al., 2013). In addition altered levels of Nrg1 and its receptor ErbB4 gene transcripts and proteins were demonstrated in the brain in schizophrenia patients (Barakat et al., 2010; Bertram et al., 2007; Chong et al., 2008; Kang et al., 2012; Law et al., 2007)

In post-mortem brain studies, Barakat et al (2010) found a decrease in Nrg1 C-terminal fragments in the frontal cortex (Brodmann's area 6), consistent with the finding of Bertram et al (2007) where Nrg1 (isoform alpha) was decreased in the white matter in the PFC in patients. Chong et al (2008) demonstrated that Nrg1 (53kDa protein) and the full length ErbB4 protein were increased in the cytoplasmic fraction of PFC patient samples, while cleaved ErbB4 products were decreased in the same fractions. Further,

Marballi et al (2012) found no change in the full length membrane bound Nrg1 protein in the prefrontal cortex or the hippocampus of schizophrenia patients while an increase in the extracellular cleavage product of Nrg1 was seen in the prefrontal cortex with no change in the hippocampus. Marballi (2012) also demonstrated a decrease in a 50kDa Nrg1 fragment in both the prefrontal cortex and the hippocampus which the authors speculate is an intracellular cleavage product. Overall Marballi (2012) suggests that altered Nrg1 may be cleavage specific in schizophrenia patients. These findings clearly show alterations in Nrg1 exist in patients, however they do appear conflicting.

Also a meta-analysis suggested the evidence for Nrg1 as a candidate gene for schizophrenia vulnerability to be inconclusive (Gong et al., 2009). The presented inconsistencies however, have previously been attributed to population differences in allelic and haplotype frequencies (Gardner et al., 2006; Harrison and Law, 2006), and support for the involvement of Nrg1 in schizophrenia risk remains (Gatt et al., 2015; Greenwood et al., 2012; Weickert et al., 2012). The mechanisms by which Nrg1 confers an increased risk to develop schizophrenia symptoms, however, remain inadequately understood (Mei and Xiong, 2008; Stefansson et al., 2004; Weickert et al., 2012).

c) Neuregulin 1 animal models

Based on the initial findings, genetic animal models with mutations of the Nrg1 gene were developed and have underlined the relevance of Nrg1 to schizophrenia vulnerability. These models include heterozygous mutations in the transmembrane domain region (Duffy et al., 2008, 2010; Karl et al., 2007), type I/II (Deakin et al., 2009, 2011) and type III (Chen et al., 2008) Nrg1. These mutant mice show different, but overlapping phenotypes, with previous work showing that heterozygous Nrg1 transmembrane domain (Nrg1 Het) mutant mice exhibit an inborn vulnerability to

develop behavioural abnormalities resembling schizophrenia. These behavioural abnormalities include hyper locomotion, deficits in sensorimotor gating and object recognition (Duffy et al., 2008, 2010; Karl et al., 2007; O'Tuathaigh et al., 2008; Stefansson et al., 2002). Further, mice exposed to excess Nrg1 in the neonatal period, or those constitutively overexpressing Nrg1 were shown to have several behavioural impairments, further confirming the potential involvement of Nrg1 in schizophrenia pathology (Abe et al., 2011; Kato et al., 2010; Mouri et al., 2013).

There is some criticism of the model in the literature, based on the inconsistent human gene studies and evidence that the timing and significance of behavioural abnormalities in mutant mice has been shown to be protocol specific and in some cases is not consistent (van den Buuse et al., 2009; Karl et al., 2011). The mouse model utilised in the present study are the Nrg1 Het mutant mice (target allele: Nrg1tm2Zhou), which have been described previously (Stefansson et al., 2002). This model was established to represent individuals at genetic risk for the development of schizophrenia characteristics (Karl et al., 2007). Whereas some phenotypes of the Nrg1 Het mice are very consistent, others were rather observed with secondary challenges, emphasising their state as a vulnerability model (Boucher et al., 2007a; Karl et al., 2011; Long et al., 2012, 2013).

While studies on Nrg1 and other susceptibility genes have increased our knowledge of schizophrenia development, they have clearly demonstrated that genetic alterations are also not the singular cause of the disease. Population based studies of patients and non-affected family members have demonstrated that having a genetic alteration in any of the candidate susceptibility genes does not confer schizophrenia, only a vulnerability to developing the illness. Further, no single genetic polymorphism, or consistent combination of polymorphisms, exists in all patients (Esper et al., 2006; Schizophrenia

Working Group of the Psychiatric Genomics Consortium, 2014). This apparent failure of genetics to explain the cause of the disease, has led to increasing acceptance that interactions between susceptibility genes and the environment, such as foetal stress, obstetric complications and immune insults, are most likely to result in the development of schizophrenia (Tsuang, 2000; Tsuang et al., 2001).

1.2.4 Environmental risk factors: Focus on the immune system and cytokines

A wide variety of environmental risk factors have been established in the aetiology of schizophrenia (Tandon et al., 2008b). Interestingly, many of these environmental stimuli directly or indirectly influence the immune system (Aubert, 2008; Meyer et al., 2008a). Specifically, infections and induced inflammation have been identified to play a role in schizophrenia development in studies of humans and in animal models (Meyer et al., 2008a; Muller and Schwarz, 2008; Tandon et al., 2008b). With cytokines being well-established as critical messengers of inflammation, a whole variety of cytokines and their receptors have shown variations in schizophrenia patients (detailed below in section 1.2.4e) and reviewed in meta-analyses by (Miller et al., 2011; Potvin et al., 2008). Patterson (2009), points out that many of these changes are present in both the peripheral blood and the cerebrospinal fluid, supporting a novel role for cytokines as neuromodulators.

a) The innate and adaptive immune system

The peripheral immune system is primarily a defence system evolved for: surveillance of tissues; recognition of damage caused by injury and invading pathogens; as well as cancer surveillance. The immune system generates a vast range of cells and molecules capable of differentiating and eliminating a seemingly endless number of pathogens in a

series of complex yet controlled responses. Additionally, elements of the immune system participate in ongoing tissue remodelling in response to continuously changing environmental demands. The immune system is divided into two components: the innate immune system, which is considered less specific and is the first line of defence; and the adaptive immune system, which is capable of recognising and specifically eliminating selected foreign antigens (Yirmiya and Goshen, 2011).

The innate immune system provides first line cellular and humoral defences against invading pathogens. When activated, the innate immune system initiates an inflammatory response, involving the rapid recruitment of innate immune cells, particularly macrophages and neutrophils. These cells phagocytose pathogens and produce a number of cytokines and chemokines that activate associated immune and tissue repair processes (Jones and Thomsen, 2013; Weismann and Binder, 2012; Yirmiya and Goshen, 2011). In contrast, the adaptive immune system recognises and subsequently remembers specific antigens. This enables the adaptive immune system to mount a stronger and more rapid response each time a specific antigen is encountered (Jones and Thomsen, 2013). Antigens are presented to T cells to activate a cellular immune response, with T cells activating B cells to produce antibodies for a humoral response. The T cells are generally divided into two main subsets: the T helper (T_H) and T cytotoxic (T_C) cells, although a third subset of T regulatory cells is also considered. The T_H and T_C lymphocytes are classified dependent on surface molecules (cluster of differentiation or CD): CD4+ cells generally function as T_H cells while CD8+ cells generally function as T_C cells. It is the CD4+ T_H lymphocytes that secrete the majority of cytokines in response to antigenic activation and thus determine the direction of the

immune response (Jones and Thomsen, 2013; Weismann and Binder, 2012; Yirmiya and Goshen, 2011).

The T_H cells are often further classified based on cytokine secretion as either T_H type 1 (T_H1) or T_H type 2 (T_H2) cells. T_H1 cells characteristically produce cytokines such as interleukin (IL)-2, tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ). T_H2 cells produce mainly cytokines such as IL-4, IL-6 and IL-10 (Müller et al., 2009). The T_H1 cells promote a cell-mediated immune response directed against intracellular pathogens, while the T_H2 cells promote B cell maturation and an antibody-based, humoral response directed against extracellular pathogens (Müller et al., 2009). Each cell-type cytokine profile promotes their specific immune type response while suppressing the alternative creating the so called 'polarized' immune response which is considered to be unbalanced in schizophrenia patients (Müller et al., 2009).

b) Cytokines

Cytokines are low molecular weight secreted proteins that exert a wide variety of effects on immune and other cells to regulate the duration and intensity of an immune response. One cytokine can have varied function, and redundancy across cytokines is common. Cytokines generally act locally and are therefore often only found in low levels in circulation. Single cells will respond to the local milieu of cytokines and it is the balance of different cytokines that affect the ultimate response (Simpson et al., 1997). Cytokines exert their effects by binding to specific membrane bound receptors located on a wide variety of cell types, as well as binding soluble receptors. These soluble receptors can act as either inhibitors (e.g. soluble IL-2 receptor or sIL-2R) or enhancers (e.g. soluble IL-6 receptor or sIL-6R) of activity. Further, endogenous antagonists exist,

which compete with cytokines for receptor binding such as the IL-1 receptor antagonist (IL-1RA) (Miller et al., 2011).

Terminology can further separate cytokines into pro- and anti- inflammatory types, as well as hematopoietic cytokines. The separation of cytokines into pro- and anti-inflammatory however, does not reflect the immune-type cells that produce these cytokines. Pro-inflammatory cytokines such as TNFα and IL-6 belong to the type-1 and type-2 immune response respectively, yet IL-4 and IL-10, considered anti-inflammatory, both belong to the type-2 immune response. While the hematopoietic cytokines, such as granulocyte-colony stimulating factor (G-CSF), stimulate the differentiation of hematologic progenitor cells into specific lineages of leukocytes or erythrocytes, however, G-CSF can also play a role in promoting the type-2 cytokine response (Xiao et al., 2007). With multiple roles and redundancies, many cytokines can play both pro- and anti-inflammatory roles in response to the cytokine milieu around them (Cavaillon, 2001).

In essence the cytokine network appears to be as complex as the immune system itself, perhaps akin to the complexity of the neurotransmitter networks in the brain. For this reason, the initial investigation of peripheral cytokines in the Nrg1 Het mouse model in this study was not limited to only those cytokines previously implicated in schizophrenia or those considered the major pro- and anti- inflammatory cytokines. A deliberately wide range of cytokines was included in the initial measurements with subsequent studies in the CNS narrowed by the results.

c) Cytokines as neuromodulators

Traditionally, cytokines have been viewed as modulators of peripheral immunity, regulating the duration and intensity of the immune response. They were not considered directly involved in normal brain function (Szelenyi and Vizi, 2007). However, our understanding of the role of cytokines in normal brain development and function is increasing swiftly (Jones and Thomsen, 2013). Cytokines are shown to be involved in normal neurodevelopment, synaptic function, neuroprotection, neurotoxicity, and to interact with other neuroendocrine and neuropeptide systems (Boulanger and Shatz, 2004; Boulanger et al., 2001; Schmitz and Chew, 2008; Yirmiya and Goshen, 2011). Cytokines also play a central role in the modulation of not only basic behaviours but also complex cognitive tasks (McAfoose and Baune, 2009); the underlying mechanisms, however, are yet to be resolved.

Cytokines produced in the periphery can penetrate the blood brain barrier, not just in pathological states that result in a leaky blood brain barrier, but also under basal conditions. Several mechanisms have been identified that allow this transition of peripheral cytokines into the brain, including active transport mechanisms and saturable transporters (Miller et al., 2009; Szelenyi and Vizi, 2007; Vollmer-Conna et al., 2004). More intriguing are increasing reports that not only peripheral blood cells, but also brain cells, including neurons, microglia and astrocytes, have the potential of *de novo* synthesis of cytokines. This is not only in response to infections or immunogenic agents, but also in response to antipsychotic drugs, including clozapine (Paterson et al., 2006; Sugino et al., 2009). In addition, cells of the CNS also express cytokine receptors (McAfoose and Baune, 2009; Na and Kim, 2007; Szelenyi and Vizi, 2007; Tohmi et al., 2007).

d) Cytokines in brain dysfunction and disease

Changes in cytokine levels in both blood and brain have been shown to interfere with neurodevelopment, and the detrimental effect of perinatal infections is well established (Meyer et al., 2008a). Throughout childhood and adolescence, however, synaptogenesis, synaptic pruning and myelination continue and are prone to be influenced by inflammatory stimuli (de Graaf-Peters and Hadders-Algra, 2006). Not surprisingly, brain cytokines were found to be dysregulated in psychopathologies like depression, Alzheimer's disease, and, schizophrenia (Fruntes and Limosin, 2008; McAfoose and Baune, 2009; Potvin et al., 2008). With neurons and glial cells responsive to cytokines (Miller et al., 2009), this provides the basis for the translation of peripheral inflammatory changes, such as those observed in schizophrenia patients, into dysregulation of brain function, with cytokines in the role of neuromodulators. Additional indications are that the complex interactions between the brain and the immune system may similarly be impaired in schizophrenia (Muller and Schwarz, 2008; Strous and Shoenfeld, 2006).

e) Cytokines in schizophrenia

Further to the role of cytokines in normal brain function, they have also been implicated in the symptomatology of schizophrenia (Fruntes and Limosin, 2008; McAfoose and Baune, 2009; Meyer et al., 2008a, 2008b; Potvin et al., 2008). Indeed, although the data basis is still scattered and inconclusive, recent studies clearly demonstrate that adult schizophrenia patients' display an imbalance in cytokine levels in both the CNS and the periphery (Patterson, 2009; Potvin et al., 2008). Together these findings raise the possibility that immune dysfunction, through a cytokine imbalance, is a primary or

secondary persistent state in the CNS and the peripheral immune system of schizophrenia sufferers (Patterson, 2009).

There have been numerous studies on cytokine levels in adult schizophrenia patients, uncovering a multitude of differences in different cytokines and soluble cytokine receptors. Several cytokines have been investigated in adult schizophrenia patients, uncovering alterations in IL-1, IL-1RA, IL-2, sIL-2R, IL-4, IL-6, sIL-6R, IL-8, IL-10, IL-12, IL-17 and IL-18 along with changes in TNFα, and IFNγ (Borovcanin et al., 2012; Müller, 2008; Muller and Schwarz, 2008; Nawa and Takei, 2006; Patterson, 2009; Potvin et al., 2008; Strous and Shoenfeld, 2006). A meta-analysis published by Potvin et al (2008) noted robust increases in *in vivo* serum levels of IL-1RA, sIL-2R and IL-6, while a decrease in *in vitro* IL-2 production was found in subjects with schizophrenia. These cytokine alterations are consistent with the theory of a blunted type 1 and over-active type 2 immune response, the data however remain conflicted, with other studies suggesting an enhanced type 1 response with no evidence of type 2 involvement (Miller et al., 2011; Müller et al., 2009).

This conflict may arise from differences in experimental methods. Firstly, studies have reported cytokines variously in circulating peripheral blood, cerebrospinal fluid, or as *in vitro* cytokine production following stimulation. Additionally, patient populations have been differentially selected, with samples taken from patients with first episode psychoses (medication naïve), recently withdrawn from medication (medication free), or stably medicated. Moreover, medication free or stably medicated patient samples were drawn from either chronic suffers or those suffering an acute relapse of symptoms. Interestingly, several studies demonstrated that altered cytokine levels were correlated with symptom severity, antipsychotic use or medication resistance in patients (Bresee

and Rapaport, 2009; Kim et al., 2004; Mahendran and Chan, 2004; Na and Kim, 2007; Singh et al., 2009; Zhang et al., 2002, 2009). Further, antipsychotic drugs have been shown to act directly on cytokine levels, however different medications result in different cytokine effects (McAllister et al., 1995; Xu et al., 1994).

A more recent meta-analysis by Miller et al (2011) included 40 studies conducted since 2005 and delineated patients into acutely relapsed and first-episode psychosis patients. The meta-analysis found that IL-1β, IL-6 and transforming growth factor (TGF-β) were increased similarly in both patient categories and were normalised with antipsychotic treatment. Interestingly IL-6 was the same in stably medicated outpatients compared with controls. IL-12, IFNγ, TNFα and sIL-2R were also found elevated in acute exacerbations of symptoms however, these did not normalise with treatment. Miller et al (2011) note several limiting factors in the studies used for the meta-analysis, particularly the lack of controls for confounding factors including body mass index and smoking.

1.2.5 The inflammatory hypothesis of schizophrenia

In the last decade, the inflammatory hypothesis of schizophrenia has re-gained much attention (Coelho et al., 2008; Miller et al., 2011; Müller and Schwarz, 2007; Potvin et al., 2008). This hypothesis advocates cytokines as important intermediaries for cross-talk between the immune system and the brain by modulating central neurotransmitter systems (Müller and Schwarz, 2007). As previously noted, peri-natal neurodevelopment can be severely affected by changes in blood and brain cytokine levels (Meyer et al., 2008a). With continued neuronal development through childhood, adolescence and into adulthood, CNS growth and function remains prone to cytokine influence (de Graaf-Peters and Hadders-Algra, 2006). Indeed, recent epidemiological data stated that

infections in adolescence also confer increased risk for schizophrenia development, more so when combined with a genetic predisposition (Dalman et al., 2008; Hickie et al., 2009). While there are limited studies demonstrating common mechanisms between neuronal and immune function, and few that attempt to directly link schizophrenia pathophysiology with altered immune function in a schizophrenia relevant model, these immunologically significant findings in schizophrenia patients make their existence and relevance highly likely.

1.2.6 Modelling an immune challenge in the Nrg1 mutant mouse model

Support for peripheral immune system alterations in schizophrenia patients are demonstrated in several epidemiological studies. These show that schizophrenia patients have altered incidence of certain types of cancer, rheumatoid arthritis and type II diabetes, illnesses related to a dysfunction of the inflammatory response (Ajdacic-Gross et al., 2014) and reviewed in (Catts et al., 2008; Leucht et al., 2007). Indeed, current treatments employed for metastatic melanoma include high-dose administration of recombinant cytokines such as IL-2, confirming the importance of a role for cytokines and the immune system in treating cancer (Aziz et al., 2009; Dutcher, 2002). IL-2 is also considered as a either mono- or adjunct therapy in a range of other solid tumours (Grande et al., 2006). Use of recombinant IL-2 has been linked to transient depression, hallucinations and delusions in cancer patients (Denicoff et al., 1987; McAllister et al., 1995). Further, mice treated with cytokines such as IL-2, IL-1β and IFNγ have demonstrated behavioural changes consistent with murine models of schizophrenia, including increased anxiety, anhedonia, cognitive deficits and hyper locomotion (Myint et al., 2009). These data raise the possibility of a common mechanism in the

pathophysiology of schizophrenia and an altered immune system, resulting in reduced incidences of immune mediated diseases, such as melanoma.

The C57Bl/6-specific B16F0 melanoma cells are a well-established tool to study melanoma growth and its effects on the immune system in mice (Overwijk and Restifo, 2000). Using these melanoma cells as an immune challenge in the Nrg1 mutant mouse model for schizophrenia will allow characterisation of differences in changes in cytokine levels following an immune stimulus based on the schizophrenia-relevant genotype compared to controls.

1.2.7 Brain regions: the prefrontal cortex and the hippocampus

Post-mortem studies from schizophrenia patients have consistently revealed structural and functional alterations in different brain regions, with particular focus on the prefrontal cortex (PFC) and the hippocampus (Harrison, 2000). Normal functioning of the PFC includes cognitive planning and working memory, functions that are disrupted in schizophrenia (Conklin et al., 2005). Neuroimaging studies have confirmed decreased activation of, and reduced blood flow to, the PFC in patients performing a specific working memory task (Carter et al., 1998). In addition, patients display a reduction in grey matter volume, confirming structural anomalies in the PFC (Breier et al., 1992; Harms et al., 2010). Additionally, cognitive functioning, learning and memory are normal functions of the hippocampus, which are disordered in schizophrenia patients. The anomalies found in the hippocampus in schizophrenia have been the subject of a number of detailed reviews (Harrison, 2004; Preston et al., 2005; Tseng et al., 2009). In brief, the hippocampus displays reduced volume, altered synaptic connections and neurotransmitter receptor changes in schizophrenia (Harrison, 2004). Impairments in declarative and recognition memory combined with reduced hippocampal activation

have also been demonstrated (Preston et al., 2005). Neonatal hippocampal lesions in mice result in adolescent neurochemical changes and behavioural changes relevant to schizophrenia, including deficits in social interaction and hyper locomotion, paired with disrupted neurotransmission (Tseng et al., 2009). Based on these findings, brain based investigations in the present studies focused on the PFC and hippocampus in Nrg1 Het animals and controls.

1.3 Hypothesis and significance of the study

It has been noted by Tsuang (2000) that a genotype x environment interaction can be considered from two viewpoints: environmental factors may influence gene expression; or the genetic phenotype may result in altered sensitivity to environmental factors. We hypothesized that an immune system challenge, in the form of melanoma, would result in a different immune response both in the periphery and in the brain in Nrg1 Het mice when compared with wild type-like (WT) litter mates suggesting that the genetic phenotype will alter sensitivity to the immune stimulus.

Two main questions were formulated in order to investigate this hypothesis:

1) Does a Nrg1 mutant mouse model demonstrate an altered peripheral immune response, specifically in the cytokine profile (Chapter 2)?

2) Are the peripheral cytokine alterations reflected in the brain (Chapters 3 and 4)?

A further exploratory question was investigated to gain a preliminary understanding of possible signalling pathway involvement in the differences discovered.

3) Are there any changes evident in gene expression in the signalling pathways common to Nrg1 and the altered cytokines found in the brain (Chapter 5)?

With the existing literature demonstrating a clear role for a genetic phenotype in the aetiology of schizophrenia and clear evidence for cytokine alterations in schizophrenia patients, it is remarkable that studies on whether such genetic mutations result in changes of the immune response remain limited. Despite recent increases in the number of publications on these topics, whether such changes are in fact interdependent is an important gap in psychopathological research that needs to be addressed to potentially allow the development of new approaches for novel prevention and treatment strategies.

A demonstration of neuroimmune interactions between the candidate schizophrenia vulnerability gene Neuregulin 1, and peripheral and central cytokine levels: focus on IL-6 and G-CSF

CHAPTER TWO

Tumour growth and peripheral immunology

Peta Snikeris
University of Wollongong

2.1 Tumour growth and peripheral immunology

Previous epidemiological studies have demonstrated a reduction in the lifetime incidence of melanoma in schizophrenia patients (Catts et al., 2008; Goldacre et al., 2005; Mortensen, 1994). Generally, the progression of melanoma is considered to result from the inability of the immune system to either recognise or mediate clearance of the tumour cells (von Boehmer, 2005; Goldsby et al., 2002), with current treatments including administration of recombinant cytokines (see 1.2.6). Whereas the effect of melanoma cells on the immune system is well described (Fan et al., 2009; Fang et al., 2008; Hensley et al., 1998; Kakinuma and Hwang, 2006; Li et al., 2009a; Overwijk and Schluns, 2009; Wang et al., 2009a) little is known about the effect of melanoma on cytokine levels in schizophrenia patients or those with a Nrg1 gene mutation.

2.1.1 Neuregulin 1 in tumour development and the peripheral immune system

The Nrg1 gene encodes six proteins with different signalling properties using the ErbB receptors for signalling (see 1.2.3). While Nrg1 and the ErbB receptors are shown to have an important role in nervous system development, they have also been implicated in peripheral tumour development. Both Nrg1 and the ErbB receptors have shown relevance as predictive indicators for some forms of cancer and have been investigated as possible biomarkers and potential therapeutics (Saxena and Dwivedi, 2012; Seshacharyulu et al., 2012; Yarden and Sliwkowski, 2001a). Nrg1 has been specifically implicated in the formation of new blood vessels in tumour tissue, a process tightly regulated under normal conditions but dysregulated during solid tumour growth and metastasis (Hanahan and Folkman, 1996; Nakano et al., 2004). This is in line with the critical role Nrg1 plays in cardiac development and function (reviewed in Wadugu and Kühn, 2012) demonstrated in the Nrg1 homozygous transmembrane domain knockout

mice, which display embryonically lethal cardiac insufficiencies (Stefansson et al., 2002). The main focus of these peripheral cancer studies, however, has been on the role of Nrg1 in the cancer tissue itself. Little is known about the potential for a broader Nrg1 role in the overall immune response.

Very little was found in the literature on the direct effect of Nrg1 on the peripheral immune system. Two studies on pain modulation in the spinal cord and nerve root propose Nrg1 as a cytokine (Lacroix-Fralish, 2008; LaCroix-Fralish et al., 2008). However these studies are not directly relevant to the potential role for Nrg1 in the peripheral response and the justification for the use of the term cytokine in relation to Nrg1 in these experiments is minimal. Of the limited studies indicating a peripheral immune impact resulting from a Nrg1 mutation, Desbonnet et al (2012) demonstrated immune changes in the Nrg1 Het mice following a chronic stress paradigm. Chronic stress has been previously shown to release a complex milieu of cytokines. The main interaction Desbonnet et al (2012) found between Nrg1 and chronic stress was an increase in IFNγ which is considered a T_H1 type cytokine, involved in the T cell mediated immune activation, suggesting the Nrg1 mutation has more potential to result in an altered cell mediated immune response.

These findings demonstrate that an interaction between a Nrg1 mutation and the peripheral cytokine response is highly likely to exist, and that this interaction may result in a skewed ratio of T_H1: T_H2 cytokine production. Understanding the peripheral cytokine response to a direct immune stimulus in Nrg1 Het mice will provide insight into the balance of cytokines produced and further our understanding of any potential imbalance in the polarized immune response in schizophrenia patients as discussed by Müller et al (2009).

2.1.2 Inducing and measuring the peripheral response

The aim of the following study was therefore to determine whether the Nrg1 Het mice demonstrated an altered peripheral immune response. The peripheral response was measured by investigating the basic tumour parameters, circulating immune cells and cytokines in response to the stimulus.

Nrg1 Het mice have been used as a model to study characteristics similar to individuals at genetic risk for the development of schizophrenia (Karl et al., 2007) as previously discussed (1.2.3c). In particular Nrg1 Het mice have been used as a vulnerability model; allowing exploration of the genetic risk combined with environmental risk factors (Boucher et al., 2007a; Karl et al., 2011; Long et al., 2012, 2013). In this study an immune stimulus was combined with the genetic risk to investigate peripheral responses.

Considering the epidemiological data demonstrating alteration in cancer incidence in schizophrenia patients (reviewed in Catts et al., 2008; Leucht et al., 2007), the B16F0 melanoma was chosen to model an immune stimulus (1.2.6). In brief, B16F0 tumours have been demonstrated to induce a low level innate and systemic immune response in normal mice and have been used previously to investigate tumour immune responses. In the present project the Nrg1 Het mice were challenged with a sufficiently immunologically compatible (syngeneic) melanoma so as to avoid complete tumour rejection while inducing the necessary immune response (Fan et al., 2009; Fang et al., 2008; Hensley et al., 1998; Kakinuma and Hwang, 2006; Li et al., 2009a; Overwijk and Schluns, 2009; Wang et al., 2009a). This provided a baseline for measurement of potentially altered responses in the Nrg1 Het mice, including circulating immune cells

from both the innate and systemic immune compartments and cytokine levels following tumour induction.

The use of the syngeneic B16F0 melanoma model not only provided the opportunity to measure the innate and adaptive immune responses to such a stimulus, but also to determine if the Nrg1 Het transmembrane mutation provided any inherent improvement or detriment in immune surveillance, suppression or progression. Therefore tumour growth was monitored and the size of the resulting solid tumour was measured after excision. In addition, the basic markers of angiogenesis and infiltration markers for neutrophils and macrophages were measured in the excised tumour. Since Nrg1 has been shown to increase the expression of vascular endothelial growth factor (VEGF) and measures of vascular formation (angiogenesis) can be used as a measurement of tumour aggressiveness, VEGF expression in the tumours was measured. Significant inflammation has also been observed at tumour sites, with infiltration by innate immune cells a hallmark of this inflammation (Hanahan and Folkman, 1996; Nakano et al., 2004; Xiong et al., 2001). Neutrophils are one of the first cell types recruited to the tumour microenvironment, with macrophages shown to be the most abundant over time. A strong infiltration by these two cells types has been correlated with tumour growth inhibition, although both cell types have also been implicated in promoting tumour growth and angiogenesis in some circumstances, therefore expression of markers for both cell types was measured in the tumours (Bröcker et al., 1988; Fridlender and Albelda, 2012; Navarini-Meury and Conrad, 2009; van Ravenswaay Claasen et al., 1992; Torisu et al., 2000).

Schizophrenia patients have variously demonstrated increases, decreases and no change in each of the main immune cell types although the results have been conflicting over

time (reviewed in Rothermundt et al., 2001). Immune cells derive from either the myeloid or lymphoid lineage. The two main myeloid derived cell types are the monocytes and the granulocytes, with neutrophils the most abundant of the circulating granulocytes. B cells and T cells are produced by the lymphoid precursors with additional cell subsets developing depending on location and activation status. T cells further develop into a number of additional cell types; the most well described being the T_H and T_C cells (Kondo et al., 2003; Laiosa et al., 2006). We determined the levels of each of macrophages, neutrophils, B cells and T_H and T_C cells as a basic measure of systemic immune activation; however detailed investigation into the activation status or functional subtypes were beyond the scope of this project.

Similar to the immune cell types, cytokines have also demonstrated a variety of changes in schizophrenia (see 1.2.4e) with several changes demonstrated to be consistent across meta-analyses (Miller et al., 2011; Potvin et al., 2008). Despite the findings in the Potvin et al (2008) and Miller et al (2011) meta-analyses that indicate IL-1β, IL-1RA, IL-2, sIL-2R, IL-6, IL-12, TGF-β, TNFα and IFNγ have been consistently found altered in patients, a debate remains as to whether there are other cytokines that will yet prove to be consistently changed and biologically relevant, thus a wide panel of cytokines was investigated.

2.1.3 Summary

With peripheral immune system changes associated with schizophrenia along with links to Nrg1 as a schizophrenia vulnerability gene and no evidence to date of direct Nrg1 involvement in the peripheral immune system, this study aimed to determine if the combination of gene and immune stimulus presented an aberrant peripheral immune response. As melanoma has been negatively associated with schizophrenia and the

B16F0 cell line demonstrated to induce an immune response in the mouse strain used, this was used as the immune stimulus. In order to determine if the peripheral immune response was perturbed several markers were investigated, this included: tumour size, angiogenesis and possible infiltration of macrophages and neutrophils into the tumours were measured; circulating levels of the major immune cell compartments of both the innate and adaptive immune response, as well as plasma cytokine levels.

2.2 Methods

2.2.1 Animal housing and breeding

All animals were housed in the animal facilities of the University of Wollongong under standard temperature controlled (20°C ± 2) conditions consisting of a 12:12 hour light-dark cycle with lights on at 0700 hrs. Animals had *ad libitum* access to standard lab chow and water. Breeding was performed by mating one WT female C57Bl/6 mouse with one Nrg1 Het male mouse. Thereafter, animals were separated, with each female assigned to a white plastic cage (50 x 20 cm) provided with additional nesting material. The day of birth was defined as post-natal day 0. The generation of the Nrg1 Het mice (target allele: Nrg1tm2Zhou) has been described previously (Stefansson et al., 2002).

Male offspring were separated from their dams on post-natal day 28. Animals were housed under standard conditions with two mice of corresponding genotype per cage matched by weight. Standard cage enrichment was provided (including sawdust, bedding material and a PVC tube). Experimental animals were 8-9 week old, male Nrg1 Het and WT control littermates. Female offspring were transferred to other experimental protocols. Euthanasia of animals was performed using the CO_2 slow infusion method.

All experiments were approved by and performed in accordance with the guidelines of the Animal Ethics Committee of the University of Wollongong (Breeding protocol AE10/03, experimental protocol AE09/17). Procedures complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, which conforms to International Guiding Principles for Biomedical Research Involving Animals. All efforts were made to minimise animal numbers and suffering.

2.2.2 Genotyping and weaning

Mice were ear marked for individual identification and genotyping with the ear tissue obtained during the ear marking process used for genotyping at the age of 3 weeks. The genotyping was performed using a commercially available genotyping kit (REDExtract-N-Amp PCR kit, Sigma, St. Louis, MO, USA) followed by a PCR which allowed for the simultaneous detection of the wild type and/or mutated allele. In brief, a pre-mixed preparation of Extraction Solution and Tissue Preparation Solution (4:1) was added to each tissue sample, vortexed and incubated for 10min at RT. Tissue was then incubated at 95°C for 3min before addition of Neutralization Solution B and maintained at 4°C until use. PCR amplification was conducted per manufacturers' instructions by adding 16µl of master mix (10µl of REDExtract-N-Amp PCR reaction mix, 1µl each of forward and reverse primers (8µM) (Table 2.2-1) plus 5µl RNase/DNase free H_2O per sample) to 4µl tissue extract in thin-walled PCR tubes. DNA was amplified using an Eppendorf, Mastercycler Pro S thermo cycler.

Amplified DNA was loaded directly onto a 1% agarose gel with SYBR green and run for 30min at 95V with a DNA ladder (DirectLoad PCR 100bp Low Ladder, Sigma) as well as Nrg1 mutation positive and negative controls. DNA was visualised with UV light on a GelDoc 212 Pro using Molecular Imaging Standard Edition (v5.0.2.30) software (Carestream, Rochester, NY, USA).

Table 2.2-1 Primer sequences used for Genotyping

Target	Sequence
Nrg1 WT Trinity	F: GGG CGC CCT GTT CCA CCA CA
	R:CCT CCG CTT CTG GAA AGG GGT GA
Nrg1 Neo173	F: ATG AAC TGC AGG ACG AGG CA
	R:GCC ACA GTC GAT GAA TCC AG

Primers supplied by Invitrogen, Carlsbad, CA, USA

2.2.3 B16F0 melanoma model

An *in vivo* murine melanoma model was used. Established from a spontaneously occurring C57Bl/6 melanoma, the B16F0 murine melanoma cells are a well-established *in vivo* model for challenging the immune system (Nakamura et al., 2002; Nicolson et al., 1978; Overwijk and Restifo, 2000). The B16F0 cell line were a kind gift from Dr Andrew Katsifis (ANSTO, Sydney, NSW, AU) and were originally purchased from the American Type Culture Collection (ATCC). All chemicals and reagents for B16F0 cell culture were obtained from Invitrogen (Carlsbad, CA, USA) unless otherwise stated. The B16F0 adherent melanoma cell line was cultured *in vitro* using RPMI 1640 medium (Sigma) supplemented with 10% Foetal Calf Serum and 200µM L-glutamine/ penicillin/ streptomycin in a 37°C, 5% CO_2 incubator. Culture medium was replaced every three days and cells passaged using trypsin (0.05%) at 70-80% confluent to a 1:10 dilution. Cells were harvested (passage 7-9) for injection using trypsin (0.05%) and resuspended in sterile dPBS (without Ca^{2+} and Mg^{2+}). In brief, excess media was removed, cells were rinsed with 2ml trypsin, followed by incubation in fresh 2ml trypsin at 37°C, 5% CO_2 until all cells had detached from the flask. 25ml supplemented media (passaging) or dPBS (injections) was added to dilute trypsin and the entire contents of the flask transferred to a sterile 50ml conical tube. After 5min centrifugation at 500 x g, supernatant was removed and pellet resuspended in 2ml supplement media (passaging) or dPBS (injections). For passaging, the resuspended pellet was then diluted 1:10 in sterile cell culture flasks. For injections, a small aliquot was removed for cell counting, using trypan blue, a haemocytometer and a light microscope (40x magnification). The final pellet was resuspended at $3x10^6$ cells per 1ml of PBS ($3x10^5$ cells per 0.1ml injection). Cells were maintained in a sterile tube on ice during

injections and mixed to ensure cells were adequately dispersed before charging each syringe.

