

Role of interferon-gamma and leucine-rich repeat kinase 2 in
stress-related pathology.

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Abstract

Pro-inflammatory cytokines promote stressor-like behavioural and neurochemical variations and are implicated in depression. Despite being a preferential inducer of the depression-linked inflammatory enzyme indoleamine 2,3-dioxygenase, the T-helper type-1 cytokine interferon gamma (IFN- γ) has received little attention in preclinical animal models. In the current studies we set out to elucidate the role of IFN- γ in stressor-related psychological pathology. In Chapter 2 we show that IFN- γ deficiency in mice attenuated some of the corticosterone, cytokine and brain regional dopaminergic effects of chronic stress. Similarly, in Chapter 3 we report that a lack of IFN- γ not only protected against stressor-induced memory dysfunction but also appeared to facilitate memory performance following stress. Surprisingly, however, under basal conditions the IFN- γ knockout mice actually had increased plasma corticosterone levels, heightened brain regional noradrenergic and serotonergic activity, and impaired spatial memory function, suggesting that a certain basal level of IFN- γ is required for the homeostatic regulation of these behavioural and physiological systems. Building on the findings from these two IFN- γ knockout studies we next undertook to ascertain whether the cytokine proactively affects depression-relevant pathophysiological domains. In Chapters 4 and 5 we demonstrate that systemically administered IFN- γ (25000 IU) mobilized peripheral cytokine networks, stimulated brain regional monoaminergic activity and sensitized the plasma corticosterone response to psychological stress. However, the cytokine did not alter locomotor activity or cause sickness-type behaviours in either of these studies. Additionally, in Chapter 5 we show that the monoaminergic effects of IFN- γ and bacterial lipopolysaccharide were exaggerated in mice overexpressing a mutated form of

the Parkinson's disease-linked factor leucine-rich repeat kinase 2 (i.e., LRRK2 G2019S). In this way, we provide preliminary proof-of-concept for an IFN- γ -LRRK2 signalling pathway that may be relevant for depression and other non-motor symptoms in PD. Overall the findings presented in this thesis support a role for IFN- γ in stressor-related pathology and provisionally implicate LRRK2 as a novel downstream mediator of the cytokine's depressogenic effect. Further studies are warranted to verify and extend the pathological significance of these findings.

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List of Abbreviations

3-HK, 3-hydroxykynurenine
5-HIAA, 5-hydroxyindole acetic acid
5-HT, serotonin
6-OHDA, 6-hydroxydopamine
ACTH, adrenocorticotrophic hormone
AD, Alzheimer's disease
AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANK, ankyrin repeat motif
ANOVA, analysis of variance
ARM, armadillo repeat motif
BAC, bacterial artificial chromosome
BBB, blood-brain barrier
BDNF, brain-derived neurotrophic factor
BH4, 5,6,7,8-tetrahydrobiopterin
CeA, central amygdala
CNS, central nervous system
COR, C-terminal of ROC
COX-2, cyclooxygenase-2
CREB, cyclic adenosine 3',5'-monophosphate response element binding protein
CRH, corticotropin-releasing hormone
CRP, C-reactive protein
CSF, cerebrospinal fluid
CVO, circumventricular organ
DA, dopamine
DAT, dopamine transporter
DI, discrimination index
DNA, deoxyribonucleic acid
DOPAC, 3,4-dihydroxyphenylacetic acid
EDTA disodium salt, disodium ethylenediaminetetraacetate dehydrate
ELISA, enzyme-linked immunosorbent assay
ERK-1/2, extracellular signal-regulated kinase (mitogen-activated protein kinase)-1/2
FST, forced swim test
G2019S, glycine-to-serine substitution at position 2019
GABA, gamma-aminobutyric acid
GAS, gamma activation sequence
GDNF, glial-derived neurotrophic factor
GR, glucocorticoid receptor
GS-Tg, LRRK2 G2019S transgenic
GTP, guanosine-5'-triphosphate
HC, hippocampus
HIV/AIDS, human immunodeficiency virus infection and acquired immune deficiency syndrome
HPA, hypothalamic-pituitary-adrenal
HPLC, high-performance liquid chromatography
Hsp90, heat shock protein 90