Cells were injected subcutaneously into the shaved left flank of animals at 8 weeks of age ($3x10^5$ cells per 0.1ml injection per mouse) using a sterile insulin syringe (0.5ml) charged with 0.1ml of cell suspension solution. A total of 8 Nrg1 Het and 12 WT animals received tumour injections. Tumours were not visible until day 8 post injection at the earliest (where the day of injection = day 0). Mice were euthanized by CO_2 on day 9 post melanoma injection. One WT mouse did not display a tumour when euthanized and was excluded from all further analysis.

2.2.4 Tissue collection

a) Whole blood, plasma and spleen

Blood was sampled by cardiac puncture into 1.3ml micro tubes with EDTA (Sarstedt, Nümbrecht, Germany) supplemented with 10µl aprotinin (Sigma). Whole blood was gently mixed by inverting the tube and then placed on ice. For flow cytometry, whole blood was added to 10ml ice cold flow cytometry buffer and placed on ice (see 2.2.5a) for flow cytometry protocol following blood collection). To obtain plasma, samples were centrifuged for 10min at 4^{o}C at 18000 x *g*, a minimum of 30min after collection, Plasma samples were frozen and stored at -20^{o}C until further analysis.

The spleen was collected following cardiac puncture and snap frozen in liquid nitrogen before storage at -80^{o}C. Spleens were thawed before being weighed.

b) Tumour tissue

The volume of primary tumours was determined post excision using vernier callipers. Tumour size was measured by (a) longest length (mm) and (b) its perpendicular width (mm), and volume was reported as ½(length × width^2) (mm^3) (Kostourou et al., 2013; Li et al., 2004). Following excision tumours were snap frozen in liquid nitrogen before storage at -80^{o}C.

2.2.5 Cellular analysis

a) Flow cytometry

Following blood collection, whole blood was added to 10ml of ice cold flow cytometry buffer (Table 2.2-2) and placed on ice. Cells were centrifuged 5min, 300 x g at 4°C and supernatant discarded. The pellet was resuspended in 2.5ml red cell lysis buffer (Table 2.2-2) and incubated for 10min on ice. Cells were then washed twice by centrifugation at 300 x g for 5min at 4^{o}C, discarding supernatant and resuspending in 10ml flow cytometry buffer. Following final wash cells were resuspended in 1ml flow cytometry buffer. A cell count was conducted using trypan blue and a haemocytometer to determine viable cell number. Using a 96 well plate, 1,000,000 viable cells were distributed per well and appropriate antibody added. Cells were incubated for 30min in the dark, on ice using 0.5µg antibody per sample. The antibodies (Table 2.2-3) were added in the following staining combinations:

- Stain 1: F4/80, B220, Ly6G, CD3
- Stain 2: CD8, B220, CD3, CD4

Titrations were performed to confirm 0.5µg antibody per sample well was appropriate.

Cells were washed twice by centrifugation at 300 x *g* for 3min at 4°C, discarding supernatant and resuspending in 200µl flow cytometry buffer. Samples were then transferred to 1.2ml Micro Titer Tubes (QSP, Petaluma, CA, USA) before acquisition using BD LSRII (BD Biosciences, San Jose, CA, USA) running FACSDiva software (v3.3, BD Biosciences). FlowJo v7.2.5 software (TreeStar Inc., OR, USA) was used for analysis of results, gating details are provided below.

Table 2.2-2: Solutions and Buffers

Buffers/ Solutions	**Components**
Flow cytometry buffer	1 x PBS
	0.5% Bovine Serum Albumin (BSA)
	0.05 mM NaN3
Red blood cell lysis buffer	Final 1x concentration buffer is made up immediately before use and consists of 7 parts dH20, 2 parts Solution A, 0.5 part Solution B, and 0.5 part Solution C (v/v). Where solutions A, B and C are as described below:
Solution A	35g NH_4Cl
	1.85g KCl
	1.5g $Na_2HPO_4.H_2O$
	0.12g KH_2PO_4
	5g Glucose
	0.05g Phenol Red
	1 litre dH_2O
Solution B	2.1g $MgCl_2$
	0.7g $MgSO_4$
	1.7g $CaCl_2$
	500ml dH_2O
Solution C	11.25g $NaHCO_3$
	500ml dH_2O
	Solutions A, B and C were autoclaved and stored at 4°C

Table 2.2-3: Antibodies used for flow cytometry

Specificity	Label	Amount/ sample (µg)
Rat anti-mouse F4/80	Alexa 488	0.5
Rat anti-mouse CD45R/B220	PerCp Cy5.5	0.5
Rat anti-mouse Ly6G	PE-Cy7	0.5
Hamster anti-mouse CD3e	APC	0.5
Hamster anti-mouse CD3e	PE-Cy7	0.5
Rat anti-mouse CD8a	FITC	0.5
Rat anti-mouse CD4	APC-H7	0.5

All antibodies from: BD Biosciences, San Jose, CA, USA except F4/80 (Alexa 488) from: eBiosciences Inc.,

Total leukocytes were separated from cell debris using forward and side scatter plots. Individual cell types were then determined using selective gating. The circulating monocyte/ macrophage population is presented as a percentage of total leukocytes identified firstly using forward and side scatter to select for size and inclusions (to remove granulocyte populations) and then by anti-F4/80 positive, anti-Ly6G (Lymphocyte antigen 6 complex, locus G; also known as GR1) negative fluorescence (Campisano et al., 2013; Holt et al., 2008). Neutrophils are represented by anti-Ly6G positive cells also following forward and side scatter section for granulocyte populations (Buonocore et al., 2008; Campisano et al., 2013). Total T and B cells are represented as a percentage of total lymphocytes. The lymphocyte population is selected from the forward and side scatter plot with total B cells represented by the anti-B220 positive lymphocyte population (Allman and Pillai, 2008; Ellis et al., 2001). Total T cells are represented by the anti-CD3 positive population, a pan T cell marker, with total T cell population further delineated into anti-CD4 positive (T_H cells) and anti-CD8 positive (T_C cell) populations, presented as a percentage of total T cells (see for example Figure 2.2-1) (Aniansson Zdolsek et al., 1999).

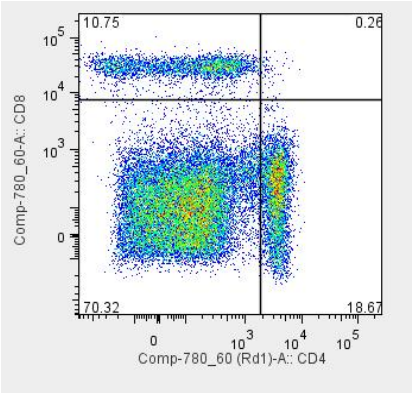

Figure 2.2-1 Example gating plot

A representative flow cytometry plot of total lymphocytes with anti-CD4 labelled (CD4+) cells on the X-axis and anti-CD8 labelled (CD8+) cells on the Y-axis after gating.

b) Immunohistochemistry

Frozen tumours were sectioned to 7µm thickness using a cryostat at -20°C and mounted on polylysine slides in series. Sections were stored at -80°C until staining.

For immunofluorescent staining, sections were fixed in -20°C acetone for 5min followed by 5min wash in PBS to remove all acetone and prevent crystallization. The PBS wash was then repeated for 2min. A PAP pen was used to draw around sections then the sections were incubated with IF block for 10min (Table 2.2-4). Sections were drained (not rinsed) before addition of primary antibody in IF blocking solution. The sections were incubated with primary antibodies in blocking solution at RT for 90min in a damp chamber. Slides were washed in TNT wash (Table 2.2-5), followed by 10mM Tris, pH 7.4. Sections were then incubated with secondary antibody in IF blocking solution for 30min at RT. The slides were washed in TNT wash buffer followed by 10mM Tris, pH 7.4 as before. Nuclei were counterstained with 1g/ml Hoechst 33342 (Sigma) for 1min and sections were covered with fluorescent mounting medium (Dako,

North America) and a coverslip with before being stored in the dark at 4°C until imaging.

Table 2.2-4 IF block

0.1M	Tris, pH 7.4	5ml of 1M
0.15M	NaCl	7.5ml of 1M
2%	Normal goat serum*	1ml
2%	normal horse serum*	1ml
Distilled water		35.5ml
Final Volume		50ml

*Hyclone, Logan, UT

Table 2.2-5 TNT wash

0.1M	Tris, pH 7.4	5ml of 1M
0.15M	NaCl	7.5ml of 1M
0.05%	Tween 20	25µl
Distilled water		35.5ml
Final Volume		50ml

Rat anti-mouse CD31 (PECAM-1; clone 390) antibody was used for detection of vasculature (2.5µg/ml; eBiosciences) followed by Alexa Fluor 594-conjugated goat anti-rat IgG (4µg/ml; Invitrogen, Carlsbad, CA) (Varney et al., 2005). For analysis of immunofluorescence, each field was imaged at 400x magnification by multichannel fluorescence microscopy for antibody staining of the cell surface marker CD31 and Hoechst 33342 nuclear stain. Images were acquired using the Nikon TE2000-E microscope (Nikon, Japan), running ImagePro-Plus software (MediaCybernetics, Rockville, MD, USA). The relative frequency of blood vessels was determined by averaging the number of positive staining vessels in 3-5 non-consecutive microscope fields from tumour sections in randomly selected Nrg1 (n=4) and WT (n=4) tumours.

2.2.6 Protein analysis

a) Luminex

Luminex is a multiplex assay which allows several analytes to be measured simultaneously in small sample volumes. This assay has shown good correlation with the more traditional ELISA assay method for cytokine measurement (see (Elshal and McCoy, 2006) for a detailed comparison). Plasma samples were analysed in duplex using a pre-mixed 32-plex mouse cytokine/chemokine 96 well plate assay kit (MILLIPLEX®Map, Millipore, Billerica, MA, USA) This kit contained the cytokines: IL-6, G-CSF, IL-12p40, IL-5, IL-13, platelet-derived growth factor-inducible protein KC (KC), monocyte chemoattractant protein-1 (MCP-1), eosinophil chemotactic protein (Eotaxin), IL-1α, interferon-gamma-inducible protein 10 (IP-10), leukaemia inhibitory factor (LIF), Cytokine LIX (LIX), monokine induced by interferon-gamma (MIG), regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein 1-alpha (MIP-1α), macrophage inflammatory protein 1-beta (MIP-1β), macrophage-colony stimulating factor (M-CSF), IL-12p70, VEGF, TNFα, granulocyte macrophage-colony stimulating factor (GM-CSF), IFNγ, IL-1β, IL-2, IL-3, IL-4, IL-7, IL-10, IL-15, IL-17 and macrophage inflammatory protein 2 (MIP-2) and was performed in accordance with the manufacturers' protocol. Note that KC, MIP-2 and LIX are murine functional homologues of human IL-8 (Hol et al., 2010). Undiluted plasma samples were used, as optimisation steps conducted prior to experimentation showed the best results were achieved without pre-dilution (data not shown). In brief, wash buffer was used to pre-wet the filters in a 96 well assay plate by 10min incubation with agitation at RT before removal of buffer. All removal of reagents was performed by vacuum. All vacuum steps were followed by blotting the bottom of

the plate to remove excess reagents. All preparation steps were completed at RT. Standards, controls and samples were loaded into 96 well plates, with samples randomly allocated to ensure even distribution of groups across the plate. Matrix solution was then added to standard and control wells followed by the addition of assay buffer and pre-mixed cytokine assay beads to all wells per manufacturer's instruction (Millipore, Billerica, MA, USA). All wells contained sample (or standard/ control) at the ratio of 1 part sample to 2 parts reagents. The plate was sealed and incubated overnight at 4°C with agitation. The following day, reagents were removed and wells washed twice with wash buffer. Detection antibodies were added into each well and incubated in sealed plate for 1 hour with agitation at RT. Following incubation, Streptavidin-PE was added to the detection antibodies and incubated for 30min, with agitation, at RT. All reagents were then removed by vacuum followed by two washes and final resuspension in 150µl of Luminex xMap sheath fluid (Luminex Corporation, Austin, TX, USA) by 5min agitation at RT on plate shaker. The plate was run on a Luminex 100™ System using xPONENT® 3.1 Software (Luminex Corporation). Data output was analysed using MasterPlex® QT: Multiplex Quantitative Analysis Curve-Fitting Software (Hitachi Solutions America Ltd, South San Francisco, CA, USA).

2.2.7 Tumour gene expression analysis

a) RNA extraction

RNA extraction was performed using the QIAshredder to homogenise tumour tissue and the RNeasy Plus Mini Kit to isolate mRNA (Qiagen, Valencia, CA, USA). The kits were used as per manufacturer's instructions. In brief, up to 700µl of lysate was add to each QIAshredder spin column, placed into an RNase free collection tube and centrifuged a maximum micro centrifuge speed for 2min. As the lysate passes through

the column it is homogenised. Extraction of mRNA was performed as a series of buffer additions and column centrifugation exactly per manufacturer's instructions, including a step to eliminate genomic DNA. Extracted RNA was eluted into 30-50µl of RNase free water. RNA concentration and quality was quantified using the NanoDrop (Thermo Scientific, NanoDrop 2000c) and accepted for use with: A260/280 ratio > 1.8 and A230/260 ratio > 2.0. Additional purification of extracted RNA was performed where required using ethanol precipitation as follows. Cold isopropanol was added to the RNA solution in equal volumes. Then 1/10 volume ammonium acetate (7.5M, pH 5.2, adjusted using glacial acetic acid) was added before vortexing and incubation at -20°C for 1 hr. Samples were centrifuged for 10min (12,000 x g, 4°C) and the supernatant discarded. Pellet was washed in 800µl cold ethanol (70%) and dislodged by inverting tubes 5 times followed by centrifugation for 5min (14,000 x g, 4°C). The wash steps were then repeated and supernatant discarded. An additional centrifugation step was performed (5min, 14,000 x g, 4°C) followed by removal of supernatant and RNA pellets were air dried. The dried pellet was resuspended in 20-30µl RNase free water before quantification on the NanoDrop as described above. A selection of 4 Nrg1 Het and 4 WT tumour samples was used. This selection was chosen based on the 4 best quality mRNA samples as determined by the NanoDrop A260/280 and A230/260 ratios.

b) cDNA synthesis

Taqman individual gene expression assays (Applied Biosystems) were used to measure relative mRNA levels in the B16F0 melanomas of Nrg1 mutant and WT mice. Following RNA extraction (2.2.7a), the QuantiTect Probe RT-PCR Kit (Qiagen) was used per manufacturers' instructions to synthesize cDNA and measure relative mRNA levels using a one-step RT-qPCR approach. In brief, an appropriate volume containing

57ng of isolated template RNA for each sample was added to 5µl 2x QuantiTect Probe RT master mix, 0.4µM primer (forward and reverse), 0.1µM probe and 0.1µl of QuantiTect RT mix per sample, with RNase-free water added to make a total sample reaction mix of 10µl. All preparation steps were completed on ice to avoid premature synthesis of cDNA.

Individual taqman primer/probes assays were purchased off the shelf from Applied Biosystems for: VEGF (marker of angiogenesis); CD68, CD163, and Arg1 (markers of macrophages) (Ellyard et al., 2010; Sullivan and Brekken, 2010; Varney et al., 2005); the cytokines G-CSF, IL-6 and normalised against Beta actin (Shi et al., 2011) as detailed in Table 2.2-6. The neutrophil marker Ly6G was custom made following the sequence in the publication of Sasmono et al (2007) with a forward primer of TGG ACT CTC ACA GAA GCA AAG and reverse primer GCA GAG GTC TTC CTT GCA ACA for a sequence product length of 128bp. The relevant probe was developed by Applied Biosystems to match the sequences above. All sequences for Ly6G (primers and probes) were checked using NCBI nucleotide blast data base with only 1 predicted result returned for the sequence used.

Table 2.2-6 Individual Taqman primer/probe assays

Gene Name	**Assay ID**
Vascular endothelial growth factor A	Mm01281449_m1
CD68 antigen	Mm03047340_m1
CD163 antigen	Mm00474091_m1
Arginase, liver	Mm00475988_m1
Colony stimulating factor 3 (granulocyte)	Mm00438334_m1
Interleukin 6	Mm00446190_m1
Actin, beta	Mm00607939_s1

Note: Applied Biosystems primer/probe sequences are commercial in confidence and sequences are not provided.

RT-qPCR was performed on a LightCycler 480 II (Roche) with the cycling conditions detailed in Table 2.2-7. Fluorescent data collection performed at the combined annealing/ extension step.

Table 2.2-7 Thermal cycling parameters: Taqman tumour tissue

Step	Temperature (°C)	Time (min:sec)	Cycles
Reverse Transcription	50	20:00	1
PCR initial heat activation	95	15:00	1
Denaturation	94	0:15	45
Combined Annealing/ extension	60	1:00	

Results are presented as fold change in expression levels (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) with untreated WT expression levels normalised to 1 ± SEM with other values presented as comparison fold change.

2.2.8 Statistics

After confirming normal distribution with the Kolmogorov-Smirnov test, body weight was analysed using a 2 (genotype) x 2 (immune challenge) x 2 (initial vs final) with repeated measures on initial vs final weight ANOVA, post-hoc paired samples t-tests were used to compare initial and final weight in each group. Tumour size was analysed using 2-tailed independent samples t-test. All other parameters were analysed using a 2-way ANOVA (factors: genotype x immune challenge), using 2-tailed independent samples t-tests to further explore group differences. Due to the exploratory nature of these studies, all p-values are presented as uncorrected p-values. Data are presented as mean ± SEM. Significance was set at $p<0.05$.

2.3 Results

2.3.1 Body weight and spleen mass unchanged in response to immune stimulus

All animals were weighed immediately prior to injection with B16F0 melanoma cells and again before sacrifice. A difference was seen between initial and final body weight within groups, ($F_{1,31}$=144.119, $p<0.001$), and paired sample t-tests confirmed body weight increased in all groups ($p<0.01$) with an average body weight gain of 9% overall. There were no other differences within or between groups in initial or final body weight with no effect of genotype, immune challenge or any interaction between the two (Table 2.3-1).

Spleens were removed and weighed; a 2-way ANOVA revealed no difference in spleen weight between groups as a result of genotype or treatment. In addition, there was no genotype x treatment interaction (Table 2.3-1).

Table 2.3-1: Mean body weight (g) and spleen weight (mg)

	Control		***Immune challenged***	
	WT	Nrg1 Het	WT	Nrg1 Het
Body Weight (g)				
Initial body weight	21.5 ± 0.3	21.3 ± 0.5	20.4 ± 0.4	21.0 ± 0.4
Final body weight	23.1 ± 0.3	23.3 ± 0.4	22.7 ± 0.4	23.0 ± 0.2
Body weight gain	1.6 ± 0.1	1.8 ± 0.2	2.3 ± 0.3	2.0 ± 0.2
Spleen Weight (mg)	64.5 ± 3.0	72.0 ± 4.6	69.2 ± 3.1	72.0 ± 3.6

Male Nrg1 Het mutant mice and WT littermates injected with subcutaneous B16F0 melanoma or saline vehicle.

The data represent mean ± SEM, IBW: initial body weight, FBW: final body weight, BWG: body weight gain, n=8-11 per group

2.3.2 Tumour growth, size and angiogenesis not influenced by genotype

Tumours for both Nrg1 Het and their WT littermates were visible on day 8 or 9 post injection. Figure 2.3-1 shows a representative tumour in-situ (A) (day 9 post injection) and following excision (B). There was no difference in tumour volume when measured with vernier callipers after excision with WT tumours 58.7± 27.9mm^3 and Nrg1 tumours 86.4± 30.0mm^3 ($p>0.05$, independent samples t-tests).

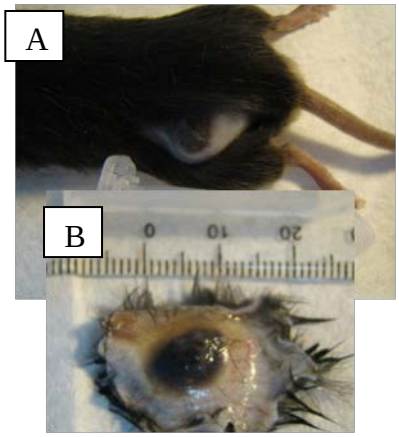

Figure 2.3-1: B16F0 Melanoma Growth.

Representative image of tumour growth in both Nrg1 Het and WT mice 9 days post injection (A) in-situ and (B) post excision. There was no difference in tumour size between genotypes.

The level of vascular infiltration into the B16F0 melanomas was measured in Nrg1 Het as compared to WT. Measurement was by immunofluorescent staining of CD31 to visualise vasculature and mRNA expression levels of VEGF. No difference was found in the number of vessels shown by immunofluorescence (Figure 2.3-2). In addition, there was no difference in the mRNA expression levels of the marker of angiogenesis (VEGF), in the B16F0 melanoma tissue following 9 days of *in vivo* tumour growth in Nrg1 Het and WT mice. VEGF mRNA expression in Nrg1 Het animal was 0.8 ±0.14 (mean fold change ± SEM, n=4 per group).

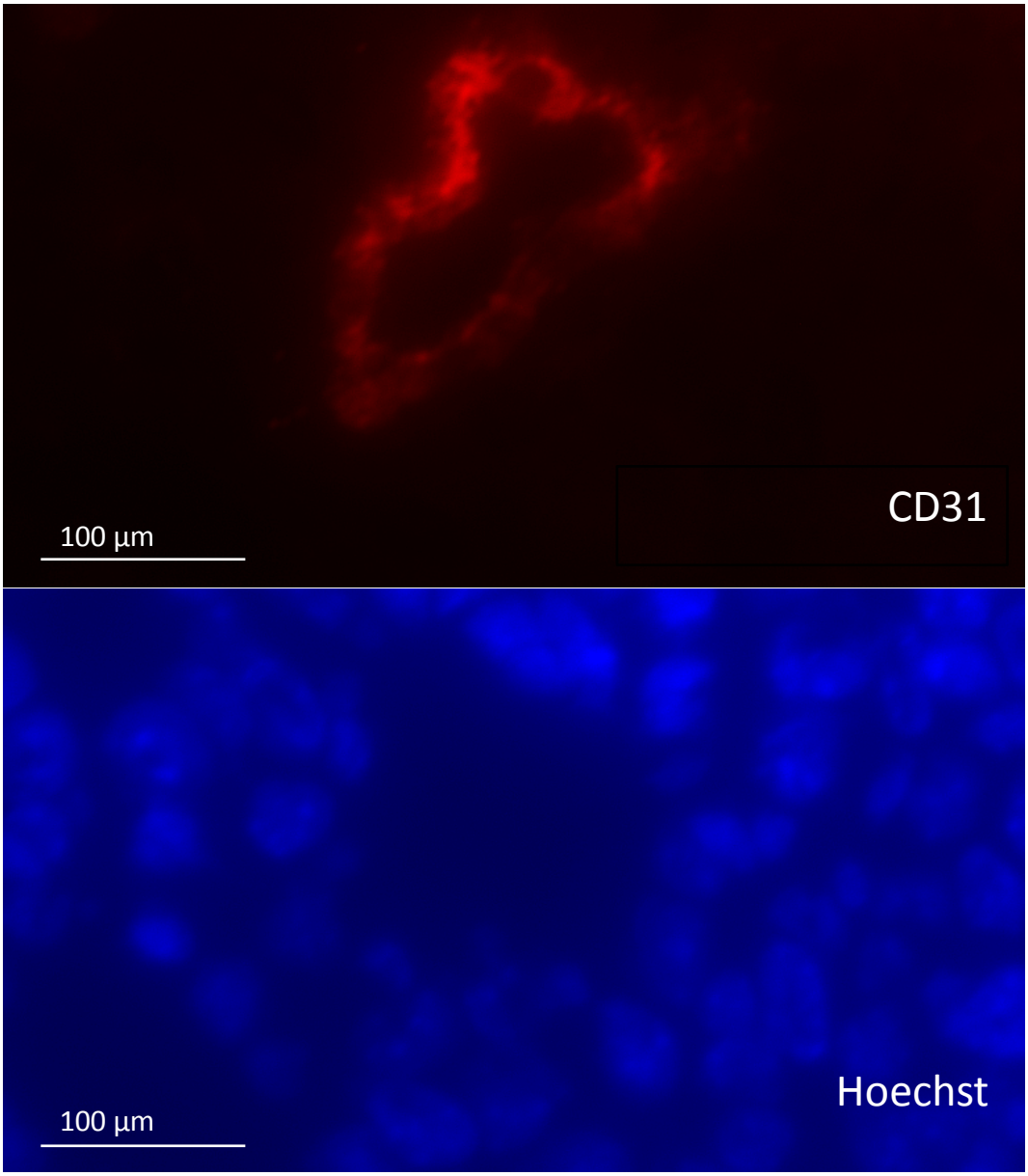

Figure 2.3-2: Representative B16F0 vasculature.

This image is representative of vascularisation in both Nrg1 Het and WT mice. Tumours were excised 9 days post injection (A) CD31 immunofluorescent staining for vascular endothelium and (B) Hoechst immunofluorescent staining for cell nuclei. There was no difference in the number of vessels visualised (n=4 per group).

2.3.3 Tumour macrophage and neutrophil population

The level of macrophage and neutrophil populations in the B16F0 melanomas was investigated by mRNA expression levels within the tumour tissue. The macrophage markers CD68, CD163 and Arg1 (Ellyard et al., 2010; Sullivan and Brekken, 2010; Varney et al., 2005) and the neutrophil marker Ly6G were measured (Sasmono et al., 2007). In addition, the mRNA expression level of the cytokines IL-6 and G-CSF (shown to have elevated protein levels in the plasma of Nrg1 Het animals subject to a melanoma, see Figure 2.3-3) were measured. There were no differences in the mRNA expression levels of CD68, CD163, Arg1, IL-6, Ly6G or G-CSF in Nrg1 tumours when compared to those from WT littermates (Table 2.3-2)

Table 2.3-2: mRNA expression levels for markers of macrophage and neutrophil infiltration in tumours

	Marker	**WT**	**Nrg1 Het**
Macrophages	CD68	1.00 ± 0.06	0.91 ± 0.07
	CD163	1.00 ± 0.48	0.88 ± 0.43
	Arg1	1.00 ± 0.25	0.69 ± 0.08
	IL-6	1.00 ± 0.49	1.04 ± 0.34
Neutrophils	Ly6G	1.00 ± 0.06	1.41 ± 0.23
	G-CSF	1.00 ± 0.23	1.07 ± 0.06

As measured in the B16F0 melanomas following 9 days of *in vivo* tumour growth in Nrg1 Het and WT mice.
The data represent mean fold change ± SEM, n=4 per group, except G-CSF where n=3 per group. WT expression levels normalised to 1 and fold change for Nrg1 Het calculated as a comparison.

2.3.4 Peripheral circulating immune cells unchanged in both Nrg1 Het and WT mice with or without the immune stimulus

Immune cells of the innate and adaptive immune response were measured in the peripheral blood. Monocyte/macrophages and neutrophils are presented as a percentage

of total leukocytes. Total T cells are presented as a percentage of total lymphocytes and CD4+ or CD8+ T cells are a percentage of total T cells. B cells are presented as a percentage of total lymphocytes (see 2.2.5a) for details of gating). No differences were found in any of the immune cell compartments measured using 2-way ANOVA (factors: genotype x immune challenge), additionally, no differences were shown with further analysis using 2-tailed independent samples t-test to compare groups ($p>0.05$) (Table 2.3-3).

Table 2.3-3: Peripheral circulating immune cells in male Nrg1 Het mice compared to WT littermates, with or without a B16F0 melanoma immune challenge

	Control				***Immune challenged***			
	WT		Nrg1 Het		WT		Nrg1 Het	
% Monocyte/Macrophage[a]	24.6	± 3.0	27.3	± 3.2	27.5	± 1.5	24.8	± 3.6
% Neutrophil[a]	14.6	± 3.7	7.1	± 2.1	17.1	± 8.0	21.2	± 9.7
% Total T cells[b]	29.6	± 1.3	31.9	± 2.1	32.7	± 4.4	30.7	± 2.1
% CD4+ T cells[c]	52.5	± 1.7	52.2	± 1.1	50.1	± 2.1	49.8	± 3.0
% CD8+ T cells[c]	39.7	± 1.8	39.6	± 1.4	40.9	± 3.2	38.3	± 0.8
% B cells[b]	32.1	± 4.7	35.4	± 3.5	31.9	± 1.7	32.7	± 4.1

The data represent mean % ± SEM, a) percentage of total leukocytes, b) percentage of total lymphocytes, c) percentage of total T cells, n=4 per group

2.3.5 Plasma cytokine profile alterations found based on genotype and/or immune stimulation

Thirty-two different cytokines were measured in the plasma to investigate the peripheral cytokine profile with or without the melanoma challenge. Twelve of the cytokines tested provided data while twenty were close to or below the detection limit using the Luminex multiplex assay (Table 2.3-4).

Table 2.3-4: Cytokine/ Chemokine 32-plex Luminex kit list

Cytokine/ Chemokine	Result
IL-6	Figure 2.3-3A
G-CSF	Figure 2.3-3B
IL-5	Figure 2.3-4
KC	Figure 2.3-4
MCP-1	Figure 2.3-5
IL-13	Figure 2.3-5
Eotaxin	Table 2.3-5
IL-1α	Table 2.3-5
IP-10	Table 2.3-5
LIX	Table 2.3-5
MIG	Table 2.3-5
M-CSF	Close to detection limit – data not shown
IL-12p40	Close to detection limit – data not shown
IL-12p70	Close to detection limit – data not shown
RANTES	Close to detection limit – data not shown
MIP-1β	Close to detection limit – data not shown
VEGF	Close to detection limit – data not shown
GM-CSF	Below the limit of detection
TNFα	Below the limit of detection
IFNγ	Below the limit of detection
IL-1β	Below the limit of detection
IL-2	Below the limit of detection
IL-3	Below the limit of detection
IL-4	Below the limit of detection
IL-7	Below the limit of detection
IL-9	Below the limit of detection
IL-10	Below the limit of detection
IL-15	Below the limit of detection
IL-17	Below the limit of detection
MIP-1α	Below the limit of detection
MIP-2	Below the limit of detection
LIF	Below the limit of detection

a) Interleukin 6 levels higher in the Nrg1 Het mice with a tumour load

Plasma IL-6 showed a genotype x treatment interaction ($F_{1,31}$=4.472, p=0.043, 2-way ANOVA) as well as a main effect of genotype ($F_{1,31}$=8.58, p=0.006, 2-way ANOVA) and treatment ($F_{1,31}$=16.96, p<0.001, 2-way ANOVA) (Figure 2.3-3 A). Unchallenged WT mice had plasma IL-6 levels of 2.6±1.4 pg/µl. Plasma levels of IL-6 were higher in Nrg1 Het mice (20.6±3.2 pg/µl) subject to the immune challenge compared to unchallenged Nrg1 Het (4.7±1.6 pg/µl, p=0.001, independent samples t-test) and challenged WT controls (7.8±2.8 pg/µl, p=0.008, independent samples t-test).

b) Granulocyte-colony stimulating factor higher in the Nrg1 Het mice with an immune stimulus

Plasma G-CSF demonstrated a treatment effect (: $F_{1,31}$=4.40, p=0.044, 2-way ANOVA) while the main effect of genotype and the genotype x treatment effect displayed a trend ($F_{1,31}$=3.71, p=0.06, $F_{1,31}$=3.103, p=0.088 respectively, 2-way ANOVA). Plasma G-CSF was higher in the Nrg1 Het immune challenged mice (659±78 pg/µl) when compared to challenged WT (371±86 pg/µl, p=0.029, independent samples t-test) and the Nrg1 Het unchallenged mice (357±84 pg/µl, p=0.020, independent samples t-test). WT unchallenged mice had a plasma G-CSF level of 344.6±38.8 pg/µl (Figure 2.3-3 B).

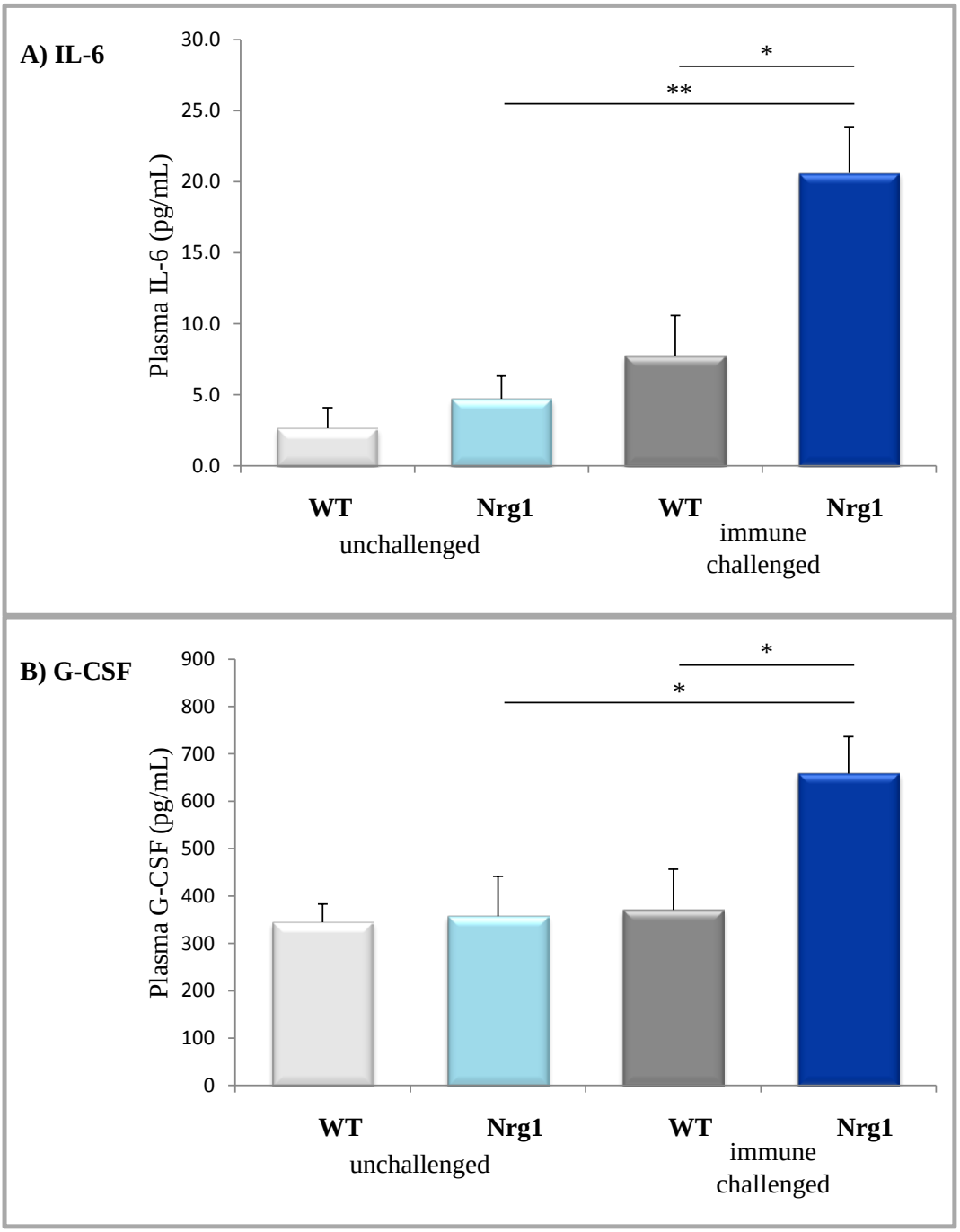

Figure 2.3-3: Plasma IL-6 and G-CSF levels in unchallenged and immune challenged Nrg1 Het mice compared to WT.

Immune challenged Nrg1 Het animals had higher levels of IL-6 and G-CSF when compared with Nrg1 Het unchallenged mice, as well as when compared with WT immune challenged animals. Plasma levels of **(A)** IL-6 and **(B)** G-CSF (pg/ml) measured using multiplex bead based Luminex assay in WT and Nrg1 Het mice injected subcutaneously with either B16F0 subcutaneous melanoma (3x105 cells in 0.1ml) or PBS control (0.1ml). Plasma was collected 9 days after melanoma injection, n = 8-11 animals per group (independent samples t-test, *$p<0.05$, **$p<0.01$).

c) Interleukin 5 and KC higher in WT animals with an immune challenge

IL-5 and KC (a murine homolog of human IL-8) plasma levels demonstrated a main effect of treatment (IL-5: $F_{1,31}$=6.10, p=0.019, KC: $F_{1,31}$=6.89, p=0.013, 2-way ANOVA). A higher level of both IL-5 and KC was seen in the WT immune challenged mice (IL-5: 16.4±1.8 pg/µl, KC: 218.3±40.9 pg/µl) compared to the WT unchallenged mice (IL-5: 9.4±2.3 pg/µl, KC: 96.9±17.3 pg/µl) (IL-5: p=0.026; KC: p=0.017, independent samples t-test; Figure 2.3-4). The Nrg1 Het unchallenged mice had 10.1±0.8 pg/µl plasma IL-5 and 133.4±58.9 pg/µl plasma KC while the Nrg1 Het immune challenged mice had 15.5±4.2 pg/µl plasma IL-5 and 241.0±44.5 pg/µl plasma KC, although no difference was determined using independent samples t-tests.

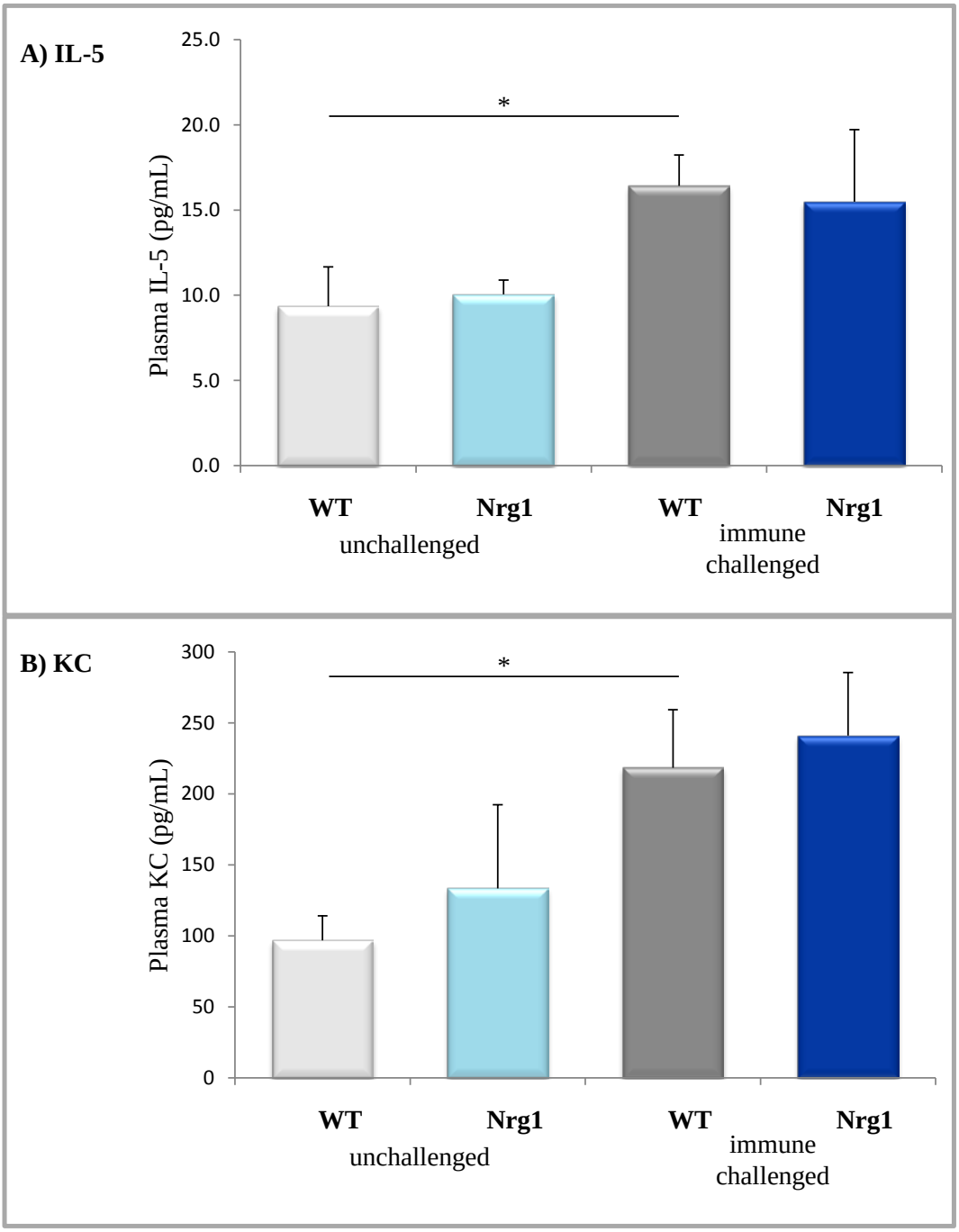

Figure 2.3-4: Plasma IL-5 and KC levels in unchallenged and immune challenged Nrg1 Het mice compared to WT.

The immune challenge increased average plasma levels of IL-5 and KC regardless of genotype (2-way ANOVA main effect of treatment, significance not shown on figure, refer to text 2.3.5c) Plasma IL-5 and KC levels were different in WT unchallenged and WT immune challenged mice. Plasma levels of **(A)** IL-5 and **(B)** KC (pg/ml) measured using multiplex bead based Luminex assay in WT and Nrg1 Het mice injected subcutaneously with either B16F0 subcutaneous melanoma ($3x10^5$ cells in 0.1ml) or PBS control (0.1ml). Plasma was collected 9 days after melanoma injection, n = 8-11 animals per group (independent samples t-test, $p<0.05$).

d) Interleukin 13 shows a genotype x treatment interaction and MCP-1 demonstrated a main effect of treatment

Further a genotype x treatment interaction was found in plasma IL-13 ($F_{1,31}$=4.779, p=0.036), and a main effect of treatment on plasma MCP-1 ($F_{1,31}$=4.21, p=0.049). Further analysis of IL-13 and MCP-1 showed no differences between the four experimental groups (independent samples t-test) (Figure 2.3-5). The WT unchallenged mice had 121.9±21.3 pg/µl plasma IL-13 and 26.8±8.8 pg/µl plasma MCP-1 while the WT immune challenged mice had 191.3±28.1 pg/µl plasma IL-13 and 60.0±15.9 pg/µl plasma MCP-1. The Nrg1 Het unchallenged mice had 203.4±47.2 pg/µl plasma IL-13 and 35.1±11.8 pg/µl plasma MCP-1 while the Nrg1 immune challenged mice had 135.3±21.7 pg/µl plasma IL-13 and 60.1±15.5 pg/µl plasma MCP-1. All additional independent samples t-tests to analyse these data between the 4 groups revealed no differences based on genotype alone when comparing cytokines in the Nrg1 Het and WT. Additionally, no differences were found in the plasma levels of the remaining detectable cytokines: Eotaxin, IL-1α, IP10, LIX, and MIG (Table 2.3-5).

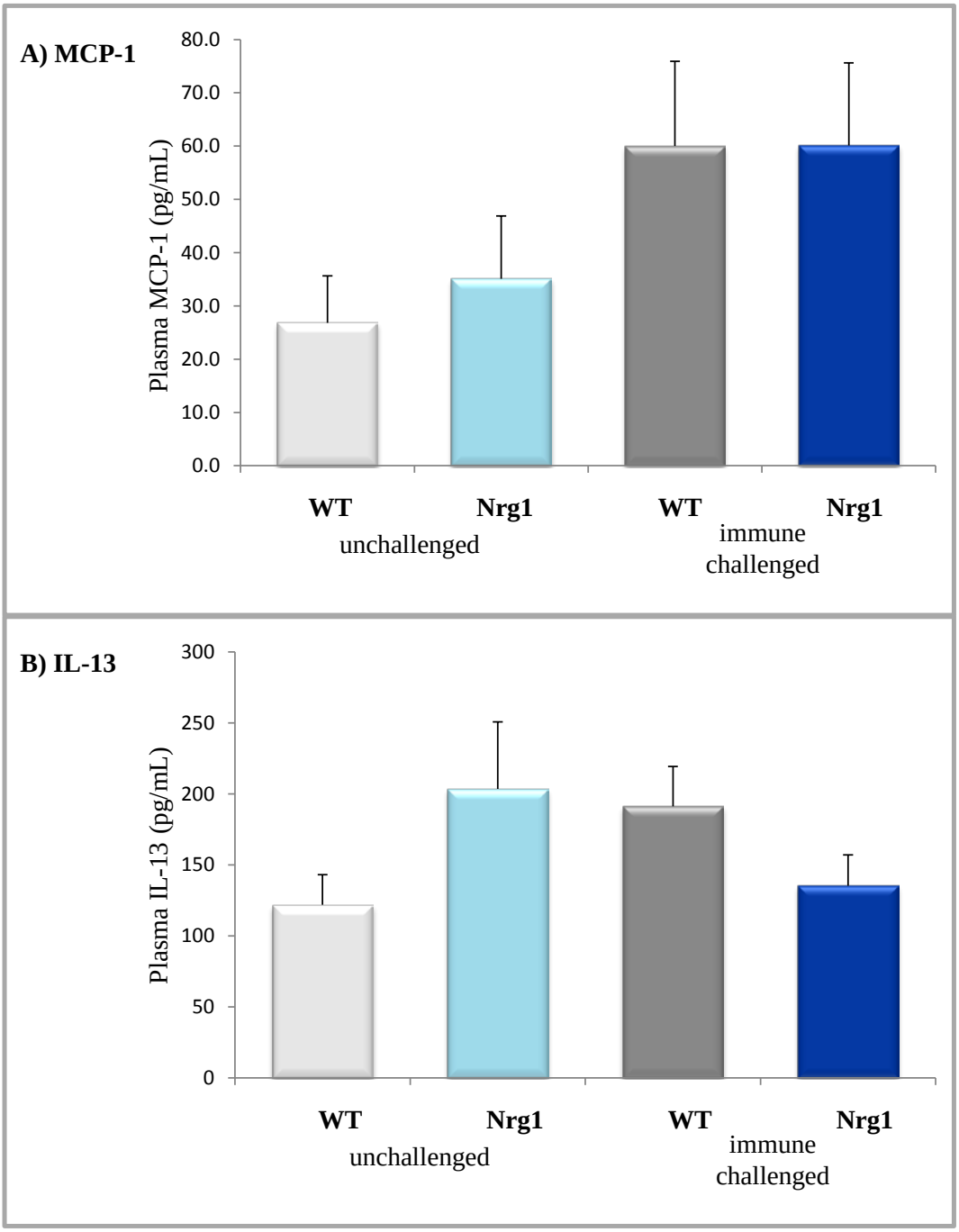

Figure 2.3-5: Plasma MCP-1 and IL-13 levels in unchallenged and immune challenged Nrg1 Het mice compared to WT.

No difference was found in plasma MCP-1 or IL-13 between experimental groups using independent samples t-tests, despite significant 2-way ANOVA findings. Plasma levels of **(A)** MCP-1 and **(B)** IL-13 (pg/ml) measured using multiplex bead based Luminex assay in WT and Nrg1 Het mice injected subcutaneously with either B16F0 subcutaneous melanoma ($3x10^5$ cells in 0.1ml) or PBS control (0.1ml). Plasma was collected 9 days after melanoma injection, n = 8-11 animals per group (independent samples t-test, $p>0.05$).

Table 2.3-5: Unchanged plasma cytokine levels (pg/ml) in male Nrg1 mutant mice and their WT littermates.

	Control		***Immune challenged***	
	WT	Nrg1 Het	WT	Nrg1 Het
Eotaxin	482.4 ± 38.3	486.9 ± 41.3	541.3 ± 59.4	525.0 ± 37.3
IL-1α	46.3 ± 3.8	53.3 ± 21.3	48.9 ± 8.9	109.7 ± 33.7
IP-10	23.5 ± 10.2	53.4 ± 17.1	56.0 ± 25.1	68.4 ± 21.1
LIX	954.9 ± 456.8	1202.4 ± 770.7	876.9 ± 211.7	979.1 ± 172.6
MIG	292.4 ± 48.9	302.5 ± 56.3	284.5 ± 43.9	267.8 ± 33.7

The data represent mean (pg/ml) ± SEM, n=8-11 per group

2.4 Discussion

This is the first experimental report to demonstrate a peripheral cytokine alteration in the Nrg1 Het mouse model in response to a direct immune challenge. It was previously shown that a Nrg1 Het mutation in mice resulted in altered cytokine production in response to stress (Desbonnet et al., 2012), however the peripheral immune response to a direct immune system stimulant was unknown. In response to the melanoma immune challenge we found differences in IL-6 and G-CSF levels in the Nrg1 Het mice.

2.4.1 Interleukin-6 levels were higher in WT immune challenged animals with further elevated levels in Nrg1 Het immune stimulated mice

The levels of circulating IL-6 seen in in the Nrg1 Het immune challenged mice were 4 times those in unchallenged Nrg1 Het mice. The difference in the IL-6 levels in Nrg1 Het mice subject to the immune stimulus was 3 times that shown in the challenged WT mice (Figure 2.3-3). The 2-way ANOVA results, demonstrating not only an interaction between genotype and immune challenge ($F_{1,31}$=4.472, p=0.043) but also a main effect of genotype ($F_{1,31}$=8.58, p=0.006), clearly show that the effect of the immune challenge was much larger in the Nrg1 mice as compared to the WT mice. Previous studies have shown higher circulating levels of IL-6 in melanoma patients (Hoejberg et al., 2012; Vereecken et al., 2012; Yurkovetsky et al., 2007), schizophrenia patients (Na and Kim, 2007; Schmitt et al., 2005; Zhang et al., 2002) and higher levels of IL-6 in families carrying a Nrg1 transmembrane domain mutation (Marballi et al., 2010). WT littermates also demonstrated apparent higher levels of IL-6 in the challenged animals compared to vehicle although this was not significant (Figure 2.3-3). With no difference in the Nrg1 Het IL-6 levels at baseline compared to unchallenged WT mice, the 3 fold higher levels of IL-6 in the Nrg1 Het challenged animal compared with WT challenged littermates

demonstrate for the first time that a direct peripheral immune stimulus on top of a Nrg1 mutant background produce a synergistic increase in IL-6 levels.

The higher levels of IL-6 found in this study are consistent with those of Desbonnet et al (2012) who showed both the Nrg1 mutation and a chronic stress paradigm increased IL-6 levels alone, with a more evident increase in IL-6 in the Nrg1 Het mice subjected to chronic stress using cultured spleen cells. Similar levels of plasma IL-6 in the unchallenged Nrg1 Het mice when compared to WT in this study appear inconsistent with Desbonnet et al (2012), where baseline spleen IL-6 levels were higher in control Nrg1 Het mice compared to WT. There are a number of possible explanations for this apparent inconsistency of findings. Firstly, the mice in the Desbonnet study were subject to a battery of behavioural tests before sacrifice, beside the chronic defeat paradigm. Such behavioural tests, although considered standard testing, increase stress levels in mice (Bailey and Crawley, 2009) and pre-conditioned stress has been shown to increase IL-6 (Cheng et al., 2015). Secondly, the mice in the Desbonnet study were older at sacrifice where age related increases in IL-6 have been seen in humans (Wei et al., 1992). With a number of possible explanations for this inconsistency between studies, the focus of the discussion is on the similar increase found following an immune stimulus.

Desbonnet et al (2012) further demonstrated that the cytokine response in Nrg1 Het animals was linked to the type of immune stimulus. The Nrg1 mutation had no additional effect on a lipopolysaccharide (LPS)-induced (B cell stimulus) cytokine release, including IL-6. These data suggest that a Nrg1 mutation does not inhibit the T_H2 cytokine response, which was enhanced by chronic stress but was not altered as a consequence of Nrg1 mutation in response to LPS. A T_H2 response promotes B cell

maturation and an antibody-based, humoral response directed against extracellular pathogens, therefore suggesting that a Nrg1 mutation would not inhibit the antibody mediated anti-tumour response. The levels of IL-6 however were increased in Nrg1 mice subject to chronic stress when subjected to Concanavalin A stimulation (T cell stimulus). Considering these data from Desbonnet el al (2012) and the current study findings demonstrating an interaction between Nrg1 mutation and a peripheral immune stimulus on IL-6 levels, a role for Nrg1 the peripheral cytokine response is evidenced.

The higher levels of IL-6 in Nrg1 Het challenged animals is also consistent with the findings of Marballi et al (2010) where a similar Nrg1 transmembrane domain mutation in humans results in peripheral immune alterations. Individuals from within 14 families carrying the Nrg1 mutation demonstrated higher plasma levels of IL-6 than unaffected individuals (Marballi et al., 2010). Further, an *in vitro* study on lymphoblastoid cell lines derived from first degree relatives of individuals diagnosed with psychosis, but having no signs of illness themselves, from these same 14 families, showed higher levels of IL-6 secretion. Messenger RNA analysis of the same cell lines showed that the expression of IL-6 was also increased (Marballi et al., 2010). These data are consistent with the findings of the current study, particularly when considering that each of these individuals could be expected to have undergone previous immune challenges, likely suffering from some childhood and other illnesses throughout development. However, this remains speculative with no information on medical background reported in the study. This further raises the possibility that the alteration of IL-6 in the Nrg1 mutant model may be a persistent state following one or more immune stimuli, as hypothesized by Patterson (2009).

IL-6 can act as a pro- or anti- inflammatory cytokine, displaying multiple functions including involvement in the type-2 immune response, haematopoiesis and acute-phase reactions. This allows for several interpretations of why the levels of IL-6 may be elevated in the Nrg1 Het stimulated animals. IL-6 is perhaps best characterized for its role in the maturation of B cells to antibody-producing cells (Hirano et al., 1986) yet no change in the percentage of circulating B cells in this study suggest elevated IL-6 is not affecting the B cell mediated immune response. However, although no change in B cell percentage was seen in the current study, a closer inspection of the maturation stage of B cells or measurement of anti-B16F0 antibody titers may elucidate differences. Alternatively, the anti-inflammatory properties of IL-6 are mediated through inhibitory effects on pro-inflammatory cytokines like reducing TNFα activity whilst simultaneously up-regulating anti-inflammatory proteins including the IL-1RA (Jones et al., 2001). In this study, both TNFα and IL-1RA were below the level of detection so determining if IL-6 is involved in an anti-inflammatory response requires further study.

The data presented in this study clearly demonstrate an alteration in the peripheral IL-6 response in Nrg1 Het mice that is consistent with existing literature. The possibility of these elevated IL-6 levels contributing to a polarised type-2 T helper cell response cannot be ruled out. While IL-6 is considered a type-2 cytokine (Müller et al., 2009), without detailed measurement of B cell activation and additional T_H2 cytokine levels below the limits of detection, there is not enough evidence to determine whether higher plasma IL-6 in immune stimulated Nrg1 Het mice is influencing a T_H2 type response. This also leaves the option of an anti-inflammatory role for these higher levels of IL-6. Nonetheless, the higher levels of IL-6 seen in immune stimulated Nrg1 Het animals, compared to both unchallenged Nrg1 Het and challenged WT mice, indicates a

dysregulation of IL-6 in the Nrg1 animals and the finding that a Nrg1 mutation results in elevated plasma IL-6 levels stands out as a robust finding.

2.4.2 Granulocyte-colony stimulating factor was elevated in Nrg1 Het immune challenged mice only

The higher level of G-CSF in Nrg1 Het mice with melanoma is a novel finding. G-CSF levels were 84% higher in Nrg1 Het immune challenged mice compared to Nrg1 Het unchallenged mice while no change was seen in challenged WT littermates compared to unchallenged WT controls (Figure 2.3-3). G-CSF stimulates differentiation and proliferation of the progenitor cells of neutrophilic granulocytes, producing mature neutrophils and stimulating release of mature neutrophils into circulation during inflammation (Nagata and Fukunaga, 1991). G-CSF is also capable of blocking apoptosis in cells of the myeloid lineage (monocytes and granulocytes) (Schneider et al., 2005a). For these reasons it has been investigated and used as a treatment to increase neutrophils in patients with neutropenia or following chemotherapy which has depleted these cells from the bone marrow (Fukunaga et al., 1991; Schneider et al., 2005b).

G-CSF has been shown increased in the serum of patients with melanoma (Yurkovetsky et al., 2007), however alterations in circulating levels of G-CSF have not previously been associated with schizophrenia patients. This cytokine is rarely reported in the schizophrenia literature excepting as a treatment for clozapine induced agranulocytosis. G-CSF plasma levels do not seem to correlate with patients who will develop clozapine induced agranulocytosis. Still, recombinant G-CSF is successfully used as an adjunct therapy with clozapine treatment in these patients (Pollmächer et al., 1997; Schuld et al., 2000). However, it remains unclear if G-CSF levels could be predictive of

agranulocytosis in patients and whether the lack of association with schizophrenia is because G-CSF is unchanged or because it has not been widely studied.

2.4.3 IL-6 and G-CSF may act together to influence the immune system

Concurrent increases in IL-6 and G-CSF have previously been demonstrated in tumour studies, including a B16F1 melanoma model (Bharadwaj et al., 2007; Yan et al., 2013). Yan et al (2013) found that IL-6 and G-CSF cooperated to inhibit neutrophil granule release in the B16F1 model, therefore increasing the pro-tumour function of this cell type. An additional study demonstrated that elevated levels of IL-6 and G-CSF, found in pancreatic cancer, were able to suppress monocyte derived dendritic cells, a common feature of this cancer type (Bharadwaj et al., 2007). Using a model of ischemia Gregory et al (2010) further demonstrated that IL-6 and G-CSF together affect monocyte signalling potential at the level of the bone marrow, resulting in impaired ability to re-vascularise following an ischemic event. Together these studies indicate that a concomitant increase in both IL-6 and G-CSF can have varied effects on multiple immune cell types, specifically those of the myeloid lineage. Therefore the higher levels of plasma IL-6 and G-CSF (Figure 2.3-3), seen in the Nrg1 Het mice subject a melanoma inoculation may be working together to alter the proliferation, maturation or release of cells of the myeloid linage primed for specific action in the periphery.

2.4.4 Immune stimulus resulted in elevated IL-5 and KC in WT mice subject to an immune stimulus

One measure of whether the melanoma model represents an adequate immune stimulus is in the changes found in the WT mice subject to the immune challenge. IL-5 and KC were 75% and 125% higher in the WT challenged animals compared to unchallenged

WT mice respectively, and although the Nrg1 Het animals displayed a similar trend (54% and 81%), this was not significantly different, most likely due to the high variability in levels between animals (Figure 2.3-4). These results, combined with the 2-way ANOVA showing a main effect of treatment (IL-5: $F_{1,31}$=6.10, p=0.019, KC: $F_{1,31}$=6.89, p=0.013) suggests that these changes are representative of a normal immune response. Consistent with these data, IL-5 and IL-8 have been shown increased in the plasma of alternate tumour types in the existing literature (Benoy et al., 2004; Ikutani et al., 2012; Lee et al., 2013; Pine et al., 2011), where IL-8 is the human homologue of KC (Hol et al., 2010). Additionally, MCP-1 and IL-13 revealed differences in the ANOVA analysis which were however, not confirmed as between group difference using independent samples t-tests. The large standard error of the mean shown in each group for these analytes has likely confounded the result and increased sample numbers or a more specific assay would be required to determine any true alterations in these cytokines. Notwithstanding these limitations, future studies may be indicated to determine if the genotype x treatment ANOVA result represents a physiologically relevant finding.

2.4.5 B16F0 melanoma demonstrated no differences based on genotype

With epidemiological studies demonstrating lower rates of melanoma in schizophrenia patients (Goldacre et al., 2005; Mortensen, 1994), it was interesting to note no difference in tumour size, angiogenesis markers or innate immune cell population within the tumour. In this experimental protocol we used an isograft model where immortalised cells from a spontaneously occurring tumour from the same genetic mouse strain were introduced into experimental animals in high numbers. This is not truly representative of how cancer establishes itself naturally in the body. Therefore, although

this result indicates that there is no difference in the immune response to a large inoculation of melanoma cells, it does not rule out the possibility of a Nrg1 mutation exerting an altered genetic influence on spontaneous cancer growth. It remains to be investigated if a less aggressive, slower growing tumour or a spontaneous tumour model may allow any subtle differences in tumour growth or the immune response within the tumour microenvironment to be elucidated over a longer time period which may be more representative of the native tumour growth environment. In addition, different techniques and a more detailed study of immune cell subtypes and locations within the tumour microenvironment may find differences. This was, however, beyond the scope of this study.

With no difference in circulating cytokine levels demonstrated between the Nrg1 Het and WT mice without an immune stimulus the Nrg1 Het mutation did not appear to result in any basal impact on the cytokine system in animals at this age. The melanoma challenge was expected to alter some cytokines in both the WT and Nrg1 Het mice as a mark of basic immune stimulation, although as a syngeneic tumour model, the magnitude of changes was not be expected to be large. Both IL-5 and KC were higher in the WT challenged animals, and although the Nrg1 Het challenged animals display a similar trend, this was not statistically different (Figure 2.3-4). This was consistent with the expectation that an immune challenge would have a basic impact on the immune system in both genotypes.

2.4.6 Circulating immune cells displayed no differences in the main cell type ratios

No difference was shown in body weight, spleen weight or the main cell types of the immune system between experimental groups. Suggesting that use of this type of

immune stimulus does not have a broader impact on the immune system, however given that the measurements were taken at a single time point and a detailed analysis of the immune cell subtypes and activation status; both circulating and in the tumour microenvironment, were beyond the scope of this project, such alterations cannot be discounted without further study. In addition, acute immune challenges, or direct stimulation of different immune response pathways may elicit different result.

2.4.7 Conclusion

This study has demonstrated that the combination of a genetic mutation in the Nrg1 gene and a peripheral immune stimulus in the form of a melanoma resulted in an altered cytokine response. This was seen in the elevated levels of IL-6 and G-CSF in the periphery of Nrg1 Het mice when inoculated with B16F0 melanoma cells. The combined elevation of these two cytokines is likely to result in an impact on cells of the myeloid linage despite the lack of findings in this model (2.4.3). More detailed study of monocyte/macrophage and neutrophil subsets in these mice would elucidate any relevant differences. The present results are consistent with the human study on a group of families with a similar Nrg1 mutation and a higher burden of psychosis diagnoses than the general population (Marballi et al., 2010). This suggests that the Nrg1 Het mice likely represent an appropriate model for further study into immune responses that may be linked to similar human Nrg1 mutations and potentially schizophrenia pathogenesis. The following chapters will focus on the response of IL-6, G-CSF and their respective signalling receptors in the brain of Nrg1 Het and WT mice with or without the B16F0 melanoma stimulus.

A demonstration of neuroimmune interactions between the candidate schizophrenia vulnerability gene Neuregulin 1, and peripheral and central cytokine levels: focus on IL-6 and G-CSF

CHAPTER THREE

Interleukin 6 in the brain

Peta Snikeris
University of Wollongong

3.1 Interleukin 6 in the brain

IL-6 has multiple roles in the peripheral immune system and is also involved in a range of brain functions, with production of the cytokine shown in both peripheral and central cell types. IL-6 has a well-established role in the acute phase hepatic response and induction of fever as well as differentiation and growth of haematopoietic stem cells, T cells and B cells (Rose-John et al., 2007). IL-6 is also shown to be important in the CNS beyond the induction of fever. This includes neurogenesis, synaptic plasticity and neurodegeneration; as relevant for emotionality, cognition and cognitive dysfunction (McAfoose and Baune, 2009; Yirmiya and Goshen, 2011). Primary production of IL-6 in the periphery comes from T cells and macrophages whereas central production is primarily from microglia (Norris and Benveniste, 1993; Sallmann et al., 2000; Straub, 2006; Van Wagoner et al., 1999). While Banks et al (1994) further demonstrated that IL-6 produced in the periphery can cross the blood-brain barrier (BBB) under basal conditions. The level of IL-6 in the brain is however, not totally reliant on microglia or peripheral production and BBB permeation. Neurons and astrocytes are also shown to produce IL-6 and express IL-6 receptors in several schizophrenia-relevant brain areas, including the PFC and hippocampus (Gadient and Otten, 1994; Jüttler et al., 2002; McAfoose and Baune, 2009; Ringheim et al., 1998; Straub, 2006). With elevated levels of plasma IL-6 found in the Nrg1 Het mice subjected to melanoma (Figure 2.3-3), the following study investigated whether a similar IL-6 alteration would be reflected in the brain.

3.1.1 IL-6 and the IL-6 signalling partners

IL-6 is an approximately 21-30kDa glycoprotein, which also exists in multimeric forms, most commonly dimers of 45-85kDa (Cayphas et al., 1987; Hirano et al., 1985; May et

al., 1991). IL-6 signalling is mediated by two receptor proteins: I) the ligand binding IL-6 receptor known as the IL-6 receptor alpha (IL-6Rα) chain, which has no intrinsic signalling capabilities; and II) the signal transduction molecule glycoprotein 130 (gp130), also known as the IL-6 signal transducer or the IL-6 receptor β chain. Expression of mRNA in the brain for both IL-6 and IL-6Rα have been demonstrated by *in-situ* in a regionally distinct manner, particularly evident in the hippocampus, hypothalamus, cortex and cerebellum (Gadient and Otten, 1994; Ringheim et al., 1998). The gp130 receptor is widely distributed in the CNS, with the distribution overlapping those areas reported to express IL-6 and the IL-6Rα (Vallières and Rivest, 1997).

a) Interleukin 6 receptor alpha

IL-6Rα is an 80kDa protein, found in both membrane bound (mIL-6Rα) and soluble (sIL-6Rα) form. The IL-6Rα does not activate intracellular signalling cascades in either form. The mIL-6Rα is mainly expressed on activated B cells, resting T cells, neutrophils, monocytes/macrophages and hepatocytes (Knupfer and Preiss, 2008; Rose-John et al., 2007). The mIL-6Rα is also expressed on neurons and glial cells (Gadient and Otten, 1994; Mathieu et al., 2010). Soluble IL-6Rα is produced endogenously in the human brain which suggests that the actions of IL-6 are not restricted by the localisation of mIL-6Rα in the CNS (Ringheim et al., 1998).

b) The gp130 receptor

The mature gp130 protein is 130kDa and is required for IL-6 signalling to occur and gp130 is a common signalling molecule for several cytokines in the IL-6 family. The membrane bound form is widely distributed in the CNS (Ringheim et al., 1998; Simpson et al., 1997) and the gp130 molecule also exists in soluble form (sgp130) (Jostock et al., 2001; Rose-John et al., 2006).

3.1.2 The classic IL-6 signalling pathway, the trans-signalling pathway, and mediation by soluble receptors

The gp130 receptor protein does not directly bind IL-6 or IL-6Rα alone, but rather binds IL-6 only when in a complex with mIL-6Rα or sIL-6Rα. (Rose-John et al., 2006; Taga et al., 1989). In the classic signalling pathway (Figure 3.1-1A) IL-6 binds to mIL-6Rα and subsequently to mgp130 to initiate intracellular signalling (Knupfer and Preiss, 2008; Rose-John et al., 2007). The IL-6/sIL-6Rα complex is able to bind mgp130 on cells lacking mIL-6Rα to initiate signalling, this is called trans-signalling (Figure 3.1-1B). Further, the sgp130 molecule is able to bind the IL-6/sIL-6Rα complex to selectively inhibit signalling by competing for membrane receptor binding (Figure 3.1-1C) (Jostock et al., 2001; Rose-John et al., 2006).