HVA, homovanillic acid
IDO, indoleamine 2,3-dioxygenase
IFN, interferon
IGF, insulin-like growth factor
IL, interleukin
iNOS, inducible nitric oxide synthase
IRF, interferon regulatory factor
ISRE, interferon-stimulated response element
Jak, janus family tyrosine kinase
JNK, c-Jun N terminal kinase
KO, knockout
KYN, kynurenine
KYNA, kynurenic acid
LC, locus coeruleus
LPS, lipopolysaccharide
LRRK2, leucine-rich repeat kinase 2
LTP, long-term potentiation
MAO, monoamine oxidase
MAPK, mitogen-activated protein kinase
MCP-1/CCL2, monocyte chemoattractant protein-1
MHC, major histocompatibility complex
MHPG, 3-methoxy-4-hydroxyphenylglycol
mRNA, messenger ribonucleic acid
Myd88, myeloid differentiation primary response protein
NAcc, nucleus accumbens
NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase
NE, norepinephrine
NET, norepinephrine transporter
NF- κ B, nuclear factor-kappa B
NK, natural killer
NMDA, N-methyl-D-aspartate
NO, nitric oxide
NSF, N-ethylmaleimide-sensitive factor
p38 MAPK, p38 mitogen-activated protein kinase
PD, Parkinson's disease
PFC, prefrontal cortex
PKB, protein kinase B/Akt
Poly I:C, polyinosinic-polycytidylic acid
QUIN, quinolinic acid
R1441G, arginine-to-glycine substitution at position 1441
ROS, reactive oxygen species
SERT, serotonin transporter
SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNc, substantia nigra pars compacta
SSRI, selective serotonin reuptake inhibitor
Stat, signal transducer and activator of transcription

Tat, HIV-1 trans-activator of transcription
Tg, transgenic
Th1/2, T-helper type-1/2
TLR, Toll-like receptor
TNF, tumor necrosis factor
TRP, tryptophan
VMAT2, vesicular monoamine transporter 2
WT, wild-type

Preface

This thesis is primarily based on the following 4 manuscripts, each of which comprises a separate research chapter in the present document. The use of copyrighted material, namely Litteljohn et al. (2010b) and Litteljohn et al. (2014), is hereby acknowledged.

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Additionally, a portion of the General Introduction (§1.2.1) is based on the below-listed publication. The use of copyrighted material (article reproduced in part with minor changes) is hereby acknowledged.

Hayley S, Litteljohn D. (2013a). Inflammatory roads to neurodegeneration: A focus on Alzheimer's and Parkinson's disease. In A Kuznecov and H Anisman (Eds.), *The Wiley-Blackwell Handbook of Psychoneuroimmunology*. Hoboken, NJ: Wiley-Blackwell, pp. 393-410.

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1 Chapter. General Introduction

1.1 The complex nature of depression

Depression is a common mood disorder that displays a high degree of co-morbidity with other medical conditions (Belmaker and Agam, 2008). Usually characterized by a combination of melancholia, anhedonia, feelings of guilt and worthlessness, impaired concentration and/or memory, psychomotor disturbances, and fatigue (though symptom profiles vary widely and several subtypes of depression are currently recognized: Harald and Gordon, 2012), depression is a major risk factor for suicide and a primary cause of disability worldwide (Dumais et al., 2005; Lépine and Briley, 2011; Vos et al., 2012). Stress is widely considered to be an important contributing factor to depression, and numerous other etiological factors ranging from genetics and personality traits to social support and substance abuse have likewise been implicated (Anisman et al., 2008a; Belmaker and Agam, 2008). Current pharmacological treatment of depression, while effective for many and generally quite safe (despite problematic side effects) (Hieronymus et al., 2016), leaves much to be desired: initial and cumulative remission rates are low (~ 60-65%) and there is typically a 2-4 week time lag before clinical response (Gelenberg and Chesen, 2000; Moncrieff and Kirsch, 2005). There is therefore a pressing need to develop new antidepressant therapies and this is inextricably linked with an improved understanding of the pathophysiology of depression.

In this regard, changes in brain monoamine and neuroendocrine systems have long been considered to play an important role (Kalin and Dawson, 1986; Lambert et al., 2000; Hamon and Blier, 2013); this is reflected in the preponderance of antidepressant medications that target these systems [e.g., the selective serotonin reuptake inhibitors

(SSRIs), serotonin-norepinephrine reuptake inhibitors, and norepinephrine and dopamine reuptake inhibitors]. More recently, alterations of neurogenesis and neurotrophin signalling have been implicated in depression (Pittenger and Duman, 2008), and the pathological relevance of processes normally associated with neurodegeneration (e.g., oxidative and nitrosative stress, glutamate-mediated excitotoxicity) is starting to gain widespread acceptance (Moynan et al., 2013; Myint and Kim, 2014). Another very active area of research, spurred on by the realization that immune abnormalities are common in depressed patients (Maes et al., 1990), concerns the potential role of inflammatory factors in depression pathogenesis. This thesis has as its primary focus the link between pro-inflammatory cytokines (immune system messenger proteins) and depression. We are particularly interested in the role of interferon-gamma (IFN- γ) given the cytokine's fundamental regulatory actions in various aspects of innate [rapid non-specific immune responses mostly involving mononuclear phagocytes and natural killer (NK) cells] and adaptive immunity (antigen-specific learned or plastic immune responses primarily involving T and B lymphocytes) (Shtrichman and Samuel, 2001).