With IL-6 signalling events reliant on the availability of membrane bound or the soluble forms of IL-6Rα and gp130 to either enhance or inhibit the final signalling product, the relative concentrations of sIL-6Rα, sgp130 and IL-6 determine their physiological effects (Scheller et al., 2011; Simpson et al., 1997). Therefore we have measured IL-6 as well as the total and soluble forms of IL-6Rα and gp130

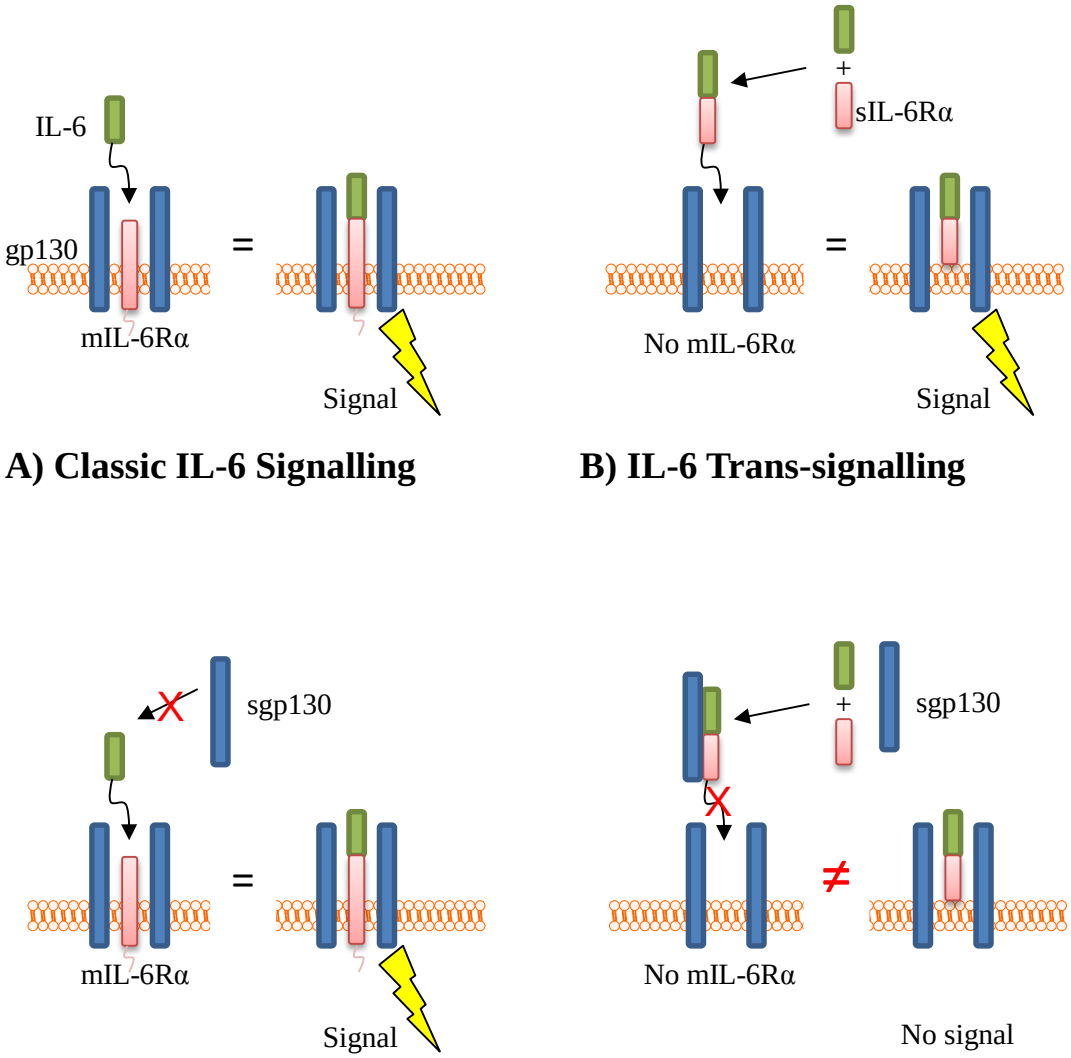

Figure 3.1-1: The IL-6 signalling complex: classic signalling, trans-signalling and selective soluble gp130 inhibition of signalling.

IL-6 signalling requires IL-6, the IL-6Rα and gp130 to form a complex in order to initiate intracellular signalling. Classic signalling occurs when IL-6 complexes with mIL-6Rα which then binds the gp130 receptor, initiating signalling **(A)**. Trans-signalling occurs when IL-6 complexes with sIL-6Rα and binds to gp130 on cells with no available mIL-6Rα to initiate signalling **(B)**. A soluble form of gp130 exists but cannot bind IL-6 directly and thus does not inhibit signalling when mIL-6Rα is present; however the sgp130 can bind the IL-6/ sIL-6Rα complex and inhibit signalling on cells where mIL-6Rα is not present **(C)**. Figure adapted from (Kallen, 2002; Rose-John et al., 2006; Scheller et al., 2011).

3.1.3 Summary

With increased peripheral IL-6 correlated with worse symptomatology, antipsychotic use and medication resistance (Na and Kim, 2007; Singh et al., 2009; Zhang et al., 2002, 2009) and the importance of the neurotransmitter systems influenced by IL-6 in the symptomatology of schizophrenia, the levels of IL-6 in the brain may play an important role in this illness. Combined with the earlier finding of elevated levels of IL-6 in the Nrg1 Het mice subjected to an immune stimulus, the hypothesis that levels of IL-6 and its signalling molecules in the brain of these mice could also be altered is strengthened.

3.2 Methods

3.2.1 Animal housing and breeding

Described in chapter 2 (2.2.1).

3.2.2 Genotyping and weaning

Described in chapter 2 (2.2.2)

3.2.3 B16F0 melanoma model

Described in chapter 2 (2.2.3). Nrg1 Het n=8, WT n=11.

3.2.4 Collection and dissection of brain tissue

Brain tissue was sampled immediately following euthanasia and cardiac puncture and fresh brains were dissected into PFC and hippocampi straight away. Mouse brains were dissected according to the mouse brain atlas (Paxinos and Franklin, 2001). Briefly, the prefrontal cortex was dissected using the olfactory bulbs, interhemispheric fissure and the corpus callosum along with visible colour differences in brain region tissue as landmarks. The hippocampus can be clearly identified as a subcortical structure. Once the prefrontal cortex was removed, the cerebellum and brain stem was removed and brains were sectioned in the mid-sagittal plane. The thalamus, septum and underlying structures were removed, leaving the hippocampus clearly visible and distinguishable for removal from the cortex. This method has been used previously (Filiou et al., 2011, 2012). Several practice dissections were made to establish landmarks and locate structural components before experimental tissue was collected.

Samples were snap frozen in liquid nitrogen before storage at -80^{o}C. Before experimental use, dissected brain tissue was approximately halved with one half used

for protein analysis and one half used for mRNA experiments. The brain tissue was weighed and randomly allocated to either a protein or mRNA buffer solution for homogenisation immediately following weighing.

It is important to note that much has been written about the potential limitations of using CO_2 as a method of euthanasia when studying the brain. Studies into the CO_2 method that specifically considering effects on the analytes measured in these experiments were not found however the current consensus is that there are limited recommendations for alternate methods of euthanasia and therefore the benefits outweigh the possible limitations (Angus et al., 2008; Conlee et al., 2005; Hawkins et al., 2006).

3.2.5 Protein analysis

a) Protein isolation and storage

Dissected mouse brain tissue was weighed and homogenised in 9 volumes ice-cold buffer containing 100µl protease inhibitor cocktail (Sigma), 100µl 50mM β-Glycerophosphate (Sigma) and 33.3µl 0.3M Phenylmethylsulfonyl fluoride (Sigma) made up in 9.8ml NP40 cell lysis buffer (Invitrogen) and briefly vortexed. Homogenised tissue was maintained on dry ice while total protein concentrations were determined for each sample using the DC assay kit (BioRad, Hercules, CA, USA) (3.2.5b). Due to the limited weight of samples allocated for protein analysis, PFC samples were pooled, with 2 animals from each group randomly pooled together to create 4 pooled total protein samples per group.

b) Protein quantification

Samples were quantified using the DC protein assay (BioRad) following manufacturers' instructions. In brief, a standard curve was determined using a BSA dilution of 0, 200,

400, 600, 800, 1000, 1200µg/ml. Using a 96 well flat bottom plate standard and sample tubes were mixed then 5µl was added per well. Next 25µl reagent A' (where A' = 20µl of reagent S into each 1ml of reagent A) was added and mixed gently by agitating plate by hand. Then 200µl reagent B was added and mixed gently by agitating plate by hand. All steps were performed at RT. All bubbles were removed and the plate was incubated for 15min at RT. Results were obtained from SpectraMax 384 Plus (Molecular Devices) plate reader (750nm) using a linear standard curve. The amount of protein used per experimental protocol is listed in the appropriate methods section.

c) Western blot

Western blotting was used to determine relative differences in the protein levels of IL-6, IL-6Rα and gp130 in the PFC and the hippocampus. A total of 5µg of total protein per well was used for both the PFC and the hippocampus for the three antibodies tested. The number of samples was 8 per group for the hippocampus and due to tissue limitations previously noted, the PFC experiments were conducted on 4 pooled samples per group (3.2.5a)3.2.5b). Each sample was tested in triplicate.

Protein samples were mixed with XT sample and reducing buffers (Bio-Rad), as per manufacturers' instructions. Samples were heated to 95°C for 5min then cooled on ice and briefly centrifuged to collect condensation. Proteins were electrophoresed in pre-cast 4-12% bis-tris gels (Bio-Rad) (200V for 50min); in 1x electrode running buffer (XT-MOPS, BioRad). Precision Plus Protein™ WesternC™ Standards (2µl) were run alongside samples. Samples were then transferred on to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) (100V, 1 hour) using 1x transfer buffer (150ml 10x Tris/Glycine buffer, 300ml methanol, 1050ml dH^2O, used ice cold). The MemCode™

Reversible Protein Stain Kit – for PVDF Membranes (Pierce Biotechnology, Rockford, IL, USA) was performed to confirm transfer of protein.

Membranes were blocked with 5% BSA or skim milk powder (BioRad) in TBS-Tween (0.01%) for 1h at RT, based on previous optimisation testing. Membranes were blotted with primary antibody diluted in 0.5% BSA or skim milk in TBS-Tween at 4°C overnight on an orbital shaker. Membranes were washed 3 times with TBS-Tween at 5min intervals at RT, with agitation. Samples were then incubated with the specific secondary antibody diluted in 0.5% BSA or skim milk powder in TBS-Tween with agitation for 1h at RT. Secondary antibody was washed 3 times with TBS-Tween at 5min intervals at RT, with agitation. Primary and secondary antibodies were used as detailed in Table 3.2-1

Membranes were developed in Amersham ECL™ Western Blotting Detection Reagents (GE HealthCare, Buckinghamshire, UK). The membranes were then exposed to Kodak BioMax films (Sigma) for up to 10min or captured on CCD Camera (Carestream, Gel Logic 2200 PRO) depending on strength of immunoreactive signals. Developed films (X-ray film processor; AGFA, CP1000, AGFA Healthcare, Rydalmere, NSW, Australia) were scanned using a GS-690 Imaging Densitometer (Bio-Rad) and quantified with Molecular Imaging Standard Edition software (v5.0.2.30, Carestream). CCD images were quantified using Molecular Imaging Standard Edition software (v5.0.2.30). The net intensity for each band was determined. A mouse anti-actin antibody was used as an internal standard (#MAB1501, 1/50000; Millipore) and each band quantified was normalised to the respective net intensity of the actin band. Data points were checked for triplicate outliers (standard deviation greater than 30% of the

mean) and individual data points removed. Triplicates (or duplicates where outliers were removed) were then averaged across the repeats.

Membranes were stripped of antibody using a buffer of Glycine (1.88g) plus SDS (15g) made up to 1 litre using distilled water (pH 2.0). Blots were incubated at RT in 10ml buffer for 15min with agitation (x2) before being washed in TBS-Tween for 10min at RT, with agitation. Following stripping, membranes were re-blocked and re-blotted with primary and secondary antibodies as described above. A maximum of 3 blots per membrane were performed.

Table 3.2-1: Antibodies for IL-6 and associated receptor western blots, including secondary antibody and buffer details

Primary Antibody	**Secondary Antibody**	**Blocking Buffer (5% in TSBT)**	**Incubation Buffer (0.5% in TBST)**
Rabbit polyclonal anti-IL-6 (H-183): sc-7920[a] (1/500)	Goat-anti-rabbit IgG-HRP[b] (1/2500)	Skim Milk	Skim Milk
Goat polyclonal anti-mouse IL-6Rα (#AF1830) [b] (1/500)	Donkey-anti-goat IgG-HRP[b] (1/2500)	BSA	BSA
Goat anti-mouse gp130 (#AF468) [b] (1/500)	Donkey-anti-goat IgG-HRP[b] (1/2500)	BSA	BSA
Mouse anti-mouse actin[c] (1/50,000)	Donkey-anti-mouse IgG-HRP[b] (1/2500)		

a) Santa Cruz Biotechnology Inc, b) R&D Systems Inc, c) Millipore, figure in ( ) is dilution factor

Membranes were developed then exposed to Kodak BioMax films for 10min (IL-6, IL-6Rα) or captured on CCD Camera (gp130). Developed films were scanned using a GS-690 Imaging Densitometer and quantified with Molecular Imaging Standard Edition software. CCD images were quantified using Molecular Imaging Standard Edition software.

Due to the consistent failure of one sample and insufficient protein to repeat the sample in triplicate, all western blot results in the PFC for the Nrg1 Het unchallenged groups

are based on n=3, the other PFC groups remain n=4. For a representative blot image, see Appendix B.

d) Luminex

Several analytes were measured using cytometric bead assays and flow cytometry. Brain samples were analysed using a custom duplex mouse cytokine 96 well plate assay kit to investigate sIL-6Rα and sgp130 receptor (MILLIPLEX®Map, Millipore, Billerica, MA, USA). The number of samples was 8-11 per group for the hippocampus and due to tissue limitations previously noted, the PFC experiments were conducted on n=4 pooled samples per group. The assay was performed in accordance with the manufacturers' protocol. Brain samples were homogenised as described above (3.2.5a) with 80µg total protein per well. Protocol was followed as described in chapter 2 (2.2.6a) with the following assay specific steps: Standards, controls and samples were loaded into 96 well plates, with samples pseudo-randomly allocated to ensure even distribution of groups across the plate. Homogenising buffer was added to standard and control wells followed by the addition of cytokine assay beads to detect sIL-6Rα and sgp130 receptor. On day two detection antibodies were incubated with samples in sealed plate for 1 hour with agitation at RT. Streptavidin-PE was added to the detection antibodies and incubated a further 30min with agitation at RT. The assay was then run on the Luminex 100 and analysed as previously described (2.2.6a). Where duplicate results were below the limit of detection or did not reach required bead count, the respective sample was removed from analysis resulting in a reduced number of samples for sIL-6Rα and sgp130 for statistical analysis (details in results sections 3.3.3. and 3.3.5 respectively).

3.2.6 Gene expression analysis

a) RNA extraction

Brain tissue RNA extraction was performed using the UltraClean Tissue and Cells RNA Isolation Kit (spin columns) following manufacturers' instructions (Mo Bio Laboratories Inc, CA, USA). In brief, tissue was homogenized using an RNase free micro pestle in 600µl TR1 buffer plus Beta-Mercaptoethanol (10µl per 1ml TR1, prepared immediately before use) in an RNase free tube, followed by passing through a 20 gauge RNase free needle at least 10 times. One volume (600µl) of solution TR2 was added to the homogenate and mixed with a pipette. 600µl of the homogenate mix was transferred to the spin filter in a fresh tube then centrifuged 1min at 10,000 x *g*. The flow through was discarded and the above steps repeated with the second 600µl volume. The spin filter was then washed with 500µl of solution TR3 by the same centrifugation step and transferred to a fresh tube. A second wash was performed by adding 500µl solution TR4 to spin column, centrifugation of 1min at 10,000 x *g* followed by discarding flow through and repeating TR4 wash. Following second TR4 wash and removal of flow through, the spin column membrane was dried by 2min centrifugation at 13,000 x *g*. The spin column was then transferred to a new collection tube before 30-50µl of RNase free water was added to column, incubated for 1min at RT then centrifuged for 1min at 10,000 x g to elute isolated RNA. All steps were performed at room temperature with final RNA solution stored at -80^{o}C until use. RNA concentration and quality was quantified using the NanoDrop (Thermo Scientific) and accepted for use with: A260/280 ratio > 1.9 and A230/260 ratio > 2.0. Additional purification of extracted RNA was performed where required using ethanol precipitation as described in chapter 2 (2.2.7a).

b) cDNA synthesis

The RT^2 First Strand Kit was used to make copy DNA (cDNA) from brain tissue following the manufacturer's instruction using 280ng RNA from the pooled PFC samples, or 550ng RNA from hippocampus samples per reaction, total reaction volume 20µl (SA Biosciences, MD, USA). An initial genomic DNA elimination step was performed using the included genomic DNA elimination mixture with up to 8µl isolated RNA (for 280ng PFC RNA or 550ng hippocampal RNA), 2µl GE (a 5x gDNA elimination buffer provided with kit) and RNase free water to a final volume of 10µl. The contents were gently mixed with a pipette followed by brief centrifugation. The mixture was then incubated at 42ºC for 5min then immediately chilled on ice for >1min. To synthesise cDNA, 10µl of RT Cocktail per manufacturers' instructions was added to each 10µl genomic DNA elimination mixture and mixed gently but thoroughly with a pipette. The mixture was then incubated at 42ºC for 15min followed by heating at 95ºC for 5min to stop the reaction (Mastercycler Pro S, Eppendorf). Finally 91µl of RNase free water was added to each reaction mix and the diluted product stored at -80^{o}C until further use. The resulting cDNA was used for determination of reference genes, the PCR array plate and individual taqman assays performed on brain tissue.

c) Determination of reference genes

Since brain tissue from the PFC and Hippocampi of Nrg1 mice treated with melanoma had not, to our knowledge, previously been performed, we determined appropriate RT-qPCR reference genes empirically. The geNorm Plus reference gene selection kit (PrimerDesign Ltd, Southampton, UK) was used to determine the most appropriate reference genes from a set of 6 PrimerDesign genes that are most likely stably expressed (Csnk2a2, Cdc40, UBC, Pak1ip1, Fbxw2 and Ap3d1). As per manufacturer's

instructions, a total of 16 samples were tested for reference genes, 2 samples per experimental group (4) per region (2) plus controls. In brief, primers provided were resuspended in 220µl of RNase/DNase free water then vortexed thoroughly and used immediately. Primers were added to SYBR Green master mix (FastStart, Roche, Basel, Switzerland) as per manufacturer's instructions before pipetting into 96 well plates. The cDNA template was then added to each primer mix in duplicate, including a no template control before running on the Roche LightCycler 480 II (Table 3.2-2 Thermal cycling parameters: geNorm). Data analysis was performed using the qBase Plus software provided with the geNorm kit. Reference gene details were added to the software module before import of LightCycler data was performed according to instructions. All annotations for plate and sample details were checked upon import for final well annotation including sample name, target gene and CT value with sample type "unknown" selected. Data was automatically analysed using the qBase Plus geNorm software module with results generated in graph and written form (Appendix A). The reference genes chosen for use in RT-qPCR experiments for brain tissue obtained under the specific experimental conditions of this study were Ap3d1 and Fbxw2.

Table 3.2-2 Thermal cycling parameters: geNorm

Step	Temperature (°C)	Time (min:sec)	Cycles
Enzyme activation	95	10:00	1
Denaturation	95	0:15	50
Data Collection	60	0:60	

d) Array based RT-qPCR

A customised RT^2 Profiler PCR Array Kit (SA Biosciences) based on SYBR green chemistry (FastStart, Roche) was utilised to measure cytokine gene expression in brain tissue following the manufacturers' instructions. In brief, sample cDNA was added to the RT^2 qPCR master mix and pipetted into the PCR array plate provided in the kit as

required for each gene location. The PCR was performed on the Roche LightCycler 480 II (Table 3.2-3 Thermal cycling parameters: RT^2 profiler PCR array) with CT values determined using the second derivative maximum built in software calculation.

Table 3.2-3 Thermal cycling parameters: RT^2 profiler PCR array

Step	Temperature (°C)	Time (min:sec)	Cycles
PCR initial heat activation	95	10:00	1
Denaturation	95	0:15	45
Combined Annealing/ extension	60	1:00	

Fluorescent data collection performed at the combined annealing/ extension step

Data was further analysed using web-based PCR ARRAY Data Analysis Software (SA Biosciences) as well as using the delta delta CT method (Livak and Schmittgen, 2001) and SPSS statistical analysis. The genes included on the custom array plate for IL-6-related analyses were IL6, IL6ra and IL6st, with Fbxw2 and Ap3d1 as reference genes with a positive control, reverse transcription control and mouse genomic DNA control included. Each experiment represents 3-4 biological samples tested in triplicate. Note that the IL-6 primers provided on the custom array plate were unable to detect mRNA expression within the control parameters. The RT-qPCR was therefore repeated using cDNA synthesised from the same tissue using a Taqman IL-6 individual gene expression assay described below.

e) Individual gene expression analysis

Following cDNA synthesis (2.2.7b), the QuantiFast Probe RT-PCR Kit (Qiagen) was used per manufacturers' instructions to measure relative mRNA levels of IL-6. In brief, 5µl 2x QuantiFast Probe PCR master mix, 0.4µM primer (forward and reverse), 0.2µM probe and diluted cDNA template up to a total sample reaction mix of 10µl were gently mixed and pipette into a 96 well place. All preparation steps were completed at RT.

The individual IL-6 taqman primer/probe assay was purchased off the shelf from Applied Biosystems. RT-qPCR as performed on a LightCycler 480 II using the second derivative maximum method to determine CT values (Roche) with the cycling conditions detailed in Table 3.2-4.

Table 3.2-4 Thermal cycling parameters: Taqman brain tissue

Step	Temperature (°C)	Time (min:sec)	Cycles
PCR initial heat activation	95	3:00	1
Denaturation	95	0:03	45
Combined Annealing/ extension	60	0:30	

Fluorescent data collection performed at the combined annealing/ extension step

Results are presented as fold change in expression levels (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) with untreated WT expression levels normalised to 1 ± SEM with other values presented as comparison fold change.

3.2.7 Statistics

After confirming normal distribution with the Kolmogorov-Smirnov test, 3-way ANOVA was used to analyse western blot protein levels (factors: brain region x genotype x immune challenge) followed by two-tailed independent samples t-tests to further explore differences between experimental groups. The 3-way ANOVA was used to elicit potential novel findings in this exploratory series of experiments. To ensure the data was appropriately robust, 2-way ANOVA (genotype x immune challenge) was also conducted on the brain regions separately. Main effects and interactions were consistent with those found in the 3-way ANOVA, therefore this data is not shown. RT-qPCR results were analysed using a 2-way ANOVA (factors: genotype x immune challenge) using two-tailed independent samples t-tests to further explore results. Due to different starting amounts of mRNA from the PFC and the hippocampus, direct regional

comparisons were not performed. Pearson's correlation coefficient was used to determine the strength of the relationship between the 46kDa and the 29kDa IL-6 band detected in western blots. Significance was set at $p<0.05$. All p-values are reported as uncorrected values.

3.3 Results

3.3.1 Interleukin-6 protein is lower in the prefrontal cortex but not the hippocampus of immune challenged Nrg1 Het mice

IL-6 was measured in the PFC and the hippocampus using western blot and RT-qPCR. IL-6 expression levels were not detected using the custom PCR array, therefore an additional individual taqman assay was performed for IL-6 only using the same cDNA template. IL-6 was detected using the individual taqman assay in both the PFC and the hippocampus however no differences were found in mRNA expression levels in either region (see Table 4.3-1)

Western blot bands were observed at approximately 29kDa and 46kDa representing monomeric and dimeric IL-6 (May et al., 1991), for a representative blot image, see Appendix B.. The levels of protein present in these two bands were correlated (Pearson's R=0.915, n=47, p<0.001) with the dimer approximately 4x more abundant (arbitrary units of relative net intensity) using anti-actin as a loading control (Figure 3.3-1). The correlation is also present by region: PFC Pearson's R = 0.966 (n=15, p<0.001) and hippocampus Pearson's R = 0.624 (n=32, p<0.001) (region scatterplots not shown).

Table 3.3-1: Interleukin 6 mRNA expression levels in the PFC and hippocampus of Nrg1 Het and WT mice with or without an immune stimulus.

	Control				***Immune challenged***			
	WT		Nrg1 Het		WT		Nrg1 Het	
Prefrontal cortex	1.00	±0.24	1.13	±0.31	0.91	±0.07	1.45	±0.43
Hippocampus	1.00	±0.40	1.16	±0.38	0.58	±0.08	0.73	±0.24

Data represent mean fold change ± SEM, n=3 pooled samples per group (PFC), n=4 per group (Hipp). WT expression levels normalised to 1 and fold change for other groups calculated as a comparison.

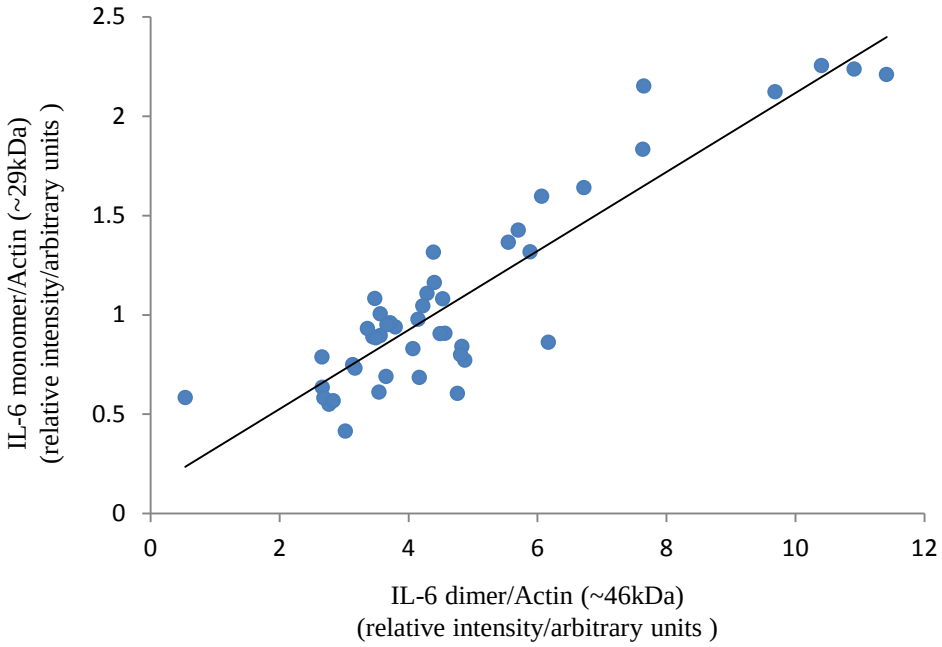

Figure 3.3-1: Correlation of the IL-6 dimer and monomer bands detected by western blot.

Both a ~29kDa band and a ~46kDa were detected by western blot using the Rabbit polyclonal anti-IL-6 (H-183): sc-7920 (Santa Cruz). Quantification and analysis of both bands determined that they were correlated (Pearson's R=0.915, p<0.001). Bands are discussed as an IL-6 monomer (~29kDa) and dimer (~46kDa) (May et al., 1991).

An interaction between region, genotype and immune challenge was found using a 3-way ANOVA (dimer $F_{1,45}$=10.76, p=0.002; monomer: $F_{1,45}$=8.01, p=0.007), as well as a genotype x immune challenge interaction (dimer: $F_{1,45}$=11.18, p=0.002; monomer: $F_{1,45}$=11.47, p=0.002). The IL-6 dimer showed an immune challenge x region interaction ($F_{1,45}$=12.91, p=0.001), whilst the monomer had a trend toward interaction ($F_{1,45}$=3.65, p=0.064).

Both the dimer and the monomer showed a main effect of region (dimer: $F_{1,45}$=29.70, p<0.001; monomer: $F_{1,45}$=51.00, p=0<001). A main effect was also seen for immune challenge in the IL-6 dimer ($F_{1,45}$=14.83, p<0.001) and the monomer ($F_{1,45}$=9.01,

p=0.005) bands. Genotype alone did not show any main effect, and no specific genotype x region interaction was seen.

Differences between groups within each region were then analysed using independent samples t-tests. Nrg1 Het mice were found to have between 52% (dimer) and 67% (monomer) less IL-6 in the PFC when subject to immune challenge compared to the unchallenged Nrg1 Het group (dimer: p=0.014; monomer: p=0.018). Although there appears to be a tendency towards increased levels of IL-6 in unchallenged Nrg1 Het in the PFC when compared to unchallenged WT mice, this was not statistically significant (dimer: p=0.093; monomer: p=0.091). No change due to the immune challenge was found for the WT groups in the PFC and no differences were seen in the hippocampus between any of the experimental groups (Figure 3.3-2).

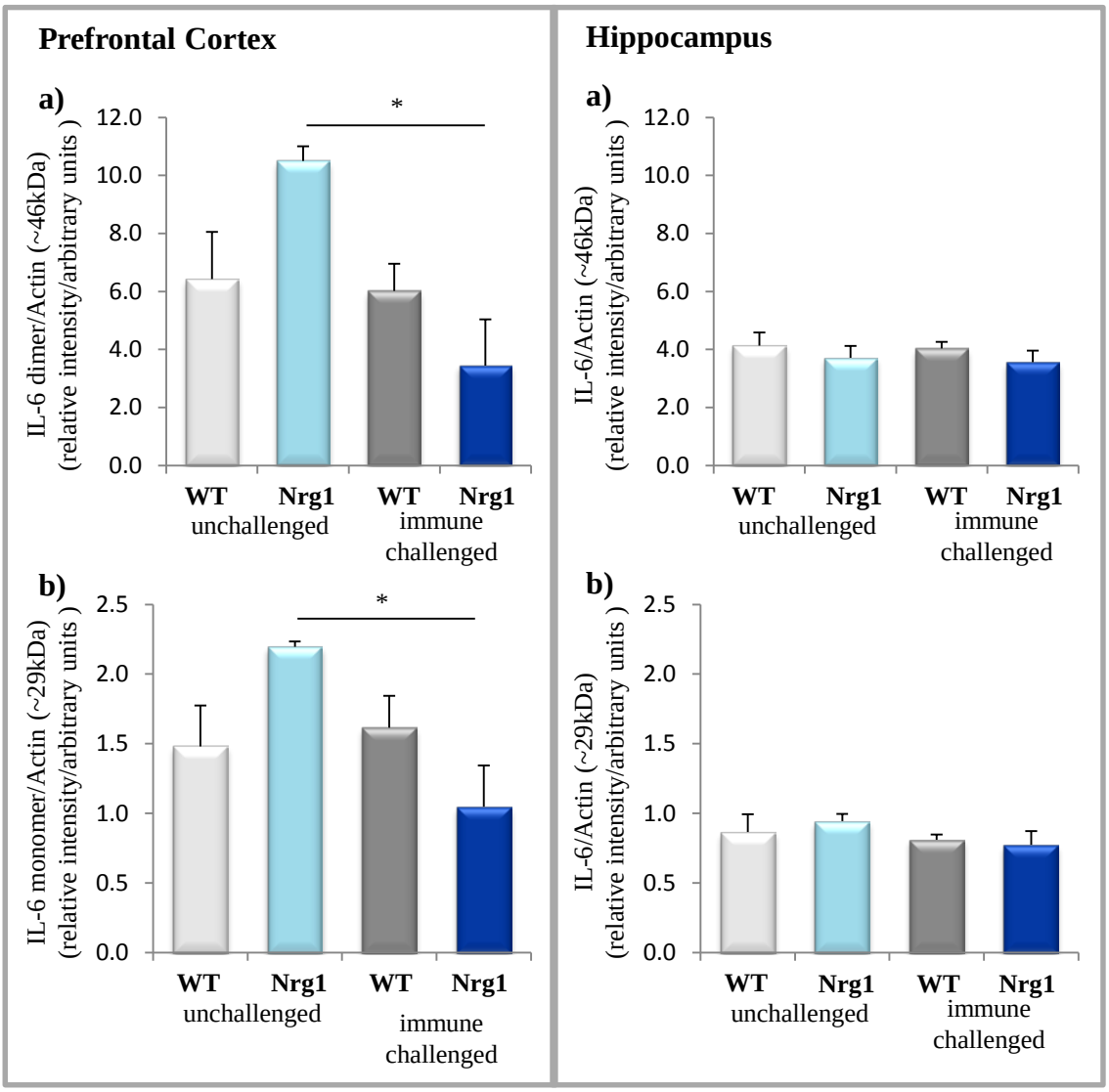

Figure 3.3-2: Lower IL-6 levels in the PFC of Nrg1 Het mice subject to an immune stimulus.

IL-6 protein levels were lower in the PFC of Nrg1 Het challenged animal when compared to Nrg1 Het unchallenged mice but not in the hippocampus. IL-6 protein levels in the PFC (left panel) and the hippocampus (right panel) where **(a)** is the relative net intensity, of the normalised IL-6 dimer and **(b)** is the IL-6 monomer in immune challenged and unchallenged Nrg1 Het and WT mice. Data represents western blot protocol: membranes were probed with anti-IL-6 antibody, stripped then re-probed with an anti-actin loading control and analysed using Molecular Imaging Standard Edition software (v5.0.2.30, Carestream). PFC n=4 (except Nrg1 Het unchallenged: n=3), hippocampus n=8 per group. *$p<0.05$ (mean ± SEM)

3.3.2 Elevated levels of total IL-6Rα in the prefrontal cortex of immune challenged Nrg1 Het mice compared with WT littermates

Total IL-6Rα was measured in brain lysate using western blot. A band was observed at approximately 73kDa and this band was analysed, for a representative blot image, see Appendix B. A main effect of region was shown using 3-way ANOVA ($F_{1,45}$=5.67, p=0.022). No other main effects or interactions were found. Using independent samples t-tests to compare experimental groups within each region, the Nrg1 Het mice had 23% higher levels of IL-6Rα in the PFC when subject to immune challenge compared to the WT immune challenged mice (p=0.001). No other differences were found between the groups in the PFC or the hippocampus (Figure 3.3-3a). The mRNA expression levels in the PFC of Nrg1 Het mice with the immune challenge was elevated compared to challenged WT mice (independent samples t-test; p=0.034; Figure 3.3-3b).

3.3.3 Soluble IL-6Rα has regional differences between the prefrontal cortex and hippocampus but was not altered by genotype or immune challenge

As the soluble form of the IL-6Rα can further enhance IL-6 signalling, the soluble form was measured with bead based flow cytometry using Luminex and is presented as pg/µg total protein. Several duplicate results were below the limit of detection or did not reach required bead count for analysis. These samples were removed from analysis resulting in: WT unchallenged n=4 (PFC), n=7 (hipp); Nrg1 Het unchallenged n=3 (PFC), n=6 (hipp); WT challenged n=4 (PFC), n=10 (hipp); Nrg1 Het challenged n=3 (PFC), n=8 (hipp).

A 3-way ANOVA (genotype x treatment x region) showed a main effect of region ($F_{1,42}$=72.47, p<0.001) with no other effects or interactions (Figure 3.3-3c).

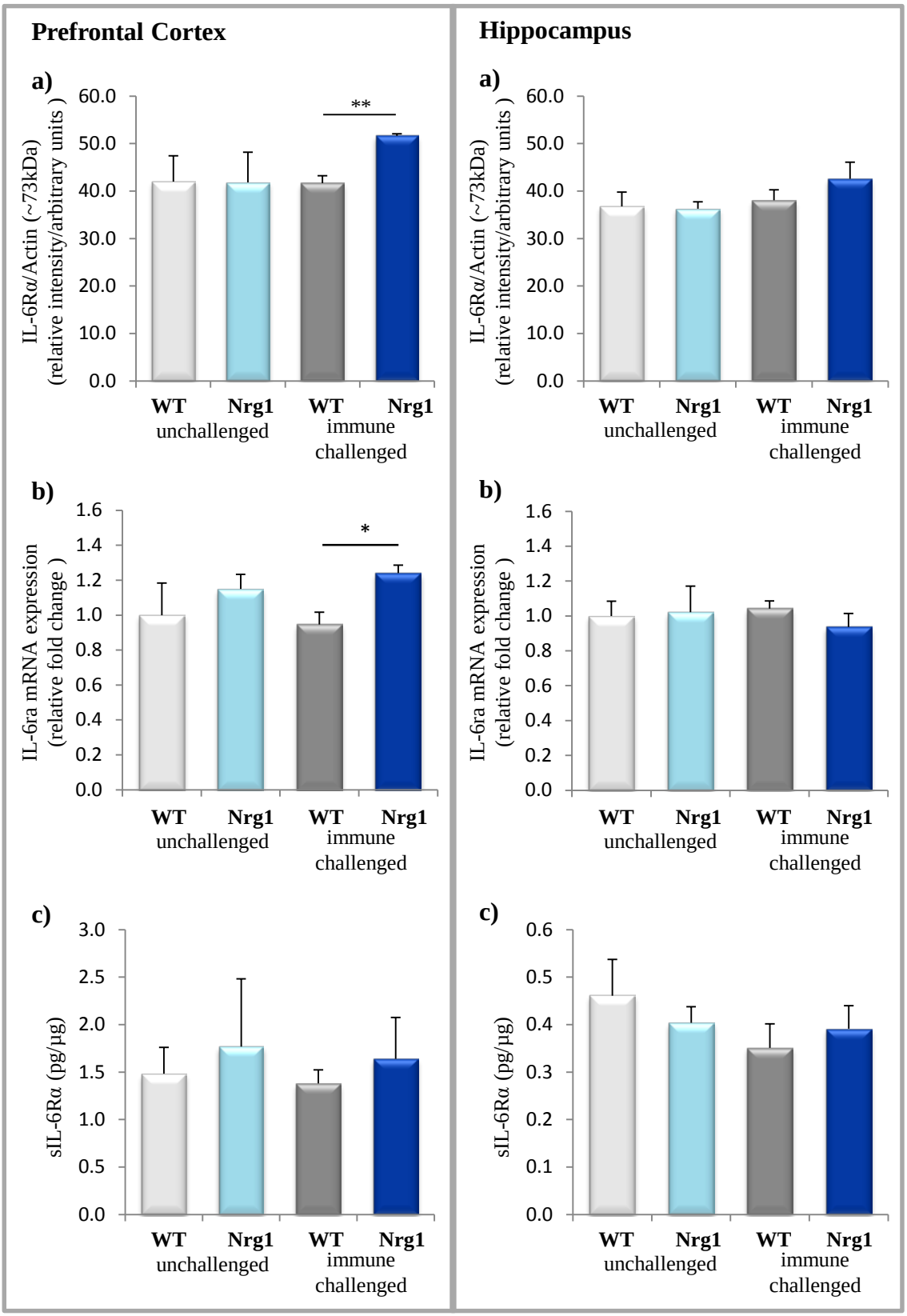

Figure 3.3-3: Elevated total, not sIL-6Rα protein levels and elevated mRNA expression in the PFC of Nrg1 Het mice subject to an immune stimulus.

Figure 3.3-3 legend continued from previous page:

Total IL-6Rα protein was higher in the PFC (left panel) of Nrg1 Het immune challenged mice compared to WT challenged controls (a) measured by relative net intensity of the normalised 73kDa band where membrane was probed with anti- IL-6Rα antibody and anti-actin loading control then analysed using Molecular Imaging Standard Edition software. Nrg1 Het immune challenged mice also demonstrated higher levels of mRNA expression in the PFC (left panel) but not the hippocampus (right panel) (b) measured as relative fold change in mRNA expression using RT-qPCR, sIL-6Rα levels measured by Luminex flow cytometry analysis show no difference between genotypes with or without immune challenge in either brain region(c). PFC n=3-4, hippocampus n=6-9 per group. **$p<0.01$ (mean ± SEM).

3.3.4 Total gp130 receptor protein demonstrated interactions between region, genotype and immune challenge in Nrg1 Het mice

Total gp130 was measured using western blot. A band was observed at approximately 130kDa and analysed using relative net intensity and anti-actin as the loading control, for a representative blot image, see Appendix B. An interaction between region, genotype and immune challenge was found using a 3-way ANOVA ($F_{1,45}$=4.57, p=0.039), as well as a region x immune challenge interaction ($F_{1,45}$=6.15, p=0.018). A main effect of region ($F_{1,45}$=81.88, p<0.001) and immune challenge ($F_{1,45}$=6.44, p<0.015) were also seen. No differences were found between groups using independent samples t-tests (Figure 3.3-4). No differences in mRNA expression levels were found using RT-qPCR.

3.3.5 Nrg1 Het mice have a lower levels of soluble gp130 in the hippocampus with an immune challenge

Since relative increases in the level of sgp130 can inhibit IL-6 signalling, the soluble form was measured separately with bead based flow cytometry using Luminex and is presented as pg/µg total protein. Several duplicate results were below the limit of detection or did not reach required bead count thus the respective sample was removed from analysis. Final experimental groups consisted of: WT unchallenged n=4, n=6 (hipp); Nrg1 Het unchallenged n=3 (PFC), n=6 (hipp); WT challenged n=4 (PFC), n=11 (hipp); and Nrg1 Het challenged n=3 (PFC), n=8 (hipp) for statistical analysis.

A 3-way ANOVA for sgp130 revealed a main effect of region ($F_{1,35}$=328.89, p<0.001) with no other effects or interactions, although there was a slight trend towards a region x immune challenge interaction ($F_{1,35}$=3.04, p=0.092). Further independent t-tests

revealed decreases in the levels of sgp130 in the hippocampus only. WT challenged mice had 55% lower sgp130 levels compared to the WT unchallenged ($p=0.007$) while the Nrg1 Het challenged animals also showed 63% lower levels of sgp130 when compared to the Nrg1 Het unchallenged groups ($p=0.002$). Additionally, the Nrg1 unchallenged animals displayed a trend towards 38% lower levels of sgp130 when compared with the unchallenged WT group ($p=0.077$) and the Nrg1 immune challenged mice displayed a similar trend for 48% lower sgp130 levels compared to their tumour challenged WT littermates ($p=0.055$). No differences were found in the PFC (Figure 3.3-4).

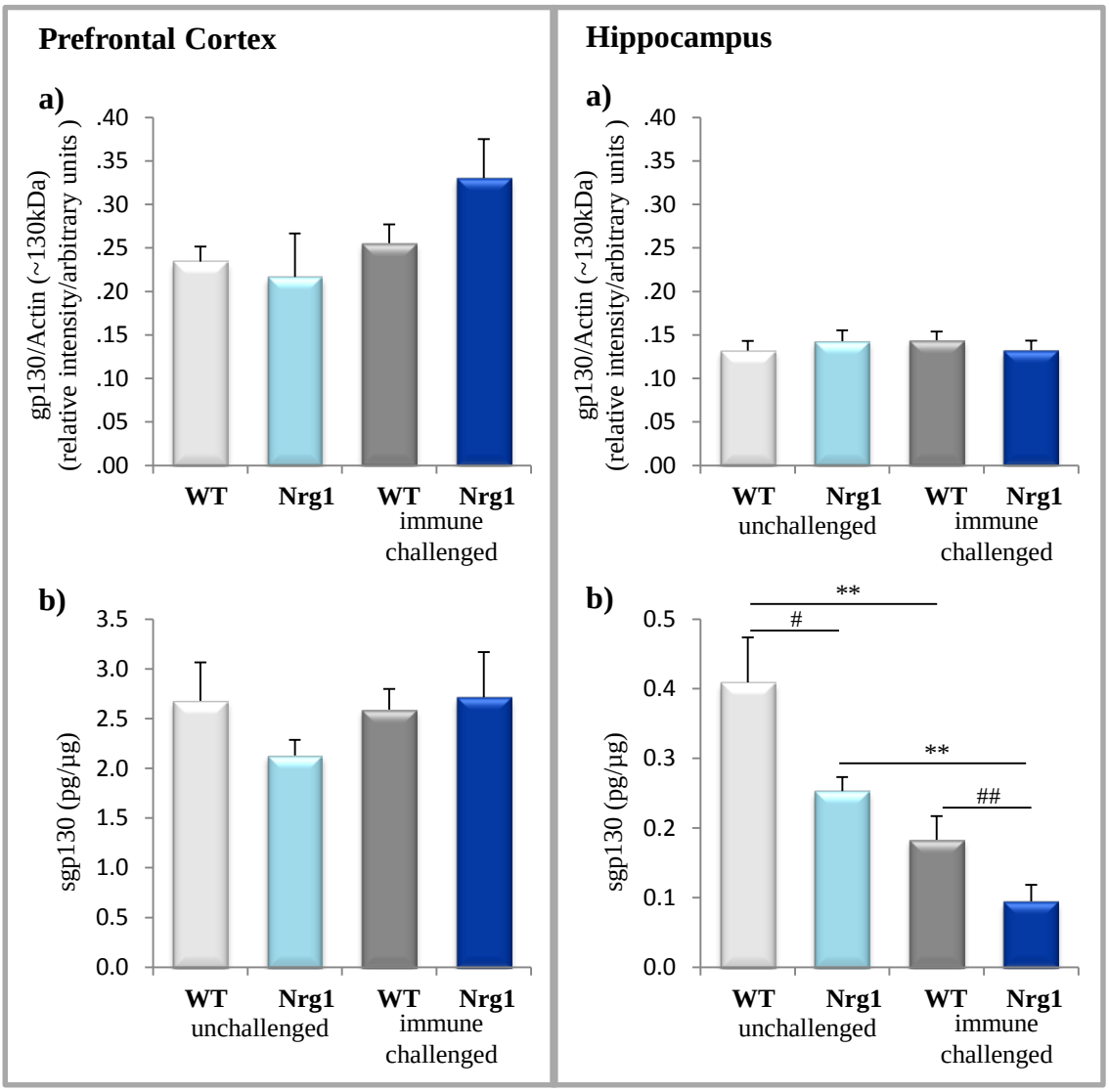

Figure 3.3-4: Soluble but not total gp130 levels were reduced in the hippocampus of unchallenged Nrg1 Het and WT mice with an immune stimulus.

Additionally, both Nrg1 unchallenged and immune challenged mice had a tendency towards reduced sgp130 when compared with their WT counterparts. Total gp130 protein levels in the PFC (left panel) and the hippocampus (right panel). Although a 3-way ANOVA revealed an interaction between region, genotype and immune challenge ($F_{1,45}$=4.57, p=0.039), independent samples t-tests did not demonstrate any difference between groups in either the PFC or the hippocampus **(a)** measured using western blot, , relative net intensity, reflecting the normalised 130kDa band. Flow cytometry analysis for sgp130 showed both WT and Nrg1 Het challenged mice had lower levels of protein than the respective unchallenged groups **(b)** using Luminex (pg/µg). Data represent mean ± SEM. PFC n=3-4, hippocampus n=8-11 (western blot); n=5-7 (Luminex) per group **p<0.01 #p=0.077, ##p=0.055

3.4 Discussion

3.4.1 IL-6 and IL-6 receptor molecules were found altered in Nrg1 Het mice when combined with an immune stimulus

With the finding of higher circulating plasma IL-6 in Nrg1 Het mice with an immune stimulus, we investigated whether these changes were similar in the brain. Alterations in Il-6 or the IL-6 signalling molecules were found in both brain regions studied; differences were also seen in the level of these molecules in the PFC and the hippocampus, while alterations in response to the immune stimulus varied between the two regions. The PFC had approximately 60% lower IL-6 levels in Nrg1 Het immune challenged compared to Nrg1 Het unchallenged mice. While total IL-6Rα protein and mRNA expression was 23% higher in the PFC of Nrg1 Het immune challenged mice compared to challenged WT mice, the sIL-6Rα did not vary. In the hippocampus, no corresponding changes were found in IL-6, total or sIL-6Rα; however sgp130 was 55% lower in the WT challenged and 63% lower in the Nrg1 Het immune challenged groups compared to their respective unchallenged controls.

3.4.2 IL-6 protein levels were lower while mRNA expression is unchanged in the prefrontal cortex of Nrg1 Het immune challenged mice

Previous studies demonstrated IL-6 is able to cross the BBB (Banks et al., 2009). We therefore anticipated that central IL-6 levels in the Nrg1 Het mice would be higher in the PFC and the hippocampus in animals subject to the immune stimulus, corresponding to elevated circulating plasma levels. However, the PFC demonstrated approximately 60% lower levels of IL-6 protein (Figure 3.3-2) in these mice with no change in mRNA expression levels (Table 3.3-1), while the hippocampus had no change in protein or mRNA (Figure 3.3-2; Table 3.3-1). This finding is consistent with the results of

Desbonnet (2012) where a chronic social defeat paradigm increased spleen IL-6 levels in Nrg1 Het mice with no corresponding increase in IL-6 in the PFC or hippocampus. Transport of IL-6 into the brain occurs via saturable transporters and has been shown to result in degradation of the intact form of IL-6 (Banks et al., 1994, 2009). This suggests that the transport across the BBB is physiologically limited and may potentiate central responses, rather than as a boost to the central response itself, this however requires further investigation.

Fillman et al (2013) found elevated expression of IL-6 mRNA in the dorsolateral PFC of post-mortem schizophrenia patients. However the authors were unable to replicate their data in a subsequent study, finding only a pattern suggesting elevated IL-6 mRNA expression in schizophrenia patients (Fillman et al., 2014). Additionally, mRNA levels for IL-6 in Nrg1 Het mice were unchanged in the PFC or the hippocampus at baseline or following a chronic stress paradigm (Desbonnet et al., 2012), consistent with the mRNA findings in the present study (Table 3.3-1).

Previous studies in yeast and human liver have shown that there is insufficient correlation between mRNA levels and protein levels to predict protein abundance directly from gene expression data and demonstrating that post-transcriptional regulation is present in various species (Anderson and Seilhamer, 1997; Gygi et al., 1999). This suggests that mRNA is not an accurate predictor of protein levels, with post-transcriptional modification mechanisms the likely effectors of differences seen between mRNA expression and protein levels.

With unchanged IL-6 gene expression and lower protein levels in the Nrg1 Het mice, production or degradation of IL-6 would appear to be affected by a Nrg1 mutation.

Although not yet well characterised, IL-6 has been shown vulnerable to post-transcriptional regulation, with molecules that can both stabilise and destabilise the transcription product (Iwasaki et al., 2011; Masuda et al., 2013; Matsushita et al., 2009). Although these modulators of IL-6 mRNA stability have not been shown directly linked to Nrg1, a mutation in Nrg1 has the potential to indirectly alter IL-6 post-translational stability. For example, degradation of the Regnase-1 protein results in increased IL-6 stability through activation of the Nuclear Factor Kappa B (NFκB) (Iwasaki et al., 2011). Nrg1 was shown to activate the NFκB pathway in schwann cells (Limpert and Carter, 2010). It is therefore plausible that a mutation in Nrg1 as seen in the Nrg1 Het mice could reduce the activation of the NFκB pathway, resulting in higher IL-6 transcript instability. These potential post-transcriptional modifications are also likely to be differentially regulated in the periphery and across brain regions as higher circulating levels of IL-6 were seen in plasma, lower protein levels in the PFC and unchanged levels in the hippocampus of the Nrg1 Het mice. With limited data available on IL-6 levels in the brain of schizophrenia patients and with mRNA expression studies showing conflicting data (Fillman et al., 2013, 2014) little can be speculated on the possible mechanism resulting in reduced IL-6 in the PFC of the Nrg1 Het mice.

It is important to note for IL-6 and its signalling proteins that results in the PFC should be interpreted with care. Due to the compromise between the amount of tissue needed, the number of analytes investigated and the restricted number of experimental animals available, along with the relatively small size of the PFC in mice, the sample size for this region is small. With a low sample number, significant interactions may not be evident in ANOVA analysis. In an attempt to overcome this limitation, the independent

student t-tests were used. It will be important to confirm all observations from the PFC in repeat studies.

3.4.3 Total but not soluble IL-6Rα levels were increased in the prefrontal cortex of Nrg1 Het immune challenged mice

The IL-6Rα was investigated to provide a more complete picture of the overall potential for IL-6 signalling to be up- or down-regulated in the Nrg1 Het mice. Total IL-6Rα protein levels in the PFC were 23% higher in the Nrg1 Het immune challenged mice, compared to WT challenged animals, with mRNA expression levels similarly elevated. Further, an increase in sIL-6Rα would suggest widespread enhanced IL-6 signalling ability in the Nrg1 Het mice; however with no change in the soluble form of the receptor in the PFC the increase in total protein is most likely the membrane bound form (Figure 3.3-3). Lower histamine induced IL-6 levels have been shown combined with elevated IL-6 receptor expression in liver cells of histamine deficient mice, demonstrating that an inverse relationship between IL-6 and the IL-6 receptor exists (Horváth et al., 2002). Although these findings in liver cells are consistent with the findings in this study, specific studies showing the relationship between IL-6 and the IL-6 receptor levels in the brain were not found, indicating that additional studies are warranted to confirm whether this inverse relationship seen in the Nrg1 Het mice is an exaggeration of a normal compensatory response.

3.4.4 The hippocampus demonstrated reduced levels of soluble gp130 in both Nrg1 Het and WT mice subject to the melanoma challenge

To complete the picture of IL-6 and IL-6 signalling partners, total and sgp130 were investigated in the PFC and the hippocampus. When considering the total levels of

gp130, the graphical representation gives the impression of a tendency towards increase in total gp130 in the PFC and possible interactions were indicated by the ANOVA analysis (4.3.4; Left panel, Figure 4.3.4a), however further analysis using student t-test did not show any alterations in protein levels by group. The mRNA expression levels for gp130 in the PFC were also unchanged. Further, there was no difference in the total gp130 protein or mRNA expression in the hippocampus. This lack of difference in total gp130 contrasts with the increased levels of IL-6Rα seen in the PFC, where the expectation was that the two receptors would be regulated in parallel (Falus et al., 1992). However different tissue types have demonstrated differential regulation of these two receptors (Nesbitt and Fuller, 1992). In particular, the expression of mRNA transcripts for these IL-6 receptors has shown regional specificity in response to an LPS immune stimulus (Vallières and Rivest, 1997). These differences could result from the small sample size but may yet represent a physiologically relevant difference; additional experiments would be required to confirm this.

Soluble gp130 was also investigated. In the hippocampus lower levels of sgp130 were found in both WT and Nrg1 Het immune challenged animals. The WT mice had 55% lower sgp130 in the hippocampus when subject to the immune stimulus compared to unstimulated WT mice. The Nrg1 Het mice showed a 63% lower level in animals subject to the immune stimulus compared to unstimulated Nrg1 Het mice. In addition, it can been seen that there is a trend toward lower levels in the Nrg1 Het mice when compared to WT both in the unchallenged and the immune challenged groups in the hippocampus (Right panel, Figure 3.3-4b). As sgp130 is a powerful inhibitor of IL-6/sIL-6Rα trans-signalling (Jostock et al., 2001), reduced levels of this protein in the immune challenged animals suggests a removal of signalling inhibition. With no other

changes in the IL-6 signalling molecules in the hippocampus, it is difficult to speculate on why inhibition would need to be reduced in this case. As gp130 is an obligate receptor for a number of other cytokines from the IL-6 family which were not measured, and sgp130 has been shown to partially inhibit the activities of these other cytokines (Jostock et al., 2001), it is possible that this alteration is not related to the IL-6 changes.

3.4.5 Regional differences and hippocampal finding limitations

Finally, each of the molecules demonstrated a main effect of region using 3-way ANOVA analysis, suggesting different levels of IL-6 and the IL-6 signalling proteins between the PFC and the hippocampus. Differential regulation of IL-6 and its signalling molecules in response to an immune challenge is consistent with previous in situ mRNA expression findings following an LPS stimulus (Vallières and Rivest, 1997). IL-6 mRNA was not detected in the basal state, but increased expression was seen in a number of brain regions following an acute immune stimulus. The receptors (IL-6Rα and gp130) were constitutively expressed in the basal state, and also selectively increased in specific brain regions following the LPS challenge. The increases in mRNA expression were not consistent in all brain regions or across all three analytes (Vallières and Rivest, 1997). Further, with the higher levels of plasma IL-6, lower levels in the PFC and unchanged levels in the hippocampus of the Nrg1 Het challenged mice found in this study, protein levels within the different brain regions may not be reliant on BBB cross over. The possible differential regulation of each analyte within specific brain regions is likely to be more important in the central response to immune stimuli, particularly in the Nrg1 Het mice. However the need to pool the PFC samples to obtain

enough protein for analysis makes this comparison difficult to interpret without further studies.

The findings in the hippocampus for these studies may also be obscured because of random allocation of hippocampal dissections to protein or mRNA studies. Differences in brain symmetry have been shown previously, in particular, lateralisation of the hippocampus. Neuron size and shape within subregions of the hippocampus, as well as the volume and symmetry of the left and right hippocampus are altered in schizophrenia patients (Zaidel et al., 1997a, 1997b). An improved experimental design using hippocampi dissected into left and right as well as dorsal and ventral segments or histological examination of the CA regions may reveal details obscured here by the random assignment of portions of the whole hippocampus from each individual animal.

3.4.6 Conclusion

IL-6 and the IL-6 signalling molecules are altered in the brain of Nrg1 Het mice subjected to a melanoma immune stimulus; although interestingly the alterations are not consistent in the two brain regions studied. Additionally, the increase in plasma IL-6 seen in the periphery was not reflected in either brain region, with PFC levels lower in the Nrg1 Het mice and hippocampal levels unchanged. Together these data suggest that IL-6 and its cognate receptors are differentially regulated in the PFC and the hippocampus with central levels of IL-6 not a simple reflection of peripheral levels. Furthermore, with mRNA expression levels inaccurate predictors of protein levels, measurement of both in exploratory studies such as this one, provide a broader understanding of potential influences requiring further study. It is worth reiterating that there are several limitations to these data, including low sample numbers for the PFC and a lack of insight into hippocampal lateralisation or details of hippocampal subregion

responses. Despite the limitations, these data provided the first evidence of approximately 60% lower IL-6 protein levels in conjunction with a 23% elevation in IL-6Rα in the PFC of Nrg1 Het mice subject to a peripheral immune stimulus. This study also demonstrated a novel 55-63% reduction in sgp130 protein in the hippocampus of mice subject to an immune stimulus, which was consistent in both genotypes. These data therefore confirm a role for IL-6 in the dysregulation of the immune response to a direct immune stimulus in the Nrg1 Het mice. This role for IL-6 is shown to be not only a disruption in the peripheral immune response, but also an aberration in the central response. Further; the importance of considering not only cytokines but likewise measuring their signalling receptors is demonstrated by the changes seen in IL-6Rα and sgp130.

A demonstration of neuroimmune interactions between the candidate schizophrenia vulnerability gene Neuregulin 1, and peripheral and central cytokine levels: focus on IL-6 and G-CSF

CHAPTER FOUR

Granulocyte-colony stimulating factor in the brain

Peta Snikeris
University of Wollongong

4.1 Granulocyte-colony stimulating factor in the brain

G-CSF is a haematopoietic growth factor with a well-defined role in the proliferation of cells of the myeloid linage. As discussed earlier (2.4.2), the maturation and release of peripheral neutrophils during inflammation is stimulated by G-CSF and a lack of G-CSF results in neutropenia. G-CSF has also been shown to influence a range of brain functions, where a deficiency in the brain results in reduced motor skill learning, impaired spatial memory formation, reduced short term recognition memory and impaired long term memory in G-CSF knockout mice (Diederich et al., 2009a). Recombinant G-CSF has been used in schizophrenia patients as a treatment for anti-psychotic induced neutropenia, with G-CSF also demonstrating anti-apoptotic characteristics in cells of the myeloid lineage (Fukunaga et al., 1991; Nagata and Fukunaga, 1991; Schneider et al., 2005a, 2005b). Although G-CSF levels have been studied in schizophrenia patients, these studies are limited in number and scope, with alterations in circulating plasma levels of G-CSF not consistent.

4.1.1 G-CSF structure, function and expression

G-CSF is a 19kDa glycoprotein produced by several peripheral cell-types with differentially regulated expression dependent on cell type or stimulus (Fukunaga et al., 1991; Nagata and Fukunaga, 1991; Schneider et al., 2005b). G-CSF is produced by bone marrow stromal cells and macrophages when stimulated by endotoxins, as well as by fibroblasts or endothelial cells when stimulated with TNFα or IL-1. LPS and several additional cytokines have also been shown to induce peripheral macrophage production of G-CSF (Li et al., 2009d). LPS significantly induces G-CSF in peripheral blood mononuclear cells and bone marrow stromal cells. In peripheral blood mononuclear cells, LPS stimulation induces G-CSF production via IL-1. IL-6 however, inhibits G-

CSF production in peripheral blood mononuclear cells. In bone marrow stromal cells, G-CSF production is also stimulated by IL-1 but is not affected by IL-6 (Hannen et al., 1999).

G-CSF expression has also been shown in neurons throughout all brain regions, including expression in the frontal cortex as well as in the hippocampus (Schneider et al., 2005a). Schneider et al (2005a) further demonstrated that G-CSF results in the differentiation of neuronal stem cells both *in vitro* and *in vivo*. Astrocyte cultures have been found to express G-CSF, dependent upon surrounding conditions, including culture medium *in vitro* or disease states *in vivo* (Li et al., 2009d; Schneider et al., 2005a; Xiao et al., 2007). While the role of microglia in the expression of both G-CSF and its receptor is yet to be confirmed (Xiao et al., 2007). Additionally, it has been demonstrated that G-CSF can pass through the intact BBB from the blood to the brain (Schneider et al., 2005a; Zhao et al., 2007). With G-CSF able to cross the BBB and be produced in the brain, as well as neuronal responses to G-CSF shown, the previous finding of higher levels of circulating plasma G-CSF in Nrg1 Het mice with a stimulated immune system suggested that the brain, particularly the PFC and the hippocampus, may reflect a similar alteration to that seen in the periphery.

4.1.2 G-CSF receptor structure, function and expression

G-CSF signals through the G-CSF receptor (G-CSFR, also known as CD114) which displays similarity with many cytokine receptor extracellular regions. In addition the G-CSFR shares homology with gp130, the IL-6 family receptor subunit that produces receptor signalling, but does not show homology with the IL-6Rα chain. However, other cytokines including IL-6 have not been seen to compete for binding to the G-CSFR. Equally, in spite of the receptor homology, gp130 does not bind G-CSF (Nagata and

Fukunaga, 1991). Studies first suggested that the G-CSFR was restricted to cells of neutrophilic granulocyte lineage in bone marrow and spleen, however it has since been found on monocytes, platelets and endothelial cells (Schneider et al., 2005b). Further, expression of the G-CSFR occurs in dopaminergic neurons in the adult brain and cell cultures (reviewed in Xiao et al. 2007). The G-CSFR is also widely distributed on rat neurons and adult neural stem cells in particular in the cortex and the hippocampus (Schneider et al., 2005a).

G-CSF production is localised to areas of G-CSFR expression in the brain and it is thought that regulation occurs through an autocrine signalling mechanism in neurons (Schneider et al., 2005a). Both G-CSF and its receptor are up-regulated following ischemia in rodents with comparable results seen in human stroke patients, suggesting rodent models are appropriate for the study of G-CSF and its receptor in the brain (Schneider et al., 2005a). Further, challenge with the bacterial mimic LPS has been shown to induce G-CSF production in rat neurons (Li et al., 2009d) which suggests that the response is not limited to physical stimuli within the brain but that G-CSF in the brain may be responsive to peripheral immune challenges.

4.1.3 Summary

With limited studies of G-CSF in schizophrenia patients, evidence of a role for G-CSF in brain functions related to schizophrenia, and a robust finding of increased levels in the periphery of the Nrg1 Het mice subject to the immune stimulus, this study focused on determining the concomitant levels of G-CSF and the G-CSFR in the brain of immune challenged mutant mice.

4.2 Methods

4.2.1 Animal housing and breeding

Described in chapter 2 (2.2.1).

4.2.2 Genotyping and weaning

Described in chapter 2 (2.2.2).

4.2.3 B16F0 melanoma model

Described in chapter 2 (2.2.3). Nrg1 Het n=8, WT n=11.

4.2.4 Collection and dissection of brain tissue

Described in chapter 3 (3.2.4).

4.2.5 Protein analysis

a) Protein isolation and storage

Described in chapter 3 (3.2.5a).

b) Protein quantification

Described in chapter 3 (3.2.5b). Protein concentration details are detailed within individual methods sections as applicable.

c) Western blot

Western blot was performed as previously described (3.2.5c). Differences in the protocol are described below.

A total of 20 µg of protein per well was used for G-CSF blotting for both the PFC and the hippocampus while 5µg of total protein per well was used for G-CSF receptor

blotting for both the PFC and the hippocampus. As for chapter 3, hippocampus n=8 while PFC n=4 pooled samples. Primary and secondary antibodies as well as blocking buffer details are shown in Table 4.2-1.

Table 4.2-1: Antibodies used for Western blotting of G-CSF and the G-CSF receptor, including secondary antibody and buffer details

Primary Antibody	**Secondary Antibody**	**Blocking Buffer (5% in TSBT)**	**Incubation Buffer (0.5% in TBST)**
Goat polyclonal anti-G-CSF (K-15): sc-49679[a] (1/250)	Donkey-anti-goat IgG-HRP[b] (1/2500)	BSA	BSA
Sheep anti-mouse G-CSF R/ CD114 (#AF6039) [b] (1/1000)	Donkey-anti-sheep IgG-HRP[b] (1/2500)	Skim Milk	Skim Milk
Mouse anti-mouse actin[c] (1/50,000)	Donkey-anti-mouse IgG-HRP[b] (1/2500)		

a) Santa Cruz Biotechnology Inc, b) R&D Systems Inc, c) Millipore. Figure in ( ) is dilution factor

A peptide competition binding assay was conducted per manufacturers' instructions to determine the correct band to analyse for G-CSF as the antibody detected several non-specific bands. The peptide (sc-49679 P) was pre-incubated with the G-CSF antibody using a 5x excess peptide concentration, for 1 hour at RT (Santa Cruz,), before the above western blot protocol was conducted. Two parallel gels were run and transferred to membranes; one incubated in G-CSF primary antibody only, the second membrane was incubated with the antibody-peptide mix. This process resulted in the disappearance of just one band at 50kDa which was subsequently analysed as previously detailed. Representative images are shown in Appendix B.

Due to the consistent failure of one sample and insufficient protein to repeat the sample in triplicate, all western blot results in the PFC for the Nrg1 Het unchallenged groups

were based on n=3, the other PFC groups remain n=4. Western blot for the hippocampus was n=8 for all groups.

4.2.6 Gene expression analysis

a) RNA extraction

Described in chapter 3 (3.2.6a).

b) cDNA synthesis

Described in chapter 3 (3.2.6b).

c) Determination of reference genes

Described in chapter 3 (3.2.6c).

d) Array based RT-qPCR

Described in chapter 3 (3.2.6d). The genes included on the custom array plate for G-CSF-related analyses were G-CSF (csf3), and G-CSFR (csf3r). Note that the G-CSF primers provided on the custom array plate were unable to detect mRNA expression within the control parameters. The RT-qPCR was therefore repeated using cDNA synthesised from the same tissue using a Taqman G-CSF individual gene expression assay described in chapter 3 (3.2.6e).

4.2.7 Statistics

After confirming normal distribution with the Kolmogorov-Smirnov test, 3-way ANOVA was used to analyse western blot protein levels (factors: brain region x genotype x immune challenge) with two-tailed independent samples t-tests to further explore differences between experimental groups. The 3-way ANOVA was used to

elicit potential novel findings in this exploratory series of experiments. To ensure the data was appropriately robust, 2-way ANOVA (genotype x immune challenge) was also conducted on the brain regions separately. Main effects and interactions were consistent with those found in the 3-way ANOVA; therefore this data is not shown. RT-qPCR results were analysed using a 2-way ANOVA (factors: genotype x immune challenge using two-tailed independent samples t-tests to further explore results. Significance was set at $p<0.05$. All p-values are reported as uncorrected values.

4.3 Results

4.3.1 G-CSF protein higher in the hippocampus of WT immune challenged mice and lower in Nrg1 Het challenged mice.

Studying G-CSF protein levels, no interactions were found using 3-way ANOVA however, a main effect of region was shown ($F_{1,43}$=5.67, p=0.006). Using independent samples t-tests to compare experimental groups within each region, the Nrg1 Het mice were found to have 24% lower levels of G-CSF in the hippocampus when subject to immune challenge compared to the WT immune challenged mice (p=0.028). In addition, WT immune challenged mice displayed 25% higher levels of G-CSF in the hippocampus when compared to the unchallenged WT animals (p=0.047; Figure 4.3-1). Representative western blot images are shown in Appendix B. Data for mRNA expression levels of G-CSF could not be obtained under the experimental conditions in this study, possibly due to a low abundance of the gene transcript, despite using both a custom PCR array plate and individual taqman assay.

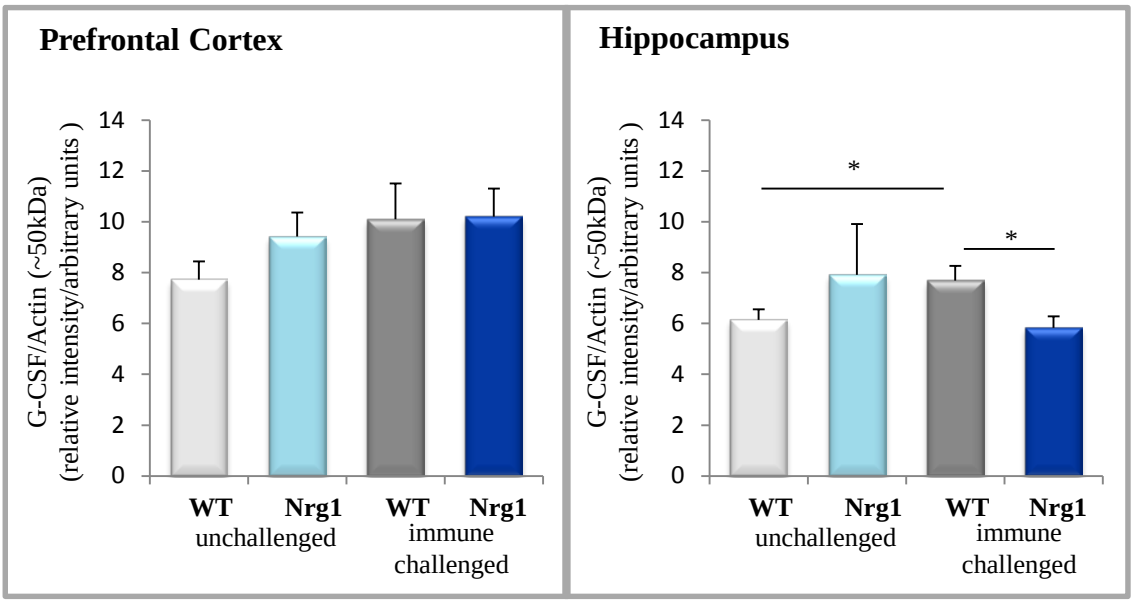

Figure 4.3-1: WT mice had increased G-CSF protein in the hippocampus while Nrg1 Het mice showed decreased levels of G-CSF with an immune challenge.