1.2 Inflammation and depression

The cytokine or immune hypothesis of depression, whose roots can be traced back to the early 1990s (first appearing as the “macrophage theory of depression”: Smith, 1991; Maes, 1995), holds that immune-inflammatory processes contribute to depression pathogenesis and are not mere epiphenomena of the disease. This is not to say that inflammation is necessarily the only player in depression or that immune dysfunction should be uniformly implicated in the disease. Indeed, as touched on above, depression is a complex disease of considerable phenotypic and etiologic heterogeneity, and one for

which a number of pathophysiological mechanisms are likely to be relevant. Still, over the past two-plus decades a great deal of evidence has accumulated to strongly implicate cytokines, and immune dysfunction more generally, in the pathophysiology of depression.

As will be discussed in the ensuing sections, the available evidence shows that: 1) depression is consistently associated with cytokine and other immune abnormalities; 2) variation in cytokine genes modifies the risk of developing depression as well as the response to antidepressant treatment; 3) therapeutic administration of cytokines induces depression-like symptoms; 4) low grade experimental endotoxemia in humans elicits sickness and depressive-like behaviours; 5) in vivo cytokine administration in animals recapitulates key neurobehavioural and pathophysiological aspects of depression whereas disruption of cytokine signalling, whether through pharmacological or genetic means, generally attenuates such changes; and 6) immunomodulation appears to be a critical mechanism underlying antidepressant responses. We then turn our attention to the question of *how* cytokines might influence depressive states, with the bulk of the evidence pointing towards cytokine-induced modulation of neuroendocrine and neurotransmitter systems as well as processes aligned with neuroplasticity and neurodegeneration. But first we broadly consider the role of immuno-inflammatory processes in health and disease, and home in specifically on the various ways in which the immune and central nervous systems (CNS) communicate.

1.2.1 Immune-to-brain signalling: an overview

In the classic sense, inflammation refers to the host response against a pathogen or foreign agent that is characterized by several obvious clinical features, including fever,

pain, edema, and redness (Hayley and Litteljohn, 2013a). The inflammatory response is mediated by a variety of immune cells, most notably circulating neutrophils, macrophages, dendritic cells, and NK cells (i.e., leukocytes). Activation of leukocytes following immune challenge or injury occurs rapidly, leading to the mobilization and trafficking of these cells throughout the body. Once at a site of injury or infection, leukocytes secrete nitric oxide (NO), prostaglandins, histamine and other factors that cause blood vessels to dilate and become leaky (i.e., increased vascular permeability). Leukocytes also release soluble inflammatory messenger proteins called cytokines, which act to limit the spread of infection, further mobilize immune cells and facilitate communication with other physiological systems. Thus, cytokines are critically involved in innate and adaptive host defense mechanisms (Shtrichman and Samuel, 2001; Hayley and Litteljohn, 2013a).

It is now well established that the brain, although immune-privileged to a degree [owing to the presence of the blood-brain barrier (BBB)], engages fundamentally with the immune system (Hayley, 2011). Circulating leukocytes routinely enter the brain (albeit in limited concentrations), whereupon they perform various “housekeeping” tasks critical for immuno-surveillance (Litteljohn et al., 2011). In doing so, infiltrating immune cells come into close apposition with local glial cells, including the brain’s own specialized immunocompetent cells, microglia. Essentially analogues of peripheral macrophages, microglia constitute the brain’s first line of defense against pathogenic or other environmental threats and are thus primary mediators of neuroinflammation (Streit et al., 2004). Although microglia can act independently of peripheral immune input (e.g., mount rapid brain-based innate inflammatory responses), they are highly attuned to infiltrating

immune cells and other signals emanating from the periphery. For instance, under the direction of cytokines, microglia express major histocompatibility complex (MHC) and associated molecules that allow for direct cell-to-cell synaptic interactions with receptors on invading T lymphocytes. This can result in the priming of both lymphocytes and microglia, and the orchestration of antigen-specific immune responses (Schroder et al., 2004; Hayley and Litteljohn, 2013a).

The sizable list of recognized cytokine immunotransmitters includes the interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), chemokines (a subclass of chemoattractant cytokines), and growth and cell-stimulating factors. Generally, cytokines are classified according to major cellular source(s) (e.g., the ILs are named after the leukocyte cells from which they are primarily released), as well as key aspects of molecular structure or physiological repertoire (e.g., the IFNs *interfere* with viral replication) (Leonard and Lin, 2000; Anisman et al., 2008b). In the broadest sense, cytokines can be divided into primarily pro-inflammatory or anti-inflammatory factors, although even this distinction may be an oversimplification (i.e., due to concentration- and- developmental stage-dependent effects, as well as cytokine pleiotropy and synergism more generally: Ozaki and Leonard, 2002). While IL-1 β , IL-6, TNF- α , and IFN- $\alpha/\beta/\gamma$ are prominent members of the former group, IL-4 and IL-10 are considered representative of the latter.

Cytokines are relatively large, soluble polypeptides (up to \sim 70 kDa) that do not readily cross the BBB (Banks, 2005). Cytokines can, however, enter the brain via the circumventricular organs (CVOs), which are specialized midline brain structures that lack a fully functional BBB; e.g., the median eminence and area postrema (Blatteis, 2000;

Ganong, 2000). Additionally, saturable carrier-mediated transport systems have been described for a number of cytokines, including TNF- α , IL-1 α/β , IL-6, and IFN- γ (Blatteis, 2000; Banks, 2005). Incredibly, Louveau et al. (2015) recently demonstrated the existence of functional lymphatic vessels lining the dural sinuses (connected to the deep cervical lymph nodes), which would seem to afford cytokines and other circulating immune factors direct, unencumbered access to the brain. In addition to circulatory routes, cytokines can influence central processes by way of afferent projection fibres ascending from the periphery (e.g., visceral branches of the vagus nerve) (Anisman et al., 2008b; Miller et al., 2009). Once inside the CNS, cytokines signal through receptors on cells comprising or proximal to the brain microvasculature (e.g., endothelial cells and glia), but may also penetrate deep within the brain parenchyma via neuronal projections and volume diffusion (Rivest et al., 2000; Vitkovic et al., 2000).

Notably, psychological, immunological and chemical stressors can all induce BBB disruption (Abdel-Rahman et al., 2002; Northrop and Yamamoto, 2012), which is anticipated to potentiate cytokine access to and action within the brain (Anisman et al., 2008b). For instance, corticotropin-releasing hormone (CRH) may promote the activation of brain mast cells and resident microglia, leading to the enhanced secretion of pro-inflammatory cytokines, chemokines (chemo-attractant cytokines) and growth factors [e.g., IL-6, IL-8/C-X-C motif chemokine ligand 8, monocyte chemoattractant protein-1 (MCP-1/CCL2), vascular endothelial growth factor] (Esposito et al., 2002; Theoharides and Konstantinidou, 2007). As well, stressor exposure induces bacterial translocation from the gastrointestinal tract and the activation of cytokine-producing inflammasomes in circulating myeloid cells and brain-resident glia (Zareie et al., 2006; Gustin et al., 2013).

Pro-inflammatory cytokines can themselves increase BBB permeability (e.g., via upregulating endothelial cell adhesion molecules: Wong et al., 1999), and this has the dual effect of enhancing cytokine production at vascular sites (i.e., a positive feedback loop) and augmenting T cell trafficking across the BBB (de Vries et al., 1996; Anisman et al., 2008b; Cayrol et al., 2008).

Beyond peripherally originating immune-inflammatory processes, cytokines and their receptors are expressed endogenously in the CNS; mounting evidence suggests this is largely due to *de novo* synthesis by a variety of glial cell types (Benveniste, 1998). Microglia, in particular, produce and bear receptors for a wide array of cytokines, which is consistent with a front-line role of these cells in host defense (Hanisch, 2002; Streit et al., 2004). However, glial cells are not the only source and target of cytokine signalling within the brain: neurons too are capable of synthesizing (especially in response to injury or infection) and responding to a variety of pro- and- anti-inflammatory cytokines and related immune factors (Tchelingerian et al., 1993; Ringheim et al., 1995; Benveniste, 1998).

The general picture that has emerged over the past 20-30 years is one in which pro-inflammatory cytokines are viewed as deleterious factors within the brain. This is supported certainly by the strong link between chronically and/or very high levels of TNF- α , IL-1 β and IFN- γ and the incidence of brain pathology (and reactive microgliosis) (Bate, 2006; McCoy and Tansey, 2008; Hayley and Litteljohn, 2013a). Conversely, anti-inflammatory agents (e.g., minocycline, non-steroidal anti-inflammatory drugs) have generally been associated with neuroprotective consequences in a range of preclinical animal models (Maczurek et al., 2008; Ghosh et al., 2012). Yet, it's become increasingly

clear that a more nuanced approach to interpreting cytokine actions in the CNS is needed (Anisman et al., 2008b; Shaftel et al., 2008; Yirmiya and Goshen, 2011). Specifically, while high levels of pro-inflammatory cytokines can be expected to have deleterious effects on brain function (e.g., via stimulation of oxidative-inflammatory cascades and apoptotic cell death programs), it may be that low physiological levels of cytokines induce neuroprotection and adaptive neuroplasticity [e.g., via the release of free radical scavengers and trophic factors such as brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF)] (Anisman et al., 2008b; Hayley and Litteljohn, 2013a). Further complicating the picture, cytokines typically display a high degree of redundancy, pleiotropy and synergy (but also antagonism) (Brebner et al., 2000; Ozaki and Leonard, 2002; Bartee and McFadden, 2013). The complex nature of cytokine physiology has led our group and others to suggest that cytokines are best considered not in isolation but as a network of biologically active mediators whose collective output determines physiological and pathological consequences (Dantzer et al., 2008; Litteljohn and Hayley, 2012).