WT mice had increased G-CSF protein in the hippocampus with an immune challenge compared to unchallenged WT controls. While Nrg1 Het mice showed decreased levels of G-CSF with an immune challenge when compared to the WT challenged animals. G-CSF protein levels in the PFC (left panel) and the hippocampus (right panel), mean ± SEM, relative net intensity shown, reflecting the normalised G-CSF ~50kDa band probed with antibody to G-CSF and an anti-actin loading control, *$p<0.05$.

4.3.2 G-CSF receptor present at higher levels in the prefrontal cortex compared to the hippocampus with no genotype or immune stimulus differences

The G-CSF receptor was measured using western blot. The band at approximately 110kDa was analysed using relative net intensity and anti-actin as the loading control, for a representative blot image see Appendix B. No interactions were found using a 3-way ANOVA (region x genotype x immune challenged) however a main effect of region was shown ($F_{1,43}$=92.30, p<0.001). Using independent samples t-tests to compare experimental groups within each region no additional differences were found (Figure 4.3-2). Further, no differences were shown in mRNA expression levels with mRNA expression for the G-CSF receptor stable in both regions across all experimental groups (Table 4.3-1).

Table 4.3-1: G-CSF receptor mRNA expression levels in the PFC and hippocampus of Nrg1 Het and WT mice with or without an immune stimulus.

	Control				***Immune challenged***			
	WT		Het		WT		Het	
Prefrontal cortex	1.00	±0.06	1.09	±0.10	1.02	±0.07	1.11	±0.09
Hippocampus	1.00	±0.14	0.98	±0.14	0.97	±0.08	0.86	±0.05

Data represent mean fold change ± SEM., n=3 pooled samples per group (PFC), n=4 per group (Hipp). WT expression levels normalised to 1 and fold change for other groups calculated as a comparison.

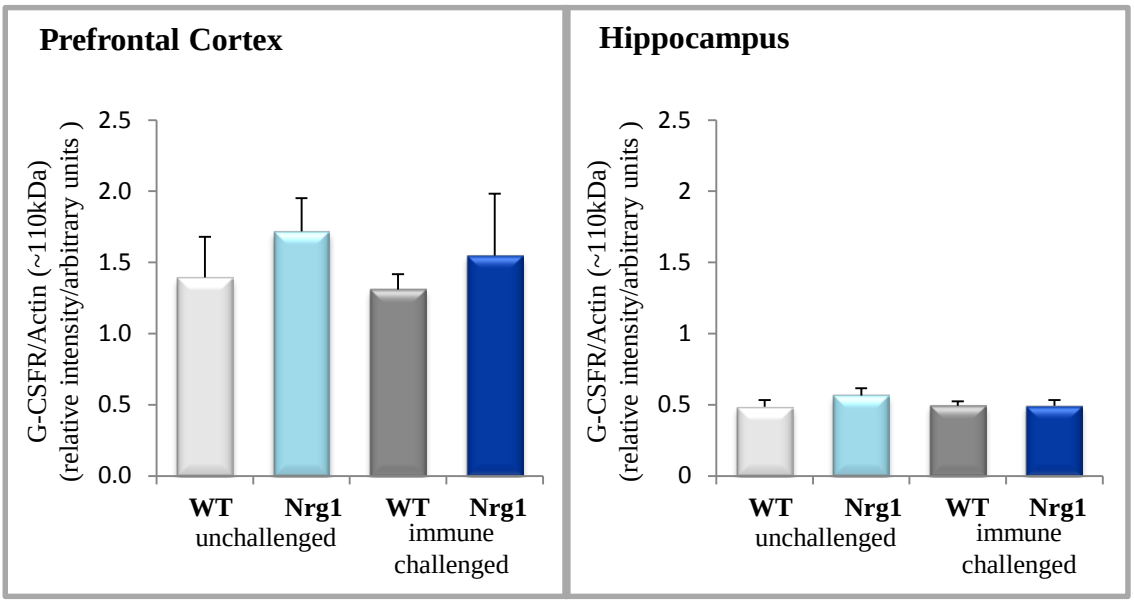

Figure 4.3-2: The G-CSF receptor protein showed no differences between Nrg1 Het and WT animals in either the PFC or the hippocampus.

G-CSFR protein levels in the PFC (left panel) and the hippocampus (right panel), mean ± SEM, relative net intensity shown, reflecting the normalised G-CSFR 110kDa band. *$p<0.05$.

4.4 Discussion

4.4.1 G-CSF protein was elevated in WT but reduced in Nrg1 Het mice when subject to melanoma immune challenge

An investigation was conducted into the levels of G-CSF and the G-CSF receptor in the PFC and the hippocampus based on the finding of elevated of G-CSF in the plasma of Nrg1 Het mice with an immune challenge. As G-CSF can cross the blood brain barrier, and the combination of genetic mutation and immune stimulus resulted in the peripheral change, it was hypothesized that increases in G-CSF would be similarly seen in these brain regions in the Nrg1 Het mice only. However, as with IL-6, the peripheral response was not directly reflected in the brain and the individual brain regions had different G-CSF responses. There was no change in G-CSF protein in the PFC, while the hippocampus displayed changes in both WT animals with the immune stimulus and Nrg1 Het mice with the immune stimulus. The WT animals demonstrated 25% higher levels of G-CSF when challenged with the melanoma, while Nrg1 Het animals had 24% less G-CSF protein. The G-CSF mRNA expression levels were not detectable and the changes seen in G-CSF levels were not reflected in the receptor as G-CSFR did not differ between groups in either protein or mRNA expression levels.

4.4.2 Elevated levels of G-CSF protein in the hippocampus of WT immune challenged mice may represent a neuroprotective mechanism

G-CSF is shown to be a neuroprotective protein in a number of circumstances, in line with the current findings of elevated G-CSF in the hippocampus of WT mice subject to the immune stimulus compared to WT control (Figure 4.3-1). Li et al (2009) demonstrated that pre-treatment of neurons with the immune stimulus LPS, increases G-CSF production in primary hippocampal neuron cultures and that these neurons were

protected from excitatory injury. A protective role for G-CSF in stroke has also been demonstrated with G-CSF able to interfere with apoptotic pathways in primary cortical rat neuronal cell cultures (Schneider et al., 2005a, 2005b). The results of the present study are therefore consistent with these previous studies, suggesting that an on-going peripheral stimulus, such as the melanoma challenged used, results in the induction of a neuroprotective mechanism in the hippocampus in WT mice.

4.4.3 Lower levels of G-CSF protein in the hippocampus of Nrg1 Het immune challenged mice may imply a disruption of the protective mechanism

In contrast to the WT animals, this study demonstrated lower levels of G-CSF protein in the hippocampus of the Nrg1 Het mice subject to the immune stimulus, suggesting a reduction in neuroprotection. It has been demonstrated, that G-CSF deficient mice show increased infarct size in experimentally induced stroke (Sevimli et al., 2009). Additionally, neurons pre-treated with anti-G-CSF in conjunction with excitatory neurotransmitters, showed reduced levels of neuroprotection, suggesting that neuronal toxicity may be potentiated by interfering with G-CSF signalling (Li et al., 2009d). G-CSF antibodies are also capable of abolishing the anti-apoptotic properties of G-CSF shown in ischemia (Schneider et al., 2005a, 2005b). Although both of these studies utilised anti-G-CSF antibody to reduce G-CSF protective capabilities, it is possible that the lower levels of the protein might result in the same effects. Further studies however, are required to confirm this in the Nrg1 Het mice.

4.4.4 G-CSF relevance to schizophrenia

With disruptions in memory function a hallmark of schizophrenia the alterations found in the performance of spatial memory tasks using G-CSF treatment in adult rats are

noteworthy (Diederich et al., 2009b). G-CSF treatment improved formation of reference memory, but not working memory during initial acquisition training in a spatial memory task. Yet in the re-acquisition phase working memory improved in the G-CSF treated animals. These authors initially suggested that new born neurons, which are reduced when G-CSF is deficient, may have influenced the memory outcomes. However, treatment with G-CSF did not significantly increase the number of new-born cells. G-CSF did however, increase survival of adult-born neurons generated during this period. Diederich et al (2009b) hypothesized therefore that the initial improvement is due to signalling pathways activated by the G-CSFR, and cannot be attributed to new born neurons. This provides some evidence to suggest that the lower levels of G-CSF in the Nrg1 Het mice could result in impaired signalling through the G-CSF receptor, potentiating neurotoxicity, or at least lessening neuroprotection, similar to those seen in Li et al (2009d) and Schneider et al (2005a, 2005b) discussed above.

G-CSF has been reported in the schizophrenia literature as an adjunct treatment in patients who develop anti-psychotic medication (clozapine) induced agranulocytosis (Pollmächer et al., 1997; Schuld et al., 2000). Although changes in neutrophil levels prior to clozapine treatment may be indicative of those at risk to develop clozapine induced agranulocytosis, G-CSF plasma levels have not been consistently correlated with development of this complication (Khan et al., 2013). Despite this lack of correlation, recombinant G-CSF is successfully used as an adjunct therapy with clozapine treatment in these patients (Khan et al., 2013; Pollmächer et al., 1997; Schuld et al., 2000). G-CSF appears to be well tolerated as a therapeutic although there are concerns long term treatment may have negative side effects (Khan et al., 2013; Welte et al., 1996). Although no correlation has been reported, it remains unclear if G-CSF

levels could be predictive of agranulocytosis in patients and whether the lack of association with schizophrenia is because G-CSF is unchanged or because it has not been widely studied.

4.4.5 Prefrontal cortex and hippocampal differences

Both G-CSF and the G-CSFR were found to have a main effect of region using 3-way ANOVA tests, suggesting a difference in protein levels between the PFC and the hippocampus. Expression of both analytes has been demonstrated previously in these brain regions, with known expression in the cortex, hippocampus and sub ventricular zone among others, although specific regional comparisons in protein levels was not performed (Schneider et al., 2005a).

As discussed in chapter 3, there are several limitations with the data in the present study, including the low sample number for PFC comparisons (3.4.2), as well as the use of randomly assignment hippocampi (3.4.5) which may obscure differences in lateralization, where hippocampal neuron size, neuron shape, volume and symmetry have previous been shown altered in schizophrenia (Zaidel et al., 1997a, 1997b). Therefore, due to these limitations, further experiments are required to determine the existence of regional differences, or lateral differences in the hippocampi.

4.4.6 Conclusion

Despite the lack of focus on G-CSF in the schizophrenia literature, the results of the present study suggest further investigation into the potential role of the interactions between immune stimuli and brain function relevant to schizophrenia. With a proven effect on neuroprotection, the 25% elevation in G-CSF levels in the hippocampus of WT mice subject to the immune stimulus is thought to represent the normal brain

response to this challenge and would be expected to confer neuroprotection from any immune stimulated damage. The demonstration of 24% lower levels in the schizophrenia vulnerability Nrg1 Het model subjected to an immune stimulus suggests that these mice may lack the necessary G-CSF provided neuroprotection to cope with on-going immune insults, resulting in neural damage. Therefore, vulnerable individuals subjected to a similar immune type stimulus may suffer immune mediated toxicity in the brain, potentially relevant to disease development, progression or symptomatology in schizophrenia.

A demonstration of neuroimmune interactions between the candidate schizophrenia vulnerability gene Neuregulin 1, and peripheral and central cytokine levels: focus on IL-6 and G-CSF

CHAPTER FIVE

An exploration of signalling molecule expression levels

Peta Snikeris
University of Wollongong

5.1 Signalling molecule expression

A limited number of studies demonstrate common signalling mechanisms between neuronal and immune function, and none attempt to directly link schizophrenia pathophysiology with altered immune function in a schizophrenia relevant model. As discussed in chapter 1 (1.2.4e), schizophrenia patients have shown various differences in multiple cytokines in the periphery, and these immunologically significant findings in patients make the existence and relevance of common signalling mechanisms highly likely. Indeed, this project has shown a link between a schizophrenia genetic vulnerability model and immune alteration by demonstrating that both peripheral and central cytokine responses are different in Nrg1 Het mice subject to inoculation with B16F0 melanoma cells. To further understand the possible signalling interactions between a Nrg1 mutation and the IL-6 and G-CSF alterations in the brain, a preliminary exploration of mRNA expression of signalling molecules potentially involved in both Nrg1 signalling and cytokine production was conducted.

5.1.1 Nrg1 and the ErbB receptors in signalling

The mechanisms of Nrg1/ ErbB signalling have been extensively studied over recent years (Mei and Xiong, 2008; Talmage, 2008). Most of the schizophrenia literature focusses on the Nrg1/ErbB4 interaction, since no association between ErbB2 or -3 mutations and schizophrenia-like behaviours have been shown (Mei and Xiong, 2008). Despite this focus on ErbB4, which not only signals in a homodimeric form, but also as a heterodimer with ErbB2 (1.2.3) the potential importance of these heterodimers in Nrg1 signalling should not be ignored (Yarden and Sliwkowski, 2001b). The canonical forward signalling pathway is activated following the cleavage of the Nrg1 extracellular domain with the soluble Nrg1 protein functioning as a ligand for the ErbB receptors

(Mei and Xiong, 2008). Once triggered, the ErbB kinases activate various intracellular signalling pathways including the PI3K/Akt pathway (Banerjee et al., 2010) and Jak/Stat phosphorylation (Liu and Kern, 2002).

5.1.2 Nrg1 and the PI3K/Akt pathway

Nrg1 has been shown to be a major activator of the PI3K/Akt pathway, acting through its ErbB receptors, in particular ErbB4 (Mei and Xiong, 2008). The PI3K/Akt signalling pathway also modulates the production of several cytokines, critical for immune function. With Nrg1 a candidate gene for schizophrenia susceptibility, the PI3K/Akt pathway represents an attractive candidate for integrating the neuropathology of schizophrenia and immune dysfunction (Okkenhaug and Vanhaesebroeck, 2003).

The PI3K/Akt pathway is of high complexity and is involved in a large number of intracellular signalling processes. A simplified explanation of the pathway and the molecules involved has been adopted here, with a number of the intermediate steps and pathway molecules not discussed. In brief, the PI3K/Akt signalling pathway is activated when a ligand, such as Nrg1, binds to a receptor tyrosine kinase, such as the ErbB receptors. When PI3K is activated it phosphorylates Akt following a number of intermediate steps. The main negative regulator of the PI3K/Akt pathway is phosphatase and tensin homolog on chromosome 10 (Pten) (Hemmings and Restuccia, 2012). To find an indication of a potential dysregulation in the PI3K/Akt pathway, Akt and Pten were therefore investigated for mRNA expression levels in the PFC and hippocampus.

Akt has three isoforms in both humans and mice, Akt1, Akt2 and Akt3, all demonstrating similar regulation (Masure et al., 1999; Nakatani et al., 1999). The

distribution of the isoforms, however, is not constant: Akt1 shows wide tissue distribution, including the brain; Akt2 is concentrated in muscle tissue and adipocytes; and Akt3 is mainly in the brain and the testes. This distribution is consistent with murine mutant models where Akt1 deficiency results in developmental defects, Akt2 deficiency results in defects in glucose homeostasis and Akt3 deficiency results in defective brain development (Cohen, 2013; Hers et al., 2011; for detailed review see: Manning and Cantley, 2007).

In addition to its neuronal cell functions, the PI3K/Akt signalling pathway modulates the production of several cytokines, critical for immune function, including IL-6 (Koyasu, 2003). Cytokines can also influence PI3K/Akt signalling, for example IL-6 participates in a signalling feedback loop using this pathway (Chakraborty et al., 2003; Sudheerkumar et al., 2008). Further, G-CSF was shown to induce increased phosphorylation of Akt in neurons and this increase in Akt activity that was thought to confer some anti-apoptotic properties. Blocking PI3K/Akt was able to partially block the G-CSF mediated protection against apoptosis (Schneider et al., 2005b). Although cell type, cellular environment and co-factors have to be considered, this indicates that this pathway may be common in CNS and immune system dysregulation. It is yet to be determined if a schizophrenia-relevant mutation of Nrg1 can act on the PI3K/Akt pathway and influence its interaction with the immune system.

5.1.3 The Jak/Stat signalling pathway: an intersection of IL-6 and G-CSF signalling

Given the complexity of signalling in any biological processes, a second pathway with the potential to integrate Nrg1 gene mutation resulting in schizophrenia vulnerability with perturbed immune function was explored. In this case we focused on a common

signalling pathway to IL-6 and G-CSF with the potential to be impacted by Nrg1 alteration: the Jak/Stat pathway. Upon stimulation with the IL-6/ IL-6Rα complex, gp130 becomes phosphorylated on tyrosine, which is mediated by members of the Jak family. The Jak kinases: Jak1, Jak2 and Tyk2, are constitutively associated with gp130, however, in the absence of Jak1, IL-6 cannot effectively mediate signalling via Stat phosphorylation (Guschin et al., 1995). Therefore, IL-6 signalling results in Jak1 phosphorylation, activating Stat1 and Stat3 (Fischer and Hilfiker-Kleiner, 2008; Schindler et al., 2007). It was also shown that in the absence of Jak1 certain cell types can still phosphorylate Stat3 (Kopantzev et al., 2002). The activation of Jak/Stat by IL-6 plays an important role in neurogenesis (Li et al., 2009b), suggesting this pathway could represent a link between altered IL-6 levels in the brain of the Nrg1 Het mice and the vulnerability to schizophrenia-like behaviours.

Jak1 was also demonstrated as the critical signalling molecule for the G-CSFR, with downstream activation of Stat3 also shown (Shimoda et al., 1997). In myeloid cells, G-CSF has been shown to signal preferentially via the Jak/Stat signalling pathway, although this pathway is only moderately activated in neurons where the PI3K/Akt pathway is favoured (Schneider et al., 2005b). The main negative regulator of the Jak/Stat signalling pathway favoured by both IL-6 and G-CSF is suppressor of cytokine signalling 3 (SOCS3) (Murray, 2007; White and Nicola, 2013). The details discussed above represent a summary of the available literature (for a review of this pathway see Harrison, 2012), from which the present study explored the mRNA expression levels of Jak1, Stat3 and SOCS3 in the PFC and hippocampus of the Nrg1 Het animals.

5.1.4 Summary

With evidence that both neuronal and immune system alterations demonstrated in schizophrenia may have a similar molecular basis, such as the Nrg1 mutation studied here, the potential for common signalling pathway involvement in the development of these alterations is strong. The current study focussed on the mRNA expression levels of the major signalling molecules involved in the activation of the PI3K/Akt signalling pathway via Nrg1 relevant to schizophrenia: ErbB2, -3, and 4; as well as Akt1, -2 and 3; and finally the pathway regulator Pten (Hemmings and Restuccia, 2012). Additionally, the main signalling molecules indicated by the alterations in IL-6 and G-CSF in both the periphery and the brain were considered: Jak1, Stat3 and the negative regulator of this pathway, SOCS3 (Murray, 2007).

5.2 Methods

5.2.1 Animal housing and breeding

Described in chapter 2 (2.2.1).

5.2.2 Genotyping and weaning

Described in chapter 2 (2.2.2).

5.2.3 B16F0 melanoma model

Described in chapter 2 (2.2.3).

5.2.4 Collection and dissection of brain tissue

Described in chapter 3 (3.2.4).

5.2.5 Protein analysis

a) Protein isolation and storage

Described in chapter 3 (3.2.5a).

b) Protein quantification

Described in chapter 3 (3.2.5b). Protein concentration details are detailed within individual methods sections as applicable.

c) Luminex

Protein levels of Akt and phosphorylated Akt (pAkt) were measured using Luminex cytometric bead assays targeting pan-Akt and pan-pAkt epitopes. MILLIPLEX MAP Total Akt/PKB MAPmate (MP46-605) and phosphoAkt/PKB (Ser473) MAPmate (MP46-601) beads and the MILLIPLEX MAP cell signalling buffer and detection kit

were used with assay buffer 1 following manufacturer instructions (Millipore). Total and phosphorylated analytes cannot be multiplexed, therefore Akt and pAkt were run in parallel plates, both following the same protocol and using the same reagent kit. In brief, wash buffer was used to pre-wet the filters in a 96 well assay plate by 10min incubation with agitation at RT before removal of buffer. All removal of reagents was performed by vacuum. All vacuum steps were followed by blotting the bottom of the plate to remove excess liquid reagents. All preparation steps were completed at RT. Positive controls were added onto plates as required per manufacturer instructions (Millipore). Assay buffer was added to each sample well. Beads were added to all wells as required. All wells contained sample (or control) at the ratio of 1 part sample to 2 parts reagents. The plate was sealed and incubated overnight at 4°C with agitation. The following day, reagents were removed and wells washed twice with wash buffer. Detection antibodies were added into each well and incubated in sealed plate for 1 hour in the dark, with agitation, at RT. Detection antibodies were removed by vacuum and Streptavidin-PE was added to the detection antibodies and incubated for 15min, with agitation, at RT in the dark. Amplification buffer was then added to the Streptavidin-PE and incubated for a further 15min under the same conditions. Reagents were then removed by vacuum followed by two washes and final suspension in 150µl of Luminex xMap sheath fluid (Luminex Corporation, Austin, TX, USA) by 5min agitation at RT on plate shaker. The plate was run on a Luminex 100™ System using xPONENT® 3.1 Software (Luminex Corporation). Data output was analysed using MasterPlex® QT: Multiplex Quantitative Analysis Curve-Fitting Software (Hitachi Solutions America Ltd, South San Francisco, CA, USA) with results reported in arbitrary units as relative mean fluorescence intensity. Additional information on Luminex can be found in 3.2.5d).

5.2.6 Gene expression analysis

a) RNA extraction

Described in chapter 3 (3.2.6a).

b) cDNA synthesis

Described in chapter 3 (3.2.6b).

c) Determination of reference genes

Described in chapter 3 (3.2.6c).

d) Array based RT-qPCR

Described in chapter 3 (3.2.6d). The signalling related genes included on the custom array plate were: Nrg1, Erbb2, Erbb3, Erbb4, Akt1, Akt2, Akt3, Jak1, Stat3, Pten, and Socs3 with Fbxw2 and Ap3d1 as reference genes, plus a positive control, reverse transcription control and mouse genomic DNA control. Each experiment represents n=3 (PFC) and n=4 (Hippocampus) biological samples, tested in triplicate.

5.2.7 Statistics

2-way ANOVAs were conducted on the PFC and hippocampus independently with the factors genotype and immune challenge. Independent samples t-tests were used for additional analysis. All analysis was conducted on fold change calculated using the Livak method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Significance was set at $p<0.05$. All data are presented as uncorrected p-values.

5.3 Results

5.3.1 ErbB2 mRNA expression levels higher in the prefrontal cortex of Nrg1 Het immune challenged mice

We first measured the expression levels of pan Nrg1 mRNA as well as the Nrg1 signalling receptors ErbB2, ErbB3 and ErbB4. In the PFC, the expression of Nrg1 mRNA did not show an interaction between genotype and immune challenge using 2-way ANOVA, however, genotype was found to have a main effect on expression levels (genotype, $F_{1,11}$=10.78, p=0.011). Similarly, in the hippocampus, no interaction was found, but a trend for a main effect of genotype was shown ($F_{1,13}$=4.72, p=0.055). Using independent samples t-test no significant differences were seen in Nrg1 mRNA expression between the four experimental groups in either the PFC of the hippocampus. The PFC however did display a tendency toward lower expression levels in the Nrg1 Het mice, at baseline and with the immune stimulus (p=0.079) (Figure 5.3-2).

The mRNA expression levels of the Nrg1 receptors: ErbB2, ErbB3 and ErbB4 were also measured. In the PFC, a genotype x treatment interaction was seen for ErbB2 ($F_{1,11}$=8.68, p=0.019) as well as a main effect of genotype ($F_{1,11}$=17.11, p=0.003). ErbB2 had elevated expression levels in Nrg1 Het challenged mice compared to WT challenged mice in the PFC (p<0.01; independent samples t-test). A trend towards lower expression levels in WT mice with the immune challenge compared to unchallenged WT controls was also seen in the PFC (p=0.057). The hippocampus showed no differences in expression levels for ErbB2 (Figure 5.3-2). ErbB3 and ErbB4 mRNA was not differentially expressed in either brain region (Table 5.3-1, PFC and Table 5.3-2, Hippocampus).

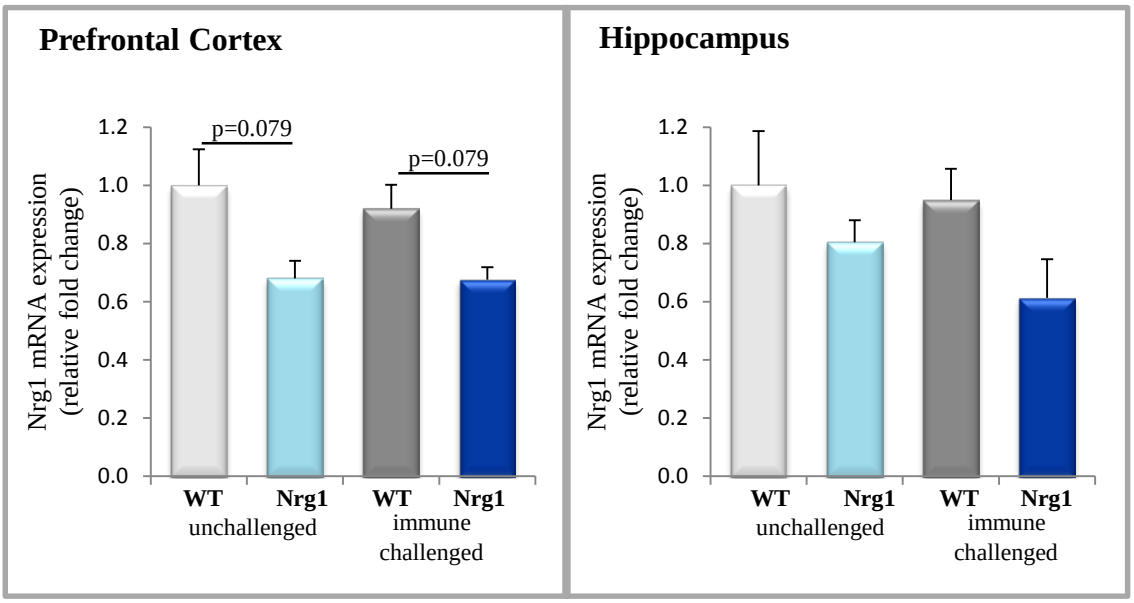

Figure 5.3-1: Nrg1 mRNA expression showed a tendency toward lower levels in the Nrg1 Het mice in the PFC

Nrg1 mRNA expression levels are shown for the PFC (left panel) and the hippocampus (right panel). 2-way ANOVA indicated a main effect of genotype in both regions, while independent samples t-test revealed only a tendency toward lower expression in the Nrg1 Het mice regardless of immune stimulation. Data represent mRNA as mean relative fold change ± SEM using RT-qPCR, n=3 pooled samples per group (PFC), n=4 per group (Hippocampus). WT unchallenged expression levels normalised to 1. (p values shown on graph; independent samples t-test).

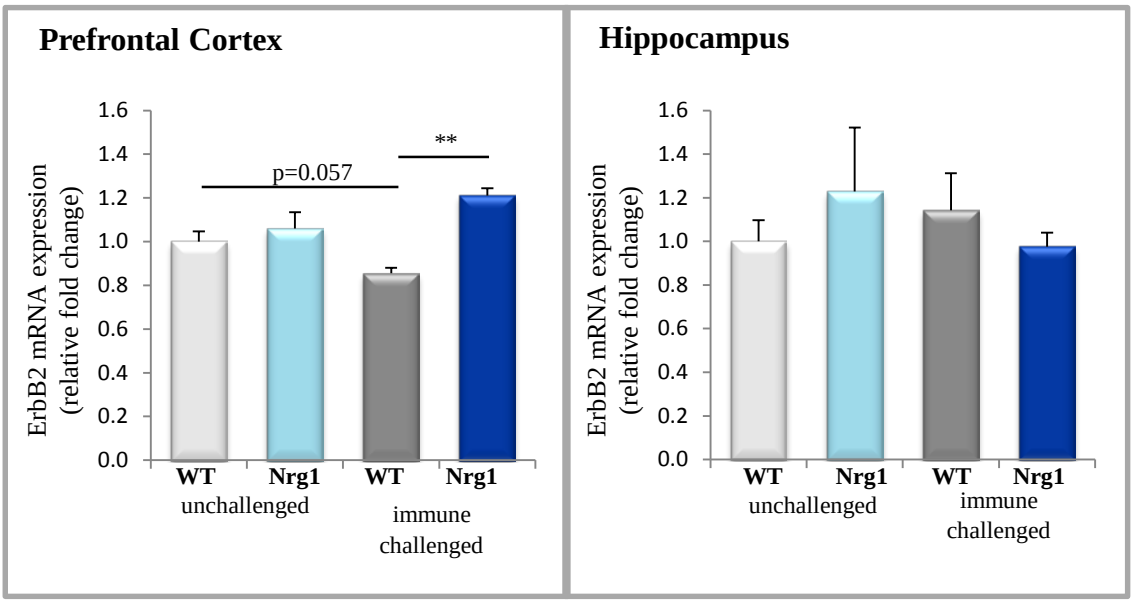

Figure 5.3-2: ErbB2 mRNA expression elevated in the PFC of immune challenged Nrg1 Het mice.

ErbB2 mRNA expression levels are shown for the PFC (left panel) and the hippocampus (right panel). A genotype x immune challenge interaction, with a main effect of genotype was indicated by 2-way ANOVA. Independent samples t-test revealed higher levels of ErbB2 in the PFC of Nrg1 Het mice with an immune challenge compared to the challenged WT, while a trend toward lower levels was seen in the WT challenged compared to WT unchallenged mice. Data represent mRNA as mean relative fold change ± SEM using RT-qPCR, n=3 pooled samples per group (PFC), n=4 per group (Hippocampus). WT unchallenged expression levels normalised to 1 (**$p<0.01$; independent samples t-test).

5.3.2 Akt mRNA levels

One of the major downstream signalling molecules of the Nrg1 pathway is Akt which is present in three main mRNA isoforms, Akt1, Akt2 and Akt3. All three isoforms were analysed using RT-qPCR. No interaction between genotype and immune challenge were seen using 2-way ANOVA, however, the PFC showed a main effect of genotype for Akt1 and Akt2 ($F_{1,11}$=10.98, p=0.011; $F_{1,11}$=7.99, p=0.022 respectively). No interactions or effects were seen in the mRNA expression levels in the hippocampus using 2-way ANOVA.

Further analysis shows Akt1 mRNA expression is higher in the PFC of unchallenged Nrg1 Het mice compared to unchallenged WT littermates ($p<0.05$; independent samples t-test). Both Akt1 and Akt 2 also show a trend towards higher mRNA expression levels in the PFC of Nrg1 Het mice subject to immune challenge compared to WT challenged mice (p=0.099; p=0.057 respectively; independent samples t-test). No differences between the groups were seen in the hippocampus when analysed with independent samples t-tests (Figure 5.3-3). Further, Akt3 expression levels remained consistent across both brain regions when analysed with 2-way ANOVA or independent samples t-test (Table 5.3-1, PFC and Table 5.3-2, Hippocampus).

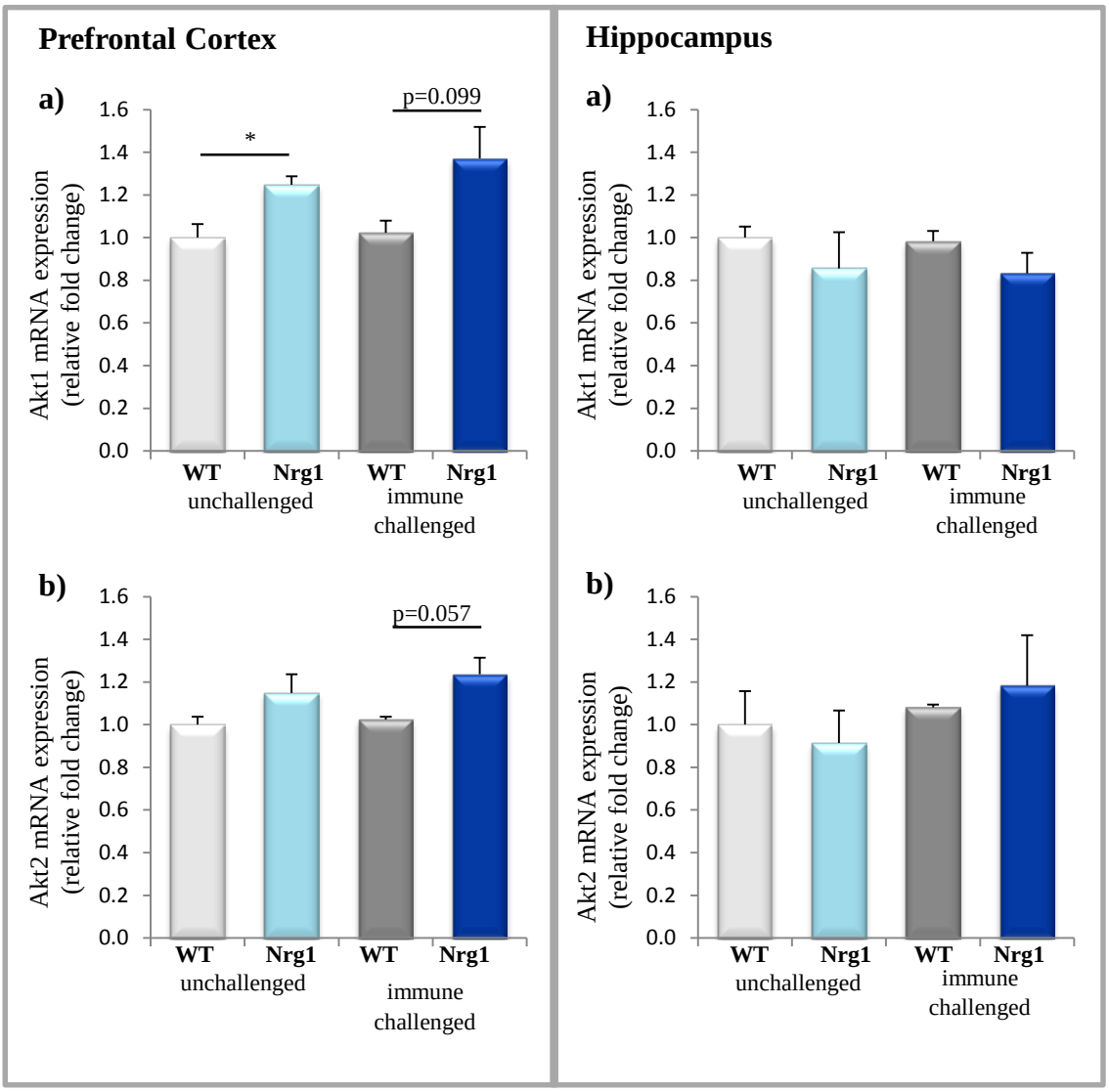

Figure 5.3-3: Higher levels of Akt1 mRNA expression at baseline in Nrg1 Het mice compared to WT littermates in the PFC with no change in Akt2.

mRNA expression levels are shown for the PFC (left panel) and the hippocampus (right panel): **(a)** Akt1 mRNA showed higher expression levels in the Nrg1 Het unchallenged mice along with a tendency toward higher levels in the immune challenged mice; **(b)** Akt2 mRNA demonstrated a trend to higher expression levels in the Nrg1 Het immune challenged mice compared to challenged WT. Data represent mRNA as mean relative fold change ± SEM using RT-qPCR, n=3 pooled samples per group (PFC), n=4 per group (Hippocampus). WT unchallenged expression levels normalised to 1 (*$p<0.05$; independent samples t-test).

5.3.3 Phosphorylated Akt protein levels lower in the hippocampus of Nrg1 Het mice without an immune stimulus.

With pAkt representing activated signalling of the PI3K/Akt pathway, the protein level of total Akt and pAkt was measured in the hippocampus using a Luminex bead based assay. Due to tissue limitation we were unable to measure the protein levels in the PFC. In the hippocampus, a genotype x immune challenge interaction was seen in Akt protein levels using 2-way ANOVA ($F_{1,32}$=4.486, $p<0.05$) whilst pAkt showed a tendency towards a genotype x immune challenge interaction ($F_{1,32}$=3882, $p=0.058$) with a main effect of genotype ($F_{1,32}$=6.127, $p<0.05$). Phosphorylated Akt protein was shown to be 41% lower in the Nrg1 unchallenged animal compared to WT in the hippocampus ($p<0.01$; independent samples t-test) (Figure 5.3-4). No between group effects were seen in the level of total Akt protein using independent samples t-test, however there was a trend toward lower protein levels (27%) in the Nrg1 unchallenged animals compared to WT ($p=0.056$).

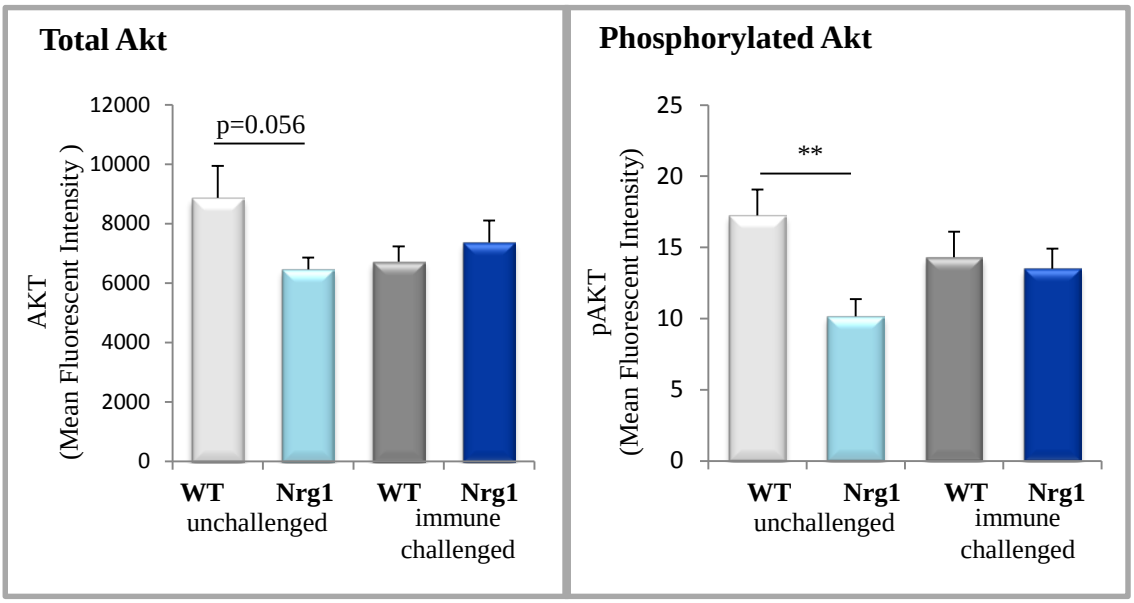

Figure 5.3-4: Total and phosphorylated Akt protein in the hippocampus

Phosphorylated Akt (right panel) was lower in the Nrg1 Het mice compared to WT without and immune challenge. Total Akt showed a trend towards similar lower levels but was not significant. Data represent mean fluorescent intensity using Luminex flow cytometry (mean ± SEM), n=8 per group except WT immune challenged where n=9 (**p<0.01; independent samples t-test).

5.3.4 Melanoma challenge results in higher Pten mRNA expression in the prefrontal cortex of Nrg1 Het mice

As a downstream effector of Akt signalling and shown to have an important role in cancer progression, we measured Pten mRNA expression levels. Analysis using 2-way ANOVA did not show any genotype x immune challenge interactions in either the PFC or the hippocampus. The hippocampus only demonstrated a trend toward a genotype main effect in Pten mRNA expression ($F_{1,13}$=4.93, p=0.051). Between groups analysis using independent samples t-test showed mRNA expression level for Pten was higher in the immune challenged Nrg1 mutant mice compared to the challenged WT mice in the PFC ($p<0.05$, independent samples t-test) while no differences were seen in the hippocampus (Figure 5.3-5).

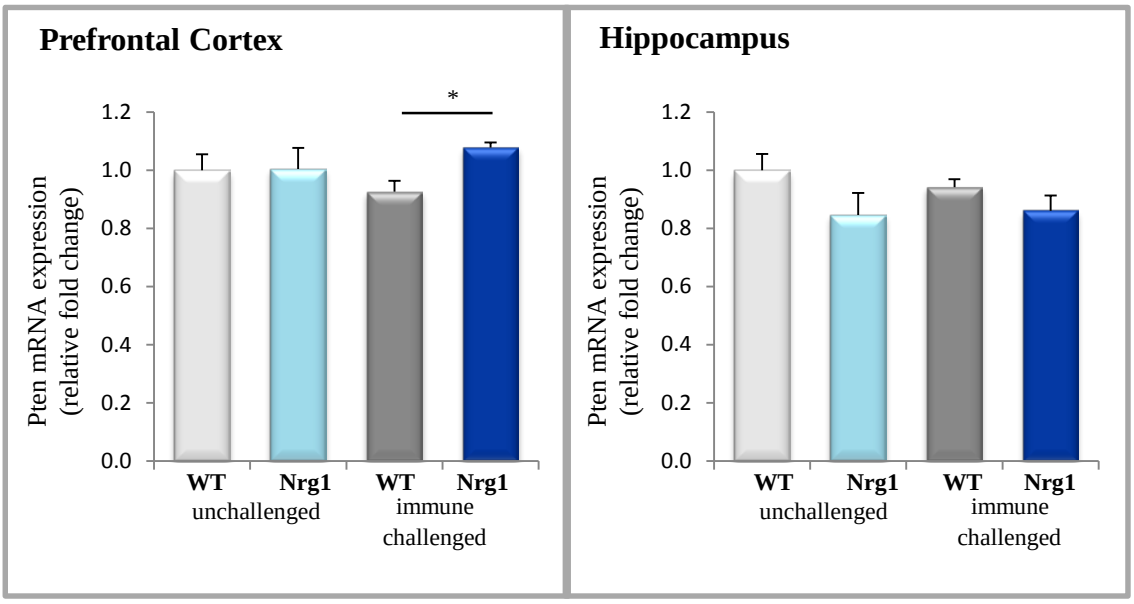

Figure 5.3-5: Elevated levels of Pten mRNA expression in the PFC of Nrg1 Het immune challenged mice.

The expression levels of Pten mRNA are shown for the PFC (left panel) and the hippocampus (right panel). Data represent mRNA as mean relative fold change ± SEM using RT-qPCR, n=3 pooled samples per group (PFC), n=4 per group (Hippocampus). WT unchallenged expression levels normalised to 1 (*$p<0.05$; independent samples t-test).

5.3.5 Jak1 mRNA expression is reduced in the hippocampus of immune challenged Nrg1 Het mice

With the Jak/Stat pathway one of the possible common pathways linking Nrg1, IL-6 and G-CSF measured Jak1 and Stat3 were. Jak1 mRNA expression did not demonstrate a genotype x immune challenge interaction in either brain region using 2-way ANOVA. However both the PFC and the hippocampus showed a main effect of genotype on Jak1 expression ($F_{1,11}$=10.85, p=0.011; $F_{1,13}$=5.54, p=0.040 respectively). Lower expression of Jak1 mRNA was found in the hippocampus of Nrg1 immune challenged mice compared to challenged WT ($p<0.05$; independent samples t-test), while a trend toward higher levels of mRNA expression in the same groups was seen in the PFC (p=0.070; independent samples t-test) (Figure 5.3-6). Stat3 mRNA was stably expressed in both regions (Table 5.3-1, PFC and Table 5.3-2, Hippocampus).

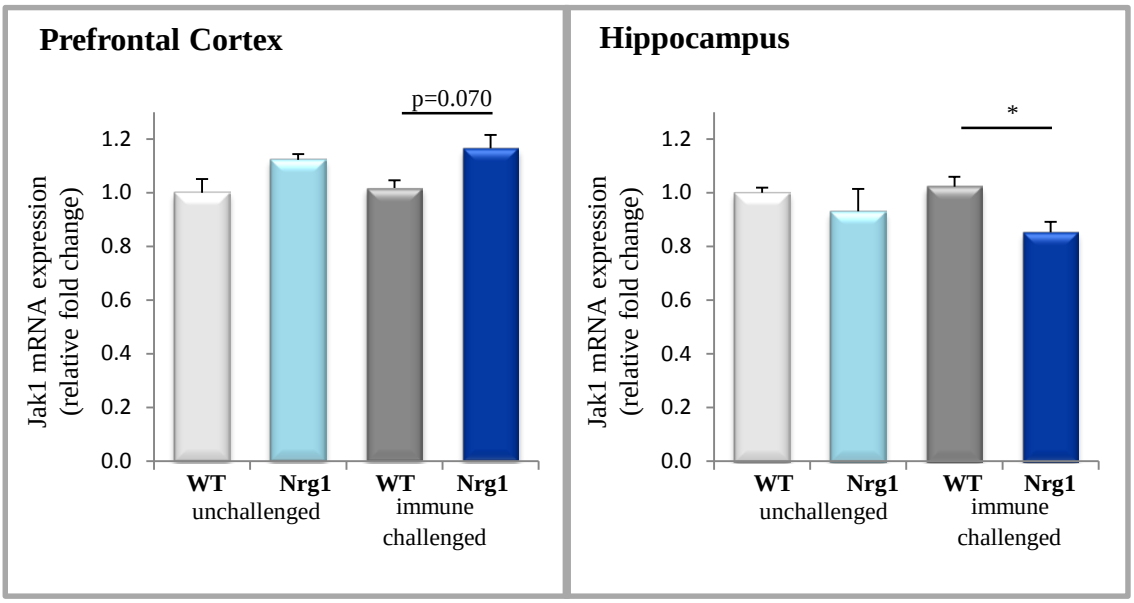

Figure 5.3-6: Lower levels of Jak1 mRNA expression in Nrg1 Het immune challenged mice compared to challenged WT in the hippocampus.

Expression levels of Jak1 mRNA are shown for the PFC (left panel) and hippocampus (right panel). Data represent mRNA mean relative fold change ± SEM using RT-qPCR, n=3 pooled samples per group (PFC), n=4 per group (Hippocampus). WT unchallenged expression levels normalised to 1 (*$p<0.05$; independent samples t-test).

5.3.6 Nrg1 transmembrane domain mutation results in elevated SOCS3 mRNA expression in the prefrontal cortex of affected mice

As a major cytokine regulator, particularly relating to the Jak/Stat pathway we also measured the mRNA expression levels of SOCS3. SOCS3 mRNA expression demonstrated a main effect of genotype using a 2-way ANOVA (genotype, $F_{1,11}$=5.33, p=0.050) in the PFC. Further between groups analysis showed SOCS3 mRNA expression to be higher in the PFC of Nrg1 Het unchallenged mice when compared to unchallenged WT animals (p=0.012; independent samples t-test). No differences were found in the mRNA expression levels of SOCS3 in melanoma challenged animals in the PFC and mRNA expression of SOCS3 was stable in the hippocampus (Figure 5.3-7)

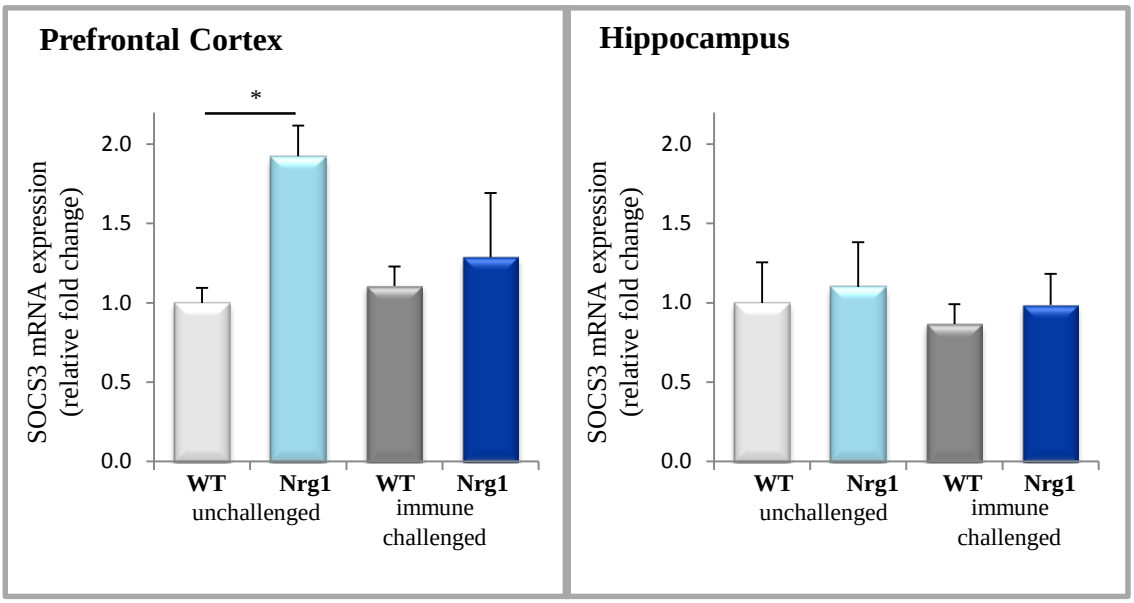

Figure 5.3-7: Elevated levels of SOCS3 mRNA expression at baseline in Nrg1 Het mice in the PFC.

The expression levels of SOCS3 mRNA are shown for the PFC (left panel) and the hippocampus (right panel). Data represent mRNA as mean relative fold change ± SEM using RT-qPCR, n=3 pooled samples per group (PFC), n=4 per group (Hippocampus). WT unchallenged expression levels normalised to 1 (*p<0.05; independent samples t-test).

Table 5.3-1: Signalling molecule mRNA expression levels in the PFC of Nrg1 Het and WT mice with or without an immune stimulus.

PFC	***Control***		***Immune challenged***	
	WT	Het	WT	Het
ErbB3	1.00 ± 0.29	1.23 ± 0.27	1.14 ± 0.09	1.26 ± 0.04
ErbB4	1.00 ± 0.10	1.10 ± 0.06	1.08 ± 0.05	1.19 ± 0.07
Akt3	1.00 ± 0.05	0.95 ± 0.01	0.92 ± 0.03	0.99 ± 0.04
STAT3	1.00 ± 0.06	1.27 ± 0.15	1.07 ± 0.03	1.23 ± 0.15

Data represent mean fold change ± SEM, n=3 pooled samples per group (PFC), n=4 per group (Hipp). WT expression levels normalised to 1 and fold change for other groups calculated as a comparison.

Table 5.3-2: Signalling molecule mRNA expression levels in the hippocampus of Nrg1 Het and WT mice with or without an immune stimulus.

Hipp	***Control***		***Immune challenged***	
	WT	Het	WT	Het
ErbB3	1.00 ± 0.23	1.12 ± 0.26	0.82 ± 0.13	0.96 ± 0.16
ErbB4	1.00 ± 0.04	1.07 ± 0.12	0.93 ± 0.01	0.96 ± 0.04
Akt3	1.00 ± 0.17	1.13 ± 0.05	1.21 ± 0.04	1.17 ± 0.04
STAT3	1.00 ± 0.14	0.99 ± 0.19	1.05 ± 0.03	1.09 ± 0.08

Data represent mean fold change ± SEM, n=3 pooled samples per group (PFC), n=4 per group (Hipp). WT expression levels normalised to 1 and fold change for other groups calculated as a comparison.

5.4 Discussion

This study analysed a number of molecules associated with the signalling pathways PI3K/Akt and Jak/Stat, as both pathways are common to a variety of Nrg1, IL-6 or G-CSF functions. This preliminary exploration, using limited sample numbers, demonstrated changes in mRNA expression levels of ErbB2, Akt1, Pten, Jak1 and SOCS3 in Nrg1 Het mice either as a consequence of the genetic mutation or in response to an immune challenge, with no change found in Nrg1 mRNA expression itself. In addition, the protein levels of pAkt also displayed alteration in the hippocampus in the presence of the immune challenge.

5.4.1 Unchanged expression of Nrg1 mRNA

The unchanged Nrg1 mRNA expression levels (Figure 5.3-1) may appear unexpected in a heterozygous mutant mouse model. However a main effect of genotype was shown using a ANOVA in the PFC, with a trend towards the same effect in the hippocampus, which is consistent with previous findings (Long et al., 2010a). In the Nrg1 Het mice, Long et al (2010a) showed reduced mRNA expression of the transmembrane and intracellular domains of Nrg1 coupled with unchanged expression of the common extracellular epidermal growth factor-like domain. The present study used a commercial primer for pan Nrg1 detection which did not target the specific Nrg1 domains targeted by Long et al (2010a). Therefore, the lack of difference shown for mRNA expression of Nrg1 using a non-specific primer design provides similar results to this found in the PFC and hippocampus in the Long et al study (2010a) and the possibility remains that the Nrg1 Het gene mutation results in an underlying change in a biologically relevant specific domain of the Nrg1 mRNA transcript. Additional data (Long et al, unpublished) show Nrg1 protein levels unchanged in both the PFC and the

hippocampus in these mice. Therefore it is suggested that the Nrg1 Het model cannot be characterised as either a simple gain or loss of function model due to the mutation (Long et al., unpublished). Further investigations measuring protein levels and functionality will elucidate the exact effect of the Nrg1 Het mutation in these mice.

Despite the lack of insight into the exact nature of the Nrg1 mutation effects, with alterations not apparent from mRNA expression studies or protein investigations (Figure 5.3-1; Long et al., 2010a; Long et al., unpublished), a range of experimental results in these mice demonstrate significant and schizophrenia-relevant alterations do occur as a consequence of the mutation. Nrg1 Het mice were shown to have alterations in both behavioural and cognitive characteristics (Chesworth et al., 2012; Duffy et al., 2010; Karl et al., 2007). Further, a series of studies on the effects of the main active constituents of cannabis (Delta(9)-tetrahydrocannabinol and cannabidiol) found distinct biological and behavioural effects in the Nrg1 Het mice (Boucher et al., 2007a, 2007b, 2010, Long et al., 2010b, 2012, 2013). In addition, Desbonnet et al (2012) demonstrated specific interactions between chronic stress paradigms and the response of the Nrg1 Het mice. These studies together with our findings of alterations in the immune response underline the continuing relevance of the Nrg1 Het mouse model in studies of schizophrenia pathophysiology, in particular when combined with additional environmental risk factors (Karl, 2013). Further, although not indicated by the mRNA expression results presented in this study, the search for alterations in Nrg1 abundance or function in the Nrg1 Het mice is indicated by the behavioural and cognitive studies discussed above. Alterations are assumed to exist but continuing studies are necessary to clarify the underlying biochemical mechanisms.

5.4.2 ErbB2 mRNA expression was elevated in the PFC of Nrg1 animals subject to immune challenge

The immune challenged Nrg1 Het animals displayed elevated levels of ErbB2 mRNA expression compared to the challenged WT with a genotype by treatment interaction seen in the PFC (Figure 5.3-2). Additionally, a tendency towards lower expression of ErbB2 was seen in the immune challenged WT challenged animals in the PFC. No changes in ErbB2 mRNA expression were seen in the hippocampus, and there were no differences in the expression of ErbB3 or ErbB4 mRNA. This suggests that the expected response to a melanoma challenge is to maintain or reduce availability of ErbB2 for signalling in the PFC and that this response is reversed in Nrg1 Het mice.

ErbB2 does not bind Nrg1 and an ErbB2 dimer cannot initiate the Nrg1 signalling cascade. A heterodimer of ErbB2 with ErbB3 or ErbB4 is required for signalling (Mei and Xiong, 2008). Ctiri and Yarden (2006) suggest ErbB2 is a "non-autonomous amplifier of the ErbB signalling network", suggesting that the higher level of ErbB2 expression seen in response to an immune challenge in the Nrg1 mice in this study could be a simple compensatory mechanism if reduced Nrg1 signalling is assumed. Despite unchanged ErbB3 and ErbB4 expression levels, additional ErbB2 may allow for more heterodimer formation, increasing signalling. In fact, Hahn et al. (2006) previously demonstrated an increase in ErbB2/ ErbB4 heterodimers in a human post-mortem study. Should subsequent studies demonstrate that Nrg1 abundance and ability to signal is not impaired in these animals, the alteration in ErbB2 mRNA expression levels would likely not represent a compensatory mechanism. In this case current literature does not provide for an alternate theory that would result in the changes seen

in ErbB2. Further experimental investigation in alternate pathways for ErbB2 up- and down- regulation would be required to interpret these data.

5.4.3 Higher level of Akt1 mRNA in the prefrontal cortex of Nrg1 Het mice at baseline

Nrg1 activation of the PI3K/Akt signalling pathway is involved in many neuronal cell functions also relevant to the development of schizophrenia, including growth, proliferation and migration (Matheny and Adamo, 2009). Therefore the mRNA levels of Akt -1,-2 and -3 were measured in the PFC and the hippocampus, with protein levels of total Akt and pAkt measured in the hippocampus only. Protein levels in the PFC were not measured in this study, as tissue limitations prevented performance of the assay.

Higher levels of Akt1 mRNA expression in the PFC were shown in Nrg1 Het mice without an immune challenge when compared to the WT unchallenged mice (Figure 5.3-3). A tendency for higher expression levels of Akt1 was seen in the immune challenged Nrg1 Het mice when compared to the challenged WT mice in the PFC (Figure 5.3-3). Additionally, Akt2 mRNA expression levels in the immune challenged Nrg1 Het mice also showed a tendency toward increase when compared to challenged WT mice in the same brain region (Figure 5.3-3).

Polymorphisms in the Akt1 gene have been associated with susceptibility to schizophrenia (Thiselton et al., 2008). Akt1 null mice have a reduced number of brain cells, although of normal size where Akt1 represents one third of the Akt isoforms present in the brain, Akt3 one half and Akt2 the remaining one sixth of Akt isoforms in the brain (Easton et al., 2005). In addition, lower levels of Akt1 protein, but not Akt2 or Akt3, were found in a post-mortem cohort study of schizophrenia patients (Emamian et

al., 2004). While these studies demonstrate a lack of or decrease in Akt1 is related to schizophrenia-like pathology, the present study found the opposite with an increase in Akt1 mRNA expression. If the changes in Akt1 in this study are a downstream effect of the presumed alteration in Nrg1 signalling, the increase, whilst not consistent with mutant models lacking complete pathway elements, may be a compensatory mechanism for changes in other pathway molecules not studied here.

5.4.4 Phosphorylation of Akt in the hippocampus was lower in the un-stimulated Nrg1 Het mice compared to WT controls

Protein levels of pAkt were lower in the hippocampus of Nrg1 Het mice without an immune challenge. The level of total Akt also tended towards lower levels in these mice (Figure 5.3-4) suggesting less overall protein availability, and therefore hypo- activity of this pathway in the hippocampus as a result of the Nrg1 mutation. Since Nrg1/ErbB signalling preferentially uses the PI3K/Akt pathway for signalling and with pAkt being a marker of pathway activation, this reduction in the hippocampus based on genotype provides an added indication that Nrg1 signalling is disrupted in the mutant mice, despite the specific change in Nrg1 remaining elusive (Keri et al., 2009; Mei and Xiong, 2008). Interestingly, when combined with an immune challenge, the levels of total and pAkt did not differ between the Nrg1 Het and the WT animals.

This reduction in pAkt, with the possible reduction of total Akt substrate, is likely to further promote a range of alterations in downstream signalling and these changes may be more likely to present the genetic mutation is combined with an environmental insult, such as the immune challenge used in this study. In particular, the return of pAkt and Akt to levels consistent with the challenged WT mice in the Nrg1 Het mice subject to an immune challenge may find a speculative mechanism from an earlier finding. In

the IL-6 and its receptor molecules chapter, it was shown that sgp130 was reduced in the hippocampus of Nrg1 Het mice not subject to the immune stimulus, with a further decrease shown following the immune challenge; this same reduction in levels was seen when comparing the WT immune stimulated mice with the un-stimulated WT controls (Figure 3.3-4). The lower levels in unchallenged Nrg1 Het mice may represent an apparent consequence of the genetic mutation; however the further reduction in challenged mice was unexplained.

With the IL-6/IL-6Rα/gp130 signalling complex capable of activating the PI3K/Akt pathway via phosphorylation of Akt and sgp130 acting as an inhibitor of IL-6/sIL-6Rα trans-signalling, a decrease in sgp130 would remove this inhibition (Jostock et al., 2001; Koyasu, 2003). This reduction in inhibition of the IL-6/IL-6Rα/gp130 pathway may potentially provide additional Akt phosphorylation activity, therefore resulting in the lower level of pAkt seen in unchallenged Nrg1 Het animals returning to the same level as the WT when both are subject to an immune challenge. The WT mice show a similar reduction in sgp130 but pAkt is unchanged between the immune challenged and unchallenged animals. This suggests that if the changes in pAkt are actually linked to sgp130 in the Nrg1 mice there must be an interaction between the Nrg1/ErbB and the IL-6/IL-6Rα/gp130 signalling pathways in an analyte that was not measured here. This speculative mechanism would require thorough investigation to determine which, if any, of these molecular changes are dependent on others in the Nrg1 Het mice.

From this we can determine that a schizophrenia-relevant mutation of Nrg1 can act on the PI3K/Akt pathway at least to the extent of mRNA expression changes in the PFC and the constitutive levels of Akt and phosphorylated Akt protein. This could potentially influence the interaction of the PI3K/Akt pathway within the immune

system, particularly in the regulation of IL-6 and the functions of G-CSF. While the present study hints at interactions, further exploration of the Akt isoforms at protein levels, along with phosphorylation and functional studies would be required to gain any detailed understanding of pathway effects and inform understanding of mechanisms or potential outcome effects.

5.4.5 Higher levels of the PI3K/Akt pathway negative regulator Pten seen in Nrg1 immune challenged mice

Also called the tumour suppressor gene, Pten is mutated in several human cancers and has also been shown to play an important role in brain development (Amiri et al., 2012; Endersby and Baker, 2008; Li et al., 1997; Williams et al., 2015). Brain defects linked to mutations in Pten include macrocephaly, seizures and mental retardation (Endersby and Baker, 2008; Orrico et al., 2009; Waite and Eng, 2002) although these defects have not been directly related with schizophrenia. In this study, the Nrg1 Het mice subject to the melanoma challenge showed elevated mRNA expression levels of Pten in the PFC when compared to WT challenged animals. As Pten is a negative regulator of the PI3K/Akt pathway (Wu et al., 1998), a higher level of mRNA expression might be expected to reduce the expression or activation of Akt if translated into functional protein. With tissue limitations preventing the measurement of total Akt and pAkt protein in the PFC, and protein levels of Pten not measured, it is not possible to draw further conclusions on the relevance of the Pten increase in Nrg1 Het challenged mice, other than that this increase is possibly a result of an overactive PI3K/Akt pathway in the PFC or that the expression on Pten had been specifically influenced by the Nrg1 mutation combined with the immune response and would result in a marked reduction in PI3K/Akt activation in this brain region. While the increased level of Pten in immune

challenged Nrg1 Het mice needs to be mentioned, further studies are required to determine the biological relevance of this elevated Pten mRNA.

5.4.6 Jak1 mRNA expression levels were decreased in the hippocampus of Nrg1 Het mice with an immune stimulus

The mRNA expression levels of Jak1, shown to be a critical factor in IL-6 and G-CSF signalling, and Stat3, the major downstream mediator of Jak1 activity, were measured in the PFC and hippocampus of Nrg1 Het and WT mice with or without an immune stimulus (Kopantzev et al., 2002; Shimoda et al., 1997). Jak1 mRNA was shown to be expressed at a reduced level in the hippocampus of Nrg1 Het mice subject to the immune challenge compared to challenged WT animals (Figure 5.3-6). No further difference in Jak1 mRNA expression was seen although there appears to be a tendency towards higher levels in the PFC of the same groups. No differences were seen in Stat3 in either brain region.

The previous study of G-CSF levels in the brain of Nrg1 Het mice (see Chapter 4) demonstrated that G-CSF levels are reduced in the hippocampus of the challenged Nrg1 Het mice compared to challenged WT littermates (Figure 4.3-1). With the G-CSF receptor representing a moderate activator of the Jak/Stat pathway in neurons (Schneider et al., 2005b), it is viable that a reduction in G-CSF signalling could result in a reduced need for Jak1, and the resulting reduction in Jak1 mRNA expression seen here. Despite the neuronal preference for G-CSF to activate the PI3K/Akt pathway over the Jak/Stat pathway (Schneider et al., 2005b), no differences were found in mRNA expression or protein levels of Akt in the same group in the hippocampus, indicating that the reduced G-CSF levels may result in aberrant Jak/Stat signalling in the Nrg1 Het mice when subject to an immune challenge. With no clear-cut picture to be observed in

our study, this interaction requires further examination in the current experimental system.

5.4.7 Basal mRNA expression levels of SOCS3 were elevated in Nrg1 Het mice

SOCS3 is a critical negative regulator of both the IL-6 and G-CSF signalling pathways (Croker et al., 2004; White and Nicola, 2013). With SOCS3 mRNA expression increased in the PFC of Nrg1 Het mice without an immune challenge, it is proposed that the Nrg1 mutation itself creates an overexpression of SOCS3 in these animals. Given the Nrg1 Het animals subject to an immune challenge demonstrated lower levels of IL-6 in the PFC and no changes in G-CSF, the focus here is on the potential interaction between this baseline increase in SOCS3 and the reduction in IL-6 following immune challenge. Under basal conditions, mRNA and protein levels of SOCS3 are highly regulated (Starr et al., 1997). The expression of SOCS3 mRNA can however, be rapidly induced by a number of cytokines. The rapid induction of SOCS3 by multiple cytokines initially led to the understanding of SOCS3 as an anti-inflammatory, immunosuppressive agent (White and Nicola, 2013; Yasukawa et al., 2003). Further study using knockout mouse models has elucidated more specific functional roles for SOCS3. Although the full body SOCS3 knock is embryonically lethal, targeted cell specific deletion models have proven viable (Roberts et al., 2001; White and Nicola, 2013). Deletion of SOCS3 in neurons led to faster recovery from acute spinal injury while deletion in oligodendrocytes provided protection from drug-induced demyelination and apoptosis (Emery et al., 2006; Okada et al., 2006). However, using deletion models does not provide direct evidence for the role of increased levels of SOCS3 under basal conditions.

SOCS3 is rapidly induced by increased IL-6 in a negative feedback loop where increased SOCS3 limits the IL-6 response, similarly, impaired SOCS3 enhances IL-6 signalling (Croker et al., 2003; Fischer et al., 2004; Schmitz et al., 2000). While SOCS3 can inhibit the IL-6 response, this has been shown to occur with different potencies in different tissues (Croker et al., 2003). Given the elevated level of SOCS3 mRNA expression in the PFC of Nrg1 Het mice without an immune stimulus, and the concurrent lower level of IL-6 protein in the PFC of Nrg1 Het mice subject to the immune challenge, it is conceivable that the Nrg1 mutation itself interferes with SOCS3 expression and the addition of an immune stimulus results in the inability of IL-6 to maintain protein levels as seen in the WT animals with or without the immune stimulus in the PFC.

It is also interesting to note the elevated levels of Pten mRNA expression in Nrg1 Het mice subject to the immune stimulus. Previous studies have demonstrated that the combined deletion of SOCS3 and Pten results in synergistic promotion of axonal regeneration (Luo and Park, 2012; Sun et al., 2011). This suggests possible links between SOCS3, Pten and IL-6 levels, demonstrating that the findings of this study are consistent with existing hypotheses of the analytes acting together.

5.4.8 Conclusion

Overall this study represents a preliminary investigation of the potential signalling pathways involved in the IL-6 and G-CSF alterations shown in the PFC and the hippocampus of Nrg1 Het mice. This study presents mRNA expression comparison data and a single protein investigation into total and pAkt in the hippocampus. Alterations were shown in the mRNA expression of a number of the pathway molecules studied, however, given the exploratory nature of the study, the low sample number, and the

focus on mRNA, limited conclusions can be drawn and the role of each mRNA expression level change described can only be considered speculative.

As noted in earlier chapters, mRNA levels and protein levels are often not correlated and in most cases the prediction of protein abundance from gene expression data is insufficient, and this remains true for the molecules discussed here (Anderson and Seilhamer, 1997; Gygi et al., 1999). Still, alterations in mRNA levels indicate alterations in cell activation and give reason to further studies. Additionally, the changes in ErbB2, Akt1, Pten, Jak1 and SOCS3, as well as protein level changes in pAkt provide data to suggest further investigations is warranted in both the PI3K/Akt and the Jak/Stat pathways in the Nrg1 Het mice in conjunction with immune stimuli. In particular, the elevated expression of Akt1 and SOCS3 in the unchallenged Nrg1 Het mice suggests that the PI3K/Akt pathway may be primed to a dysfunctional response even before the immune stimulus. Further, the elevated levels of ErbB2 and Pten in the Nrg1 Het immune challenged animals further indicate a role for the PI3K/Akt signalling pathway. The lower level of Jak1 expression in the hippocampus may be a direct result of the G-CSF changes in this brain region, however, Jak 1 has also been shown to affect PI3K/Akt signalling.

To determine a more complete picture and gain insight into the potential signalling mechanisms, future investigations would need to include repeat mRNA studies as well as protein and functional phosphorylation assays. Such studies may also consider the possible differences between long-term immune stimulation as presented here, and acute pathway stimulation. Therefore, while beyond the scope of this study, investigations into protein and functionality are the next step in understanding the Nrg1 gene and immune stimulation interactions.