1.2.2 Evidence of cytokine involvement in depression

1.2.2.1 Cytokine abnormalities in depression

A large number of studies have indicated that depressed patients have increased peripheral blood and/or cerebrospinal fluid (CSF) levels of inflammatory and immune factors (Miller et al., 2009; Haapakoski et al., 2016). Included here are certain types of immune cell (e.g., monocytes, neutrophils), acute phase proteins [e.g., haptoglobin and C-reactive protein (CRP)], inducible inflammatory enzymes [e.g., indoleamine 2,3-dioxygenase (IDO)], prostaglandins (e.g., PGE₂), and various pro-inflammatory

cytokines (Song et al., 1994; Levine et al., 1999; Danner et al., 2003; Ford et al., 2004; Kling et al., 2007). The cytokines that have most consistently been found to change in depression, and in some cases even to predict symptom severity and/or treatment resistance, are TNF- α , IL-1 β and IL-6 (Maes et al., 1997; Mikova et al., 2001; Eller et al., 2008; Dowlati et al., 2010; Liu et al., 2012; Yoshimura et al., 2013; Young et al., 2014). Alterations in the levels of myriad other cytokines, including IL-2, IL-5, IL-7, IL-8, IL-18, IFN- γ , MCP-1/CCL2, and granulocyte-colony stimulating factor, have also been described (Dahl et al., 2014; Al-Hakeim et al., 2015). As well, cytokine circadian rhythms and inter-relations appear to be disturbed in depression. For instance, Alesci et al. (2005) described a 12-h shift in the normal IL-6 circadian rhythm while Dhabhar et al. (2009) noted that the usually strong positive correlation between plasma IL-6 and IL-10 levels was completely absent in depressed patients. In the latter study blood levels of IL-10 were actually diminished among depressed patients while IL-6 concentrations trended towards being increased, suggesting that the loss of a counter-balancing, immunoregulatory association between the two cytokines may contribute to inflammation-related depression symptoms (Dhabhar et al., 2009).

While the evidence for peripheral immune alterations in depression is undeniable, data implicating central inflammatory changes have been much harder to come by; this is largely due to technological and logistical constraints but also contradictory research findings (Hannestad et al., 2013). There is nevertheless an intriguing case to be made. To begin, Shelton et al. (2011) described an upregulation of multiple pro- and anti-inflammatory cytokine messenger ribonucleic acids (mRNAs) in postmortem prefrontal cortex (PFC) of depressed patients just as Dean et al. (2010) reported an increase in TNF

trans-membrane protein levels (a non-soluble precursor of TNF- α). In addition, postmortem studies in depressed individuals who died by suicide yielded histological evidence of brain regional microglial priming and activation [e.g., upregulated expression of human leukocyte antigen and quinolinic acid (QUIN)], as well as enhanced monocyte recruitment (Steiner et al., 2008, 2011; Torres-Platas et al., 2014). Similar findings, including brain region-specific microgliosis, increased expression of pro-inflammatory cytokines, and the potentiated brain trafficking of inflammatory T cells, have been observed in stressor-based animal models of depression (Audet et al., 2011; Hinwood et al., 2012; Beurel et al., 2013; Couch et al., 2013; Kreisel et al., 2014; Liu et al., 2015a; Reader et al., 2015).

Intriguingly, a recent positron emission tomography (PET) imaging study using translocator protein density as a marker of microglial activation revealed that depressed patients currently experiencing a major depressive episode had markedly increased levels of neuroinflammation in the PFC, insula and anterior cingulate cortex (Setiawan et al., 2015). Moreover, depression severity correlated with the degree of microglial activation in the latter brain region (Setiawan et al., 2015). These groundbreaking in vivo data are at first glance contradicted by the earlier negative PET translocator protein findings of Hannestad et al. (2013). Yet, it's important to note that in the latter study imaging was performed on individuals with mild -to-moderate depression (i.e., Montgomery-Åsberg Depression Rating Scale scores ranging from 5 to 30) whereas in the more recent study of Setiawan et al. (2015) all subjects were verified at the time of imaging to have *at minimum* moderately severe depression [i.e., a minimum score of 17 on the 17-item Hamilton Depression Rating Scale (mean \pm standard deviation: 20.8 \pm 3.8), which can be

considered the lower cutoff score for moderate depression (Zimmerman et al., 2013)]. As previously mentioned, circulating cytokine alterations tend to be more pronounced with increasing depression severity or treatment resistance (Maes et al., 1997; Eller et al., 2008; Cattaneo et al., 2013), and this raises the question of whether immune-based stratification might prove useful in depression research. Taking this line of reasoning a step further, one might also consider the merits of endorsing an inflammatory endophenotype for depression (Hayley, 2011; Audet and Anisman, 2013; Fornaro et al., 2013; Hughes et al., 2016).