A demonstration of neuroimmune interactions between the candidate schizophrenia vulnerability gene Neuregulin 1, and peripheral and central cytokine levels: focus on IL-6 and G-CSF

CHAPTER SIX

Conclusion and future directions

Peta Snikeris
University of Wollongong

6.1 Conclusion and future direction

This study aimed to determine if the mutation of a gene originally identified as a schizophrenia vulnerability gene would influence the peripheral immune response. Further, the study proposed that such a mutation would also change the production of cytokines in the brain in the presence of a peripheral immune stimulus. In order to examine this hypothesis, the Nrg1 Het mutant mouse model, a model of schizophrenia-like genetic vulnerability, was subjected to a direct immune stimulus in the form of a B16F0 melanoma. Following the immune challenge, a range of peripheral immune measures were obtained, with a focus on cytokine responses. With alterations determined in IL-6 and G-CSF in the periphery, these two cytokines were further investigated in the brain. The levels of IL-6 and G-CSF in the PFC and the hippocampus were measured, along with their obligate signalling receptors and displayed a range of alterations. Finally two pathways, PI3K/Akt and Jak/Stat, common to Nrg1, IL-6 and G-CSF signalling were subject to a preliminary investigation of mRNA expression levels to determine if the dysfunction in protein could be linked to a shared signalling pathway. The results shown in this thesis provide evidence of my original contribution to science, demonstrating the following:

1) A Nrg1 mutation resulted in higher levels of IL-6 in the periphery in response to a direct peripheral immune stimulus in mice (Chapter 2). Importantly, similar elevations in IL-6 levels were shown in human schizophrenia patients and first degree relatives with a Nrg1 mutation (Marballi et al., 2010);

2) Immune stimulation resulted in various alterations of IL-6 and its receptors in the PFC and the hippocampus of Nrg1 mutant mice dependent on brain region or analyte studied (Chapter 3);

3) For the first time, an alteration in circulating plasma G-CSF levels and G-CSF in the hippocampus was shown in response to a peripheral immune stimulus in Nrg1 Het mice (Chapters 2 and 4);

4) A preliminary analysis of two signalling pathways involved in Nrg1, IL-6 and G-CSF functioning in the brain suggests further investigation of signalling molecules that were found altered. Although with limitations, the data provide indication that activation and function in the common pathway PI3K/Akt is warranted (Chapter 5).

Although animal models, including our Nrg1 mutant mice, do not entirely reflect human psychopathologies, our studies are a fundamental step in identifying neuroimmune mechanisms that may contribute to the translation of the genetic vulnerability conferred by a Nrg1 mutation into immune system dysfunction and the psychopathological disruption of brain signalling potentially relevant for schizophrenia.

6.1.1 The results reflect gene; immune; and gene x immune interactions

With the underlying premise that genetic vulnerability combined with an immune stimulus will lead to disease development or worse disease outcomes, the results can also be reconsidered as they relate to genotype; immune stimulus; and the genotype x immune stimulation interaction. A general overview of all findings from the present study is presented in (Table 6.1-1).

Table 6.1-1 Overview of thesis findings

Group wise comparison	**Nrg1 no ic *vs* WT no ic (A)**	**WT ic *vs* WT no ic (B)**	**Nrg1 ic *vs* Nrg1 no ic (C)**	**Nrg1 ic *vs* WT ic (D)**
Plasma cytokines			↑IL-6	↑IL-6
			↑G-CSF	↑G-CSF
		↑IL-5		
		↑KC		
Prefrontal cortex			↓IL-6 (p)	
				↑IL-6Rα (p+m)
				↑ErbB2 (m)
	↑Akt1 (m)			
				↑Pten(m)
	↑SOCS3 (m)			
Hippocampus		↓sgp130 (p)	↓sgp130 (p)	
		↑G-CSF (p)		↓G-CSF(p)
	↓pAkt (p)			
				↓Jak1 (m)
Proposed influence	Genotype	Immune stimulation	Genotype x immune	Genotype x immune

ic = immune challenged, no ic = without immune challenge, p = protein measurement, m = mRNA expression level measurement. Only changes with significant p values from t-test analysis shown.

Changes shown in the unchallenged Nrg1 Het mice, when compared with unchallenged WT mice could be considered to result from the genetic mutation. From this perspective, with Akt1 and SOCS3 mRNA higher in the PFC, yet lower pAkt levels in the hippocampus, the PI3K/Akt signalling pathway is implicated as a potential aberrant pathway effecting later changes revealed in conjunction with the immune stimulus (Table 6.1-1: A). Since all three molecules are related to the PI3K/Akt signalling

pathway, it may be considered that these brain regions could be primed to respond differently to any stimulus that will influence the activity of this pathway in the presence of this Nrg1 mutation.

When WT mice are stimulated with the melanoma challenge, the immune response demonstrated compared to un-stimulated WT can be taken to represent a normal immune response under these experimental conditions (Table 6.1-1: B). The increase in IL-5 and KC in the plasma confirms an effective peripheral immune response to the B16F0 melanoma challenge. The reduction in sgp130 and the increase in G-CSF in the hippocampus validate that the peripheral immune stimulus is able to alter cytokines in the brain without the influence of a genetic mutation.

Further evidence was provided to suggest that genotype plays a significant role in the immune response to a peripheral immune challenge in both the periphery and the brain. If it is assumed from the baseline results that the Nrg1 Het mutation has primed the PI3K/Akt pathway for an altered response, the decrease in IL-6 in the PFC and the decrease in sgp130 in the hippocampus could both potentially result from, or contribute to, aberrant signalling (Table 6.1-1: C). Interestingly the sgp130 receptor was reduced in both WT and Nrg1 Het mice subject to an immune stimulus when compared to unchallenged WT and Nrg1 Het mice respectively. In this case the lower levels seen in the Nrg1 Het mice may be consistent with a normal immune response, or may represent a similar alteration but occur as a result of a completely different mechanism. The PI3K/Akt pathway also has the potential to influence both IL-6 and G-CSF production in the periphery, however as no pathway molecules were measured in peripheral cells any interaction is purely speculation. The differences between immune challenged and the unchallenged Nrg1 Het mice therefore represent an irregular immune response in the

context of the gene mutation, providing strong evidence for the pathological potential of a gene x immune interaction in this model.

The final comparison considered was between the immune challenged Nrg1 Het and immune challenged WT mice where the differences point to potentially divergent outcomes of the genotype x immune challenge. The brain outcomes vary noticeably between the expected response to an immune stimulus shown in the WT mice and the immune response in the Nrg1 Het mice subject to the immune challenge (Table 6.1-1: D). In particular, additional support can be found for aberrant PI3K/Akt signalling with higher mRNA expression levels of the PI3K/Akt inhibitory molecule, Pten in the PFC. The hippocampus on the other hand, provides the first evidence for involvement of a potential second signalling pathway with reduced levels of Jak1 mRNA expression. It is possible, that the reduced level of Jak1 expression is a direct consequence of the lower levels of G-CSF in the hippocampus. Alternatively, as G-CSF preferentially utilises the PI3K/Akt pathway in the brain and Jak1 can directly interact with PI3K/Akt, the possibility remains that lower expression of Jak1 is related to the PI3K/Akt pathway alterations thought to exist in this model. Altogether, these data confirm a critical pathological potential of a Nrg1 mutation to prime the peripheral immune and the neuroimmune system for changes relevant to the development of schizophrenia.

6.1.2 Potential interactions with neurotransmitters: Relevance of changes in CNS cytokine levels in response to a peripheral immune stimulus to the study of schizophrenia

Although alterations in IL-6 and G-CSF in the PFC and the hippocampus of Nrg1 Het mice with an immune stimulus may not appear directly linked to schizophrenia, there are several lines of evidence to show that these changes could have impacts on

neurotransmission, relevant to the disease. Not only Nrg1, but also IL-6 and G-CSF have been shown to influence schizophrenia-relevant neurotransmitter systems, including dopamine, glutamate, GABA and serotonin.

IL-6 is a critical neuronal survival factor during development and adulthood, however IL-6 and schizophrenia relevant neurotransmission interactions are also demonstrated (Bauer et al., 2007; Dunn, 2006; McAfoose and Baune, 2009). In particular, low IL-6 concentration in dopaminergic neuronal cell cultures had protective effects while increasing doses of IL-6 resulted in neurotoxicity (Li et al., 2009c) The effect of IL-6 on glutamatergic neurons appears to demonstrate a 'U' shaped curve *in vitro*; with both high and low doses proving neurotoxic while a moderate level of IL-6 had neuroprotective effects (Wang et al., 2009b) Further, age related loss of GABAergic interneurons can be prevented by targeted deletion of IL-6 while an overexpression of IL-6 in mice resulted in degeneration of these interneurons (Dugan et al., 2009; Samland et al., 2003). These existing findings, together with the increased levels of plasma IL-6 yet reduced levels of IL-6 protein found in the PFC of Nrg1 mice show it is possible dopamingergic, glutamatergic and GABAergic neurons are affected by a peripheral immune stimulus. Any potential effects may be dependent on age, type of immune stimulus (innate, adaptive, humoral or cellular, peripheral or central, acute or chronic) and the intensity of response; however the likelihood of an interaction is high.

G-CSF has been extensively studied for the neuroprotective functions (Li et al., 2009d; Schneider et al., 2005a, 2005b). G-CSF knockout mice, show reduced NMDA-R density in the dendritic layers of the hippocampus (CA3 and dentate gyrus) with enhanced GABAa receptor binding density in the same areas of the hippocampus and the dentate gyrus (Diederich et al., 2009a). This would suggest a potential reduction in

excitatory signalling in these areas of the hippocampus, consistent with the glutamatergic hypofunction hypothesis of schizophrenia (Olney and Farber, 1995). The higher levels of G-CSF found in the hippocampus of WT mice subject to the immune stimulus are therefore potentially representative of a functional protective mechanism in line with previous literature. On the other hand, the reduced level of G-CSF found in the hippocampus of Nrg1 Het mice with the immune stimulus might reflect a lack of neuroprotection, resulting in reduced excitatory neurotransmission as shown in the G-CSF knockout mice, consistent with the glutamatergic hypothesis of schizophrenia.

6.1.3 Method overview, limitations and experimental recommendations

To test our hypothesis, male Nrg1 Het mice were challenged with a 9 day stimulus from B16F0 melanoma cells injected in the flank. The first consideration was the peripheral immune response, measuring circulating immune cells and cytokine levels following the immune stimulus using flow cytometry techniques. Tumour growth in the periphery was monitored and the tumour excised for measurement of size. In addition, markers of angiogenesis and of innate immune cell infiltration were investigated using fluorescence microscopy and real time reverse transcription quantitative PCR (RT-qPCR).

Central levels of those cytokines altered in the periphery were considered next, with the investigation conducted in the PFC and the hippocampus and extended to include the cytokine receptors. Western blotting, RT-qPCR and flow cytometry methods were used for these studies. The central response was studied in the PFC and the hippocampus, two brain regions considered relevant in schizophrenia. Finally we conducted a preliminary exploration of potential common signalling pathway molecules using RT-qPCR and flow cytometry methods.

These exploratory studies were conducted with male Nrg1 Het mice only as sex is known to influence schizophrenia susceptibility in the general population and in relation to genetic risk (Goldstein et al., 2013; van der Werf et al., 2014; Zhang et al., 2012). Sex has also been shown to influence schizophrenia-like traits in the Nrg1 Het mouse model used (Chesworth et al., 2012). Thus additional studies in female mice would provide further insight into the sex differences in this mouse model and inform future human patient studies on the need for adequate male and female subject numbers to ensure accurate interpretation of results.

Measurement of cytokines used a multiplex Luminex assay for 32 cytokines combined may have resulted in a loss of sensitivity for some of the included cytokines. In schizophrenia patients changes in cytokines such as TNFα and IL-2 were shown previously (Borovcanin et al., 2012; Miller et al., 2011). While the Luminex assay had these cytokines included, they were below the limit of detection in the assay used. Although it has been shown that such multiplex kits show good correlation with other measurement methods such as ELISA, it is possible for the quantitative results to differ between methods where different capture and reporting beads are used (Elshal and McCoy, 2006). Therefore, validation of all findings using individual ELISA kits should be performed, and these or individual Luminex assays may yield more unequivocal results for additional cytokines. While these validation studies could not be performed in the course of present studies owing to technical difficulties resulting in an inability to obtain further tissue, they represent a productive avenue for follow-up studies. Due to the exploratory nature of this project, statistical results were chosen to be reported without correction for multiple testing, which represents a potential limitation in the analysis and interpretation of results.

Tumour growth was monitored before excision and measurement of size, angiogenesis markers and innate immune cell infiltration. As the tumour and tumour microenvironment were not the focus of these studies, only limited measurements were taken and no differences found. This does not rule out the possibility of changes in the tumour microenvironment and more detailed studies may elucidate differences. For example, utilising a slower growing tumour, or injecting a smaller number of tumour cells would allow monitoring over a longer period and may reveal differences in growth rate. Additionally, a cross breeding program of Nrg1 Het mice with mice that spontaneously develop tumours may provide a more realistic setting to determine genetic influences on tumour growth and immune response.

Cytokines found altered in the periphery were then investigated in the PFC and the hippocampus, with obligate cytokine receptors included in these studies. As discussed in chapter 3 and relevant to all subsequent chapters, the small physical size of the PFC in mice and the limitations this presented in the amount of tissue available resulted in the pooling of PFC samples. Although initially representing 8 animals, the result of pooling is statistical analysis on only 3-4 samples. For technical reasons, no more animals could be added to this analysis. In this case the results are therefore rather descriptive, hence the analysis and interpretation of the resulting data must be considered with care. Future studies to account for tissue limitation could increase the sample number and pooling samples to analyse multiple protein and mRNA analytes. Alternatively, focussing future studies on single analytes such as IL-6 alone could better utilise the limited tissue obtained. Repeat studies should be conducted to confirm the data presented herein on the PFC.

Similarly, the findings in the hippocampus for these studies may mask additional alterations since hippocampal dissections were randomly allocated to experimental protocols. Brain lateralisation variances have been demonstrated in the hippocampus and future studies could dissect the hippocampi specifically into left and right as well as dorsal and ventral segments to overcome this limitation. Additionally, histological examination of the hippocampal sub regions could be performed to reveal details concealed by the random assignment of hippocampal dissections.

In addition, the limitation presented by tissue availability in the PFC and in the brain overall resulted in the requirement to change from luminex assays, which need a large input of total protein for analyte analysis and work best with liquid rather than solid tissue, to western blotting which uses far less total protein input to obtain valid results for the number of follow-up analytes investigated. Repeat studies, focussing on a limited number of analytes and using either ELISA or western blot for both the peripheral and the brain measurements will need to be performed. Additionally, increased sample numbers in follow up studies may also give explicit insights into potential correlations between the peripheral and central analytes and their changes due to immune stimulation.

The final signalling molecule study presented mRNA expression comparison data. The use of a commercially available mRNA expression assay designed for high analyte throughput with low sample numbers in this exploratory study means that all mRNA expression findings should be confirmed with higher sample numbers, repeat experiments, and those in conjunction with protein and functional phosphorylation analysis, which were, however, beyond the scope of this study.

As noted above, animal models, including our Nrg1 mutant mice, only partially reflect human schizophrenia symptomatology or pathogenesis, and there are limitations affecting the validity and full interpretation of some results. However, the present studies were a fundamental step in identifying neuroimmune interactions that may contribute to the translation of a genetic vulnerability conferred by Nrg1 into immune system dysfunction and psychopathological disruptions in brain signalling, particularly with a focus on the IL-6 system.

6.1.4 Future directions: Investigations of the interaction of peripheral immune stimuli; the central cytokine alterations; and, schizophrenia relevant neurotransmission

Nrg1 is a candidate gene for schizophrenia development. The present study has shown that a Nrg1 mutation also represents a vulnerability factor for an individual's neuroimmune system, allowing an altered response to an immune stimulus. The altered CNS immune response in Nrg1 Het mice is further hypothesised to facilitate disruption of already aberrant neurotransmitter systems, which could impact on onset, symptom progression or severity in schizophrenia. It is therefore important to continue providing new evidence of the role for genetic vulnerability and immune system interactions in schizophrenia pathology.

In order to further the present findings, Nrg1 Het mice could be subjected to a range of peripheral immune stimuli, including a repeat of this melanoma stimulus at different intensity and time frames, and acute or long term challenges with LPS and the viral mimic polyinosinic-polycytidylic acid (PolyIC). In addition, direct injection of IL-6, G-CSF or a combination of both could be employed in both Nrg1 Het and WT mice to look for similar responses in the brain. Following any of the stimuli above, IL-6, G-CSF

and the PI3K/Akt pathway analytes could be further examined for mRNA expression changes, protein level alterations, and functional impairments.

Additional to the cytokine, mRNA and protein measurements performed in this study, neurotransmitter receptor density assays for dopamine, glutamate and GABA could be performed. Microdialysis techniques could be employed to measure real time neurotransmitter and cytokine release in freely moving mice to determine the direct effects of a peripheral immune challenge *in vivo* both in the periphery and the brain. The schizophrenia relevance of these could be confirmed with the administration of anti-psychotic drugs to see if the alterations are ameliorated. At the same time, schizophrenia-relevant behavioural and cognitive testing will be required, including open field testing for hyper locomotion; and pre-pulse inhibition, to shed light on the pathophysiological impact of the respective immune stimulation. Such future studies can confirm the validity of this gene x immune model and elucidate the detail of the links between alterations of cytokine levels, neurotransmitter signalling, and reflections in pathological behaviour.

6.1.5 Final conclusion

The results of the present study show a clear interaction between a genetic mutation and an immune stimulus relevant to schizophrenia, with a particular focus on IL-6 and G-CSF. The results demonstrated in our Nrg1 Het mice are similar to findings in human studies for peripheral cytokine changes in schizophrenia patients, and in particular are consistent with findings in humans carrying a similar Nrg1 genetic mutation. We could therefore establish the Nrg1 Het mice as a valuable model to study gene x immune interactions as potentially relevant to psychopathology. Future studies based on these

current findings will provide further insight into the role of peripheral and central cytokine changes in schizophrenia development, progression and treatment.

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APPENDIX A

GeNorm qBase plus software generated results for selection of reference genes to use in brain tissue based RT-qPCR (high resolution figures are unavailable from this software).

A1) geNorm qBase plus software generated graph (geNorm M): Average stability of remaining reference targets

This graph demonstrates the increasing expression stability of each tested reference gene ranked from left to right across the graph. The genes on the far right-hand side of the graph are the most stably expressed: i.e. Ap3d1 followed by Fbxw2

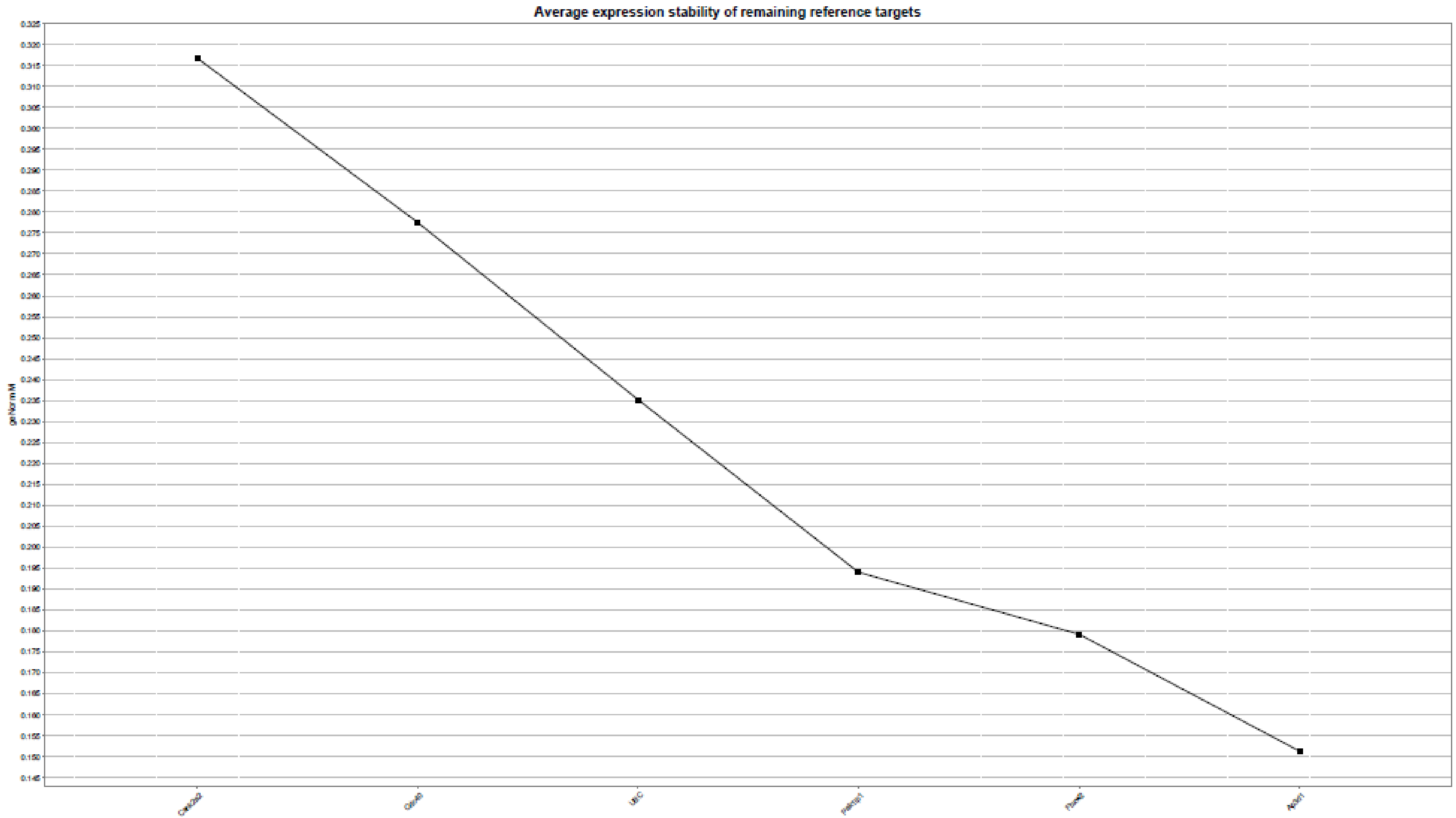
A2) geNorm qBase plus software generated graph (geNorm V).

This graph is generated to depict the optimal number of reference genes to use in subsequent experiments. The software uses a “pairwise variation, V(n/n+1)” calculation to determine the optimal number of reference genes. An appropriate number of reference genes is reached with V(n/n+1) is below 0.15.

In this experiment it can be seen that the two most stably expressed genes demonstrate a V(n/n+1) less than 0.075 with limited gain from adding additional reference genes, therefore two genes were used: Ap3d1 and Fbxw2

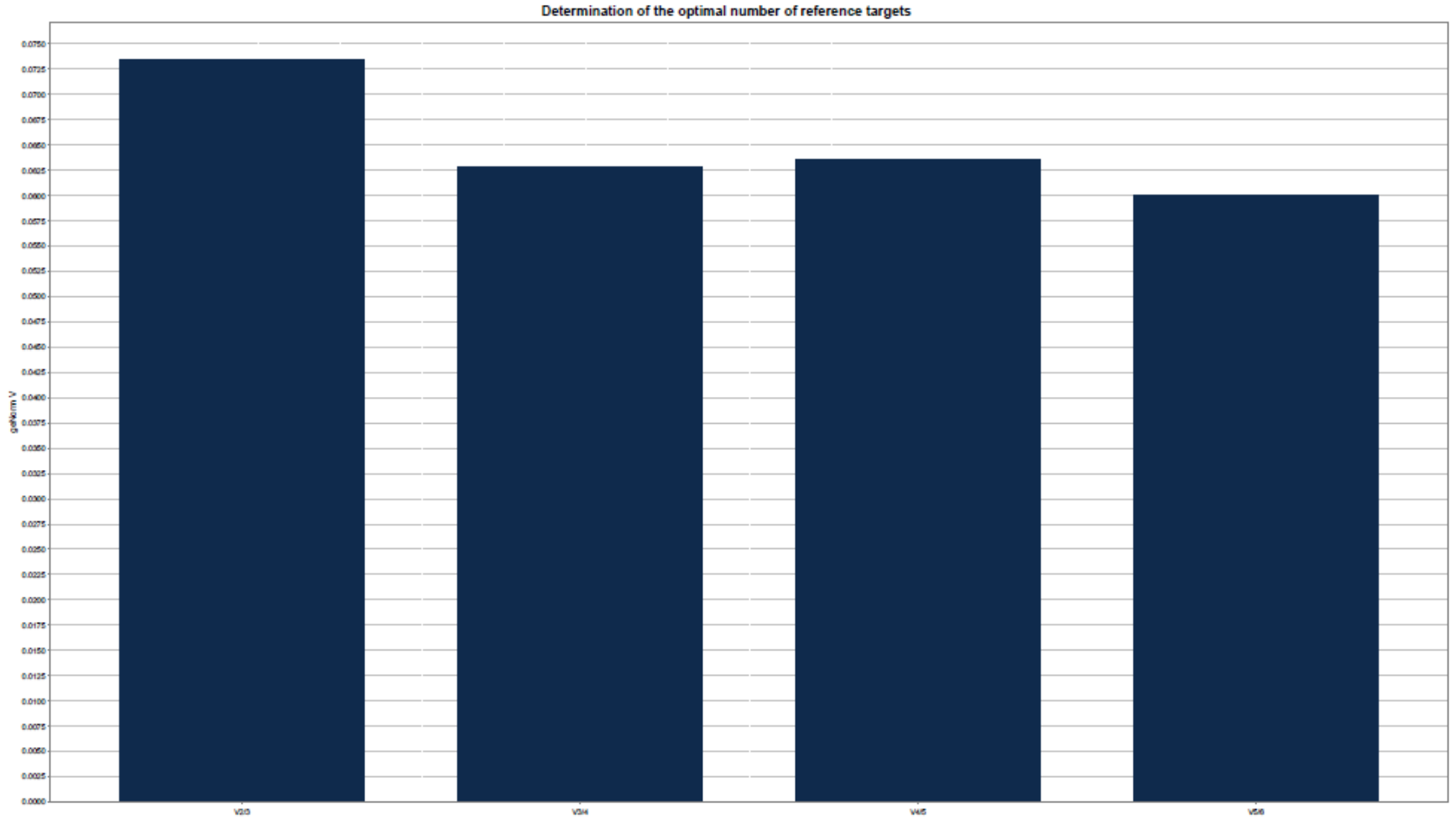
A3) the geNorm qBase plus software generated written report is included as a “screen capture” to demonstrate the analysis of the noted graphs.

A1)

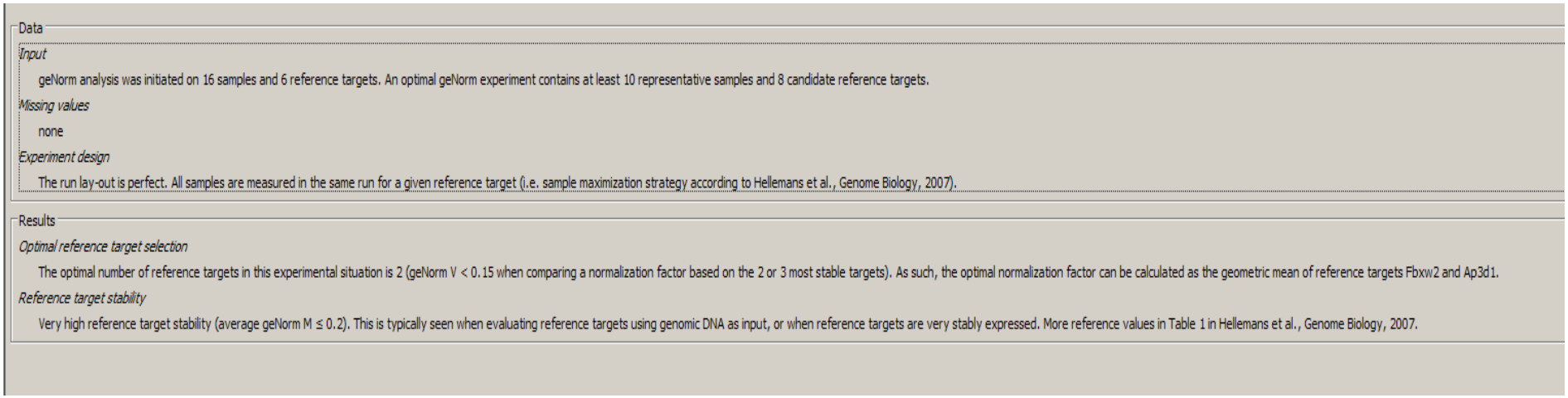


Average expression stability of remaining reference targets
geNorm M
UBC

A2)


Determination of the optimal number of reference targets
0.0750
0.0725
0.0700
0.0675
0.0650
0.0625
0.0600
0.0575
0.0550
0.0525
0.0500
0.0475
0.0450
0.0425
0.0400
0.0375
0.0350
0.0325
0.0300
0.0275
0.0250
0.0225
0.0200
0.0175
0.0150
0.0125
0.0100
0.0075
0.0050
0.0025
0.0000

A3)


Data
Input
geNorm analysis was initiated on 16 samples and 6 reference targets. An optimal geNorm experiment contains at least 10 representative samples and 8 candidate reference targets.
Missing values
none
Experiment design
The run lay-out is perfect. All samples are measured in the same run for a given reference target (i.e. sample maximization strategy according to Hellemans et al., Genome Biology, 2007).
Results
Optimal reference target selection
The optimal number of reference targets in this experimental situation is 2 (geNorm V < 0.15 when comparing a normalization factor based on the 2 or 3 most stable targets). As such, the optimal normalization factor can be calculated as the geometric mean of reference targets Fbxw2 and Ap3d1.
Reference target stability
Very high reference target stability (average geNorm M ≤ 0.2). This is typically seen when evaluating reference targets using genomic DNA as input, or when reference targets are very stably expressed. More reference values in Table 1 in Hellemans et al., Genome Biology, 2007.

APPENDIX B

Figure representing western blot results for each protein measured, showing one representative animal from each group in each of the brain regions as well as competitive binding assay for G-CSF.

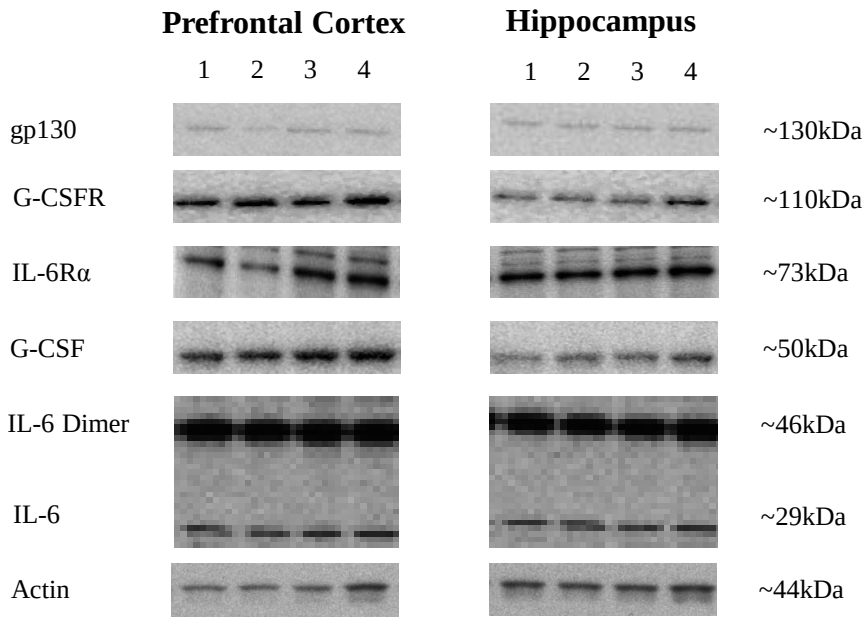
a) gp130 (Sections 3.2.5c, 3.3.4 and Figure 3.3-4

b) G-CSFR (Sections 4.2.5c, 4.3.2 and Figure 4.3-2

c) IL-6Rα (Sections 3.2.5c, 3.3.2 and Figure 3.3-3)

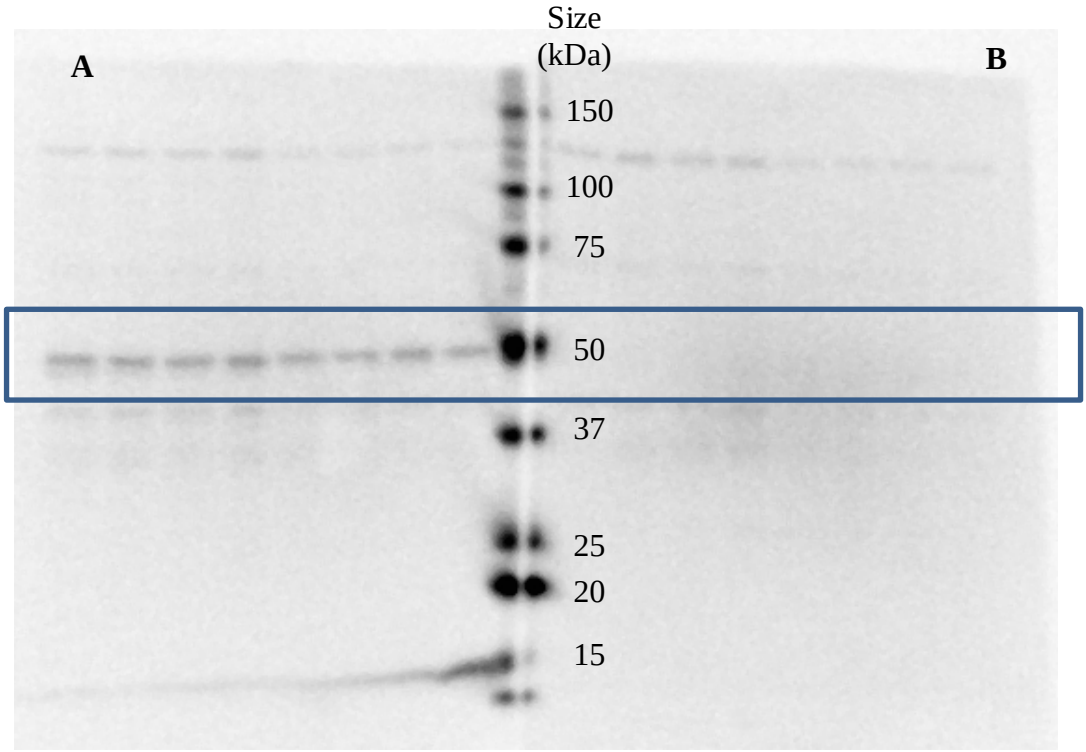
d) G-CSF (Sections 4.2.5c, 4.3.1 and Figure 4.3-1

e) IL-6 (Sections 3.2.5c, 3.3.1 and Figure 3.3-2)



1 = WT unchallenged, 2 = Nrg1 unchallenged, 3 = WT immune challenged, 4 = Nrg1 immune challenged

Results of competition binging assay to determine G-CSF in western blot (4.2.5c)



(A) is G-CSF (K-15): sc-49679 (Santa Cruz) at 1:250 o/n at 4 degrees with BSA block (1 hour) and (**B)** is is G-CSF (K-15): sc-49679 pre-incubated for 1 hour before use with the G-CSF blocking peptide: sc-49679 P (Santa Cruz), 5x more concentrated than antibody.