In any case, the totality of the evidence indicates that cytokine abnormalities are germane to depression. The issue of whether or not cytokines actually contribute to depression pathogenesis is the subject of what follows next.

1.2.2.2 Cytokine gene variation and depression

Attempting to parse complex heterogeneous neuropsychiatric conditions, such as depression, into discrete genetic contributions is fraught with difficulties (Hodgson and McGuffin, 2013). Despite this, an increasing number of studies have indicated that genetic variation in specific cytokines, most notably IL-1 β , IL-10 and TNF- α , may influence depression risk, and perhaps especially so in the medically ill (Jun et al., 2003; Borkowska et al., 2011; Holtzman et al., 2012; Dunn et al., 2013; Kim et al., 2013a, b; Saad et al., 2014). In particular, Jun et al. (2003) were the first to report a possible link between polymorphism in the TNF- α gene and depression, and this was more or less confirmed in the recent genome-wide association study of Bosker et al. (2011). More recently, Holtzman and colleagues (2012) discovered that a genetic trend toward low levels of the anti-inflammatory cytokine IL-10 increased depression risk in renal failure

patients, while Haastrup et al. (2012) found that a genetic predisposition to high levels of the pro-inflammatory cytokine IL-18 augmented the likelihood of developing depression in response to stressful life events. In addition to modulating depression risk, mounting evidence suggests that cytokine gene variation may play a role in determining responsiveness to antidepressant treatment. For instance, Uher and colleagues (2010) reported that polymorphism in the genes encoding IL-11 and IL-6 predicted the clinical response to the SSRI agent escitalopram, while Baune and colleagues (2010) noted that polymorphism in the IL-1 β gene impaired not only emotion processing but also the clinical response to antidepressant treatment.

However, it stands to reason that any “real” effect of cytokine gene variation on depression risk and/or antidepressant response is likely to be very small and must surely be viewed in the context of multiple interacting genetic and environmental influences (Hodgson and McGuffin, 2013). Indeed, most candidate depression genes do not hold up under hypothesis-free genome-wide association testing conditions, although the TNF- α rs76917 polymorphism looks to be an important exception (Bosker et al., 2011). Intriguingly, emerging evidence also suggests that certain cytokines and their downstream targets might be useful transcriptomic or epigenetic biomarkers for the antidepressant response [e.g., deoxyribonucleic acid (DNA) methylation status]; the data have thus far provisionally implicated TNF- α and IL-11 (Powell et al., 2013a, b). Ultimately, cytokine gene variation in depressed individuals, and perhaps also individual differences at the epigenome and transcriptome levels, can be expected to result in some modification of neuroimmune communication, and this, in turn, helps shape disease

vulnerability and/or responsiveness to antidepressant therapy (Hayley and Litteljohn, 2013a; Martin et al., 2015).

1.2.2.3 Therapeutic administration of cytokines

An important line of evidence bolstering the case of cytokines in depression comes from an accounting of the behavioural effects of cytokine immunotherapy; i.e., the application of exogenous cytokines, most commonly the IFNs, to treat medical illness. In particular, cancer and hepatitis C patients undergoing IFN- α immunotherapy often develop prominent neuropsychiatric symptoms, including a depressive-like syndrome that is largely amenable to antidepressant treatment (Capuron et al., 2000; Kraus et al., 2008; Schaefer et al., 2012). Of note, there appears to be a high degree of clinical overlap between iatrogenic cytokine/IFN-induced depression and idiopathic unipolar depression, although the former tends to be associated with more pronounced physical symptoms (e.g., psychomotor retardation and weight loss) but less severe feelings of guilt (Capuron et al., 2009).

As we've seen already in the case of idiopathic depression, evidence is beginning to suggest that functional gene polymorphism in certain cytokines or their receptors, including IL-6, TNF- α , IL-28B, IFN- γ , and IFN- α/β receptor 1, may influence vulnerability to and/or the clinical presentation of IFN-induced depression (Felger and Lotrich, 2013). So far probably the most compelling data have been described for IL-6, with both Bull et al. (2009) and Udina et al. (2013) reporting that individuals genetically predisposed to produce higher levels of this cytokine have an increased risk of developing depression during IFN- α therapy. And it's not just variation in cytokine genes that has been found to influence IFN-induced depression risk and symptom profile:

various other immune system genes [e.g., encoding the inflammatory enzymes cyclooxygenase-2 (COX-2) and IDO-1], as well as those related to monoamine, neuropeptide and growth factor systems (e.g., the serotonin transporter 5-HTTLPR, CRH and BDNF) have also been implicated (Felger and Lotrich, 2013; Cozzolongo et al., 2015). Importantly, given the aforementioned phenotypic similarities between cytokine/IFN-induced depression and idiopathic disease, it is reasonable to suggest that advancing our understanding of the underlying pathophysiology of the one may help us better understand the other (Capuron et al., 2009).

1.2.2.4 Experimental systemic inflammation in humans

Also relevant here are studies that have sought to delineate the neurobehavioural sequelae of experimentally induced transient human endotoxemia; i.e., low-grade systemic inflammation. Generally, these investigations have indicated that low-dose endotoxemia [e.g., with bacterial lipopolysaccharide (LPS)] markedly increases pro- and anti-inflammatory cytokines, sympathetic catecholamines and glucocorticoids, whilst inducing a range of behavioural symptoms, including disturbed sleep and feeding, negative mood, anxiety, feelings of social disconnection, and cognitive impairment (Mullington et al., 2000; Reichenberg et al., 2001; Krabbe et al., 2005; Grigoleit et al., 2011; Kullmann et al., 2013, 2014). In several of these studies correlations were even noted between inflammatory cytokines and endotoxemia-induced behavioural symptoms (e.g., cognitive impairment, low mood), suggesting that cytokines may have a mediating role (Reichenberg et al., 2001; Krabbe et al., 2005).

Recently, neuroimaging approaches have been applied to experimental human endotoxemia models, with the promise of identifying candidate brain substrates of innate

immune/cytokine action (Schedlowski et al., 2014). In this regard, preliminary evidence already supports a role for the amygdala, ventral striatum/nucleus accumbens, insula, and anterior cingulate cortex (Eisenberger et al., 2009, 2010; Hannestad et al., 2012; Inagaki et al., 2012). These areas are considered to be important nodes in interoceptive, emotion and/or reward processing, and all are implicated in depression (Price and Drevets, 2011).

1.2.2.5 Basic research and preclinical animal models

While human endotoxemia models have garnered substantial recent research interest and are poised to significantly advance our understanding of brain-immune/cytokine interactions, much of what we know in this regard is thanks to basic research and preclinical animal studies. Such work has been instrumental in elucidating the behavioural, neurochemical and neuroendocrine effects of specific cytokines or their combination, and on the whole can be considered to have yielded compelling evidence for a role of cytokines in depression.

In particular, exogenous administration of pro-inflammatory cytokines in mice and rats (most notably IL-1 β and TNF- α) elicits non-specific symptoms or “sickness behaviours” that, while potentially adaptive (e.g., serving to minimize energy expenditure), bear a strong resemblance to some of the neurovegetative-type endotoxemia/depression symptoms in humans (Dantzer et al., 2006, 2008; Anisman, 2009). Included here are soporific symptoms, fatigue, diminished motor activity, reduced sexual behaviour, anorexia, fever, and curled body posture (Dantzer et al., 2006). Additionally, mounting evidence has indicated that IL-1 β , TNF- α and IFN- α can elicit depressive-like emotional and cognitive disturbances (in particular hedonic-like deficits), and in some cases these effects were noted upon administration of subthreshold cytokine

doses (for sickness) (Gibertini et al., 1995; Bluthé et al., 1997; Yirmiya et al., 2002; Merali et al., 2003; Kaster et al., 2012; Hayley et al., 2013; Neis et al., 2014). Even IL-6, which is not directly implicated in sickness behaviour (Lenczowski et al., 1999), was seen to induce depressive-like symptoms when administered centrally (Sukoff Rizzo et al., 2012). Thus, cytokines appear to be capable of inducing centrally mediated depressive-like behaviours that are at least partially dissociable from sickness responses (Anisman et al., 2008b; Dantzer et al., 2008). Consistently, evidence from human studies indicates that cytokine-associated mood worsening, whether in the context of experimental endotoxemia or some other systemic inflammatory challenge (e.g., vaccination, infection), arises independently of physical sickness symptoms (Schedlowski et al., 2014).

In addition to these behavioural alterations, cytokine administration in rodents has been linked to pronounced hormonal, monoamine and neuroplastic changes (Dunn, 2000; Dunn et al., 2005a; Anisman et al., 2008b); these recall not only the effects of psychologically relevant stressors but also the presumed pathophysiology of depression (see §1.2.3). For instance, van Heesch et al. (2013) reported that intraperitoneal injection of TNF- α provoked signs of anhedonia coupled with increased serotonin (5-HT) and dopamine (DA) metabolite accumulation within the nucleus accumbens. Similarly, Anisman et al. (2008c) noted that subchronic (7 days) administration of IL-1 β altered 5-HT and norepinephrine (NE) neurotransmission in the PFC and hippocampus (and augmented mRNA expression of several cytokines and 5-HT receptors), while Koo and Duman (2008) found that hippocampal cell proliferation was suppressed by exogenous IL-1 β .

More indirect evidence of cytokine involvement in depression comes from animal studies that have used pharmacologic or genetic strategies to target the action of specific cytokines. In this regard, an antidepressant-like phenotype has been reported for mice genetically lacking TNF- α , IL-6, IL-1 β or their respective receptors (Butterweck et al., 2003; Simen et al., 2006; Camara et al., 2013, 2015). However, it bears noting that strain-dependent variation and compensatory developmental effects can complicate the interpretation and generalizability of such data (Kalueff et al., 2007; Eisener-Dorman et al., 2009). Moreover, studies are increasingly indicating that physiological levels of pro-inflammatory cytokines are required for the maintenance of myriad normal brain functions (Yirmiya and Goshen, 2011; Kohman and Rhodes, 2013; see Chapter 3).

In addition to these phenotypic characterizations, numerous studies have revealed that genetic or pharmacologic inhibition of pro-inflammatory cytokines, and conversely the potentiation of anti-inflammatory cytokines, such as IL-10, can mitigate the depressive-like effects of LPS and various stressor preparations (e.g., chronic variable or restraint stress, social defeat) (Chourbaji et al., 2006; Ben Menachem-Zidon et al., 2008; Goshen et al., 2008; Koo and Duman, 2008; Sukoff Rizzo et al., 2012; Karson et al., 2013; Pan et al., 2013). For instance, in the aforementioned study of Koo and Duman (2008), IL-1 β signalling blockade via treatment with an IL-1 β receptor inhibitor or the use of IL-1 receptor knockout (KO) mice mitigated the deleterious effects of stress on hippocampal neurogenesis and hedonic responding. Similarly, administration of the TNF- α antagonist infliximab attenuated depression-and-anxiety-like symptoms in rats exposed to chronic mild stress (Karson et al., 2013). In contrast, Voorhees et al. (2013) showed that chronic restraint-induced behavioural despair in the mouse forced swim test (FST)

was ameliorated by exogenous application of IL-10. Consistently, Mesquita et al. (2008) reported that IL-10 null mice exhibited depressive-like behaviour that was amenable to IL-10 treatment whereas transgenic mice overexpressing this cytokine displayed an antidepressant-like phenotype.

1.2.2.6 Immunomodulation and the antidepressant response

Mounting evidence suggests that immunomodulation may contribute mechanistically to the therapeutic action of antidepressant drugs. Indeed, several commonly used antidepressants, including prominent members of the SSRI class of drugs, were reported to decrease pro-inflammatory cytokines (e.g., IL-1 β , IL-6, TNF- α) and/or increase anti-inflammatory ones (e.g., IL-4, IL-10) (Kubera et al., 2005, 2009; Brustolim et al., 2006; Diamond et al., 2006; Himmerich et al., 2010a, b). These findings have largely been recapitulated in basic and preclinical animal models (both stressor- and- immune-based) (Kubera et al., 2000a, b; Roumestan et al., 2007; Ohgi et al., 2013; Réus et al., 2013). New and emerging antidepressants, including the N-methyl-D-aspartate (NMDA) receptor antagonist ketamine, have similarly been found to possess potent immunomodulatory properties (Chang et al., 2009; Walker et al., 2013; Réus et al., 2015; Yuhas et al., 2015). Overall, these reports are consistent with other studies linking antidepressants to the modulation of the T-helper type-1 (Th1)/Th2 cytokine balance, and specifically the suppression of pro-inflammatory Th1-type cytokines (IFN- γ , IL-12) in favour of the more anti-inflammatory Th2-type ones (IL-10, IL-4) (Maes et al., 1999; Kubera et al., 2001; Diamond et al., 2006).

However, it's important to underscore that symptom improvement under antidepressant treatment does not always coincide with normalization of circulating

cytokines (Eller et al., 2009; Hannestad et al., 2011). This has resulted in an ongoing debate concerning the pathophysiological significance of peripheral cytokine variations in depression (Bruenahl et al., 2013). Despite this, or perhaps because of it, the suggestion was offered that circulating cytokines might usefully be regarded as trait markers for certain sub-types or candidate endophenotypes of this highly heterogeneous disease (e.g., treatment-resistant, increased stress sensitivity) (Litteljohn and Hayley, 2012; Audet and Anisman, 2013), and possibly even as predictors of antidepressant response (Krishnadas and Cavanagh, 2012). In keeping with this, it will be recalled that genetic variation in TNF- α , IL-6 and IL-1 β was found to influence clinical response to antidepressant treatment (Uher et al., 2010).

Just as antidepressants are understood to exert potent immunomodulatory effects, anti-inflammatory agents are increasingly being recognized for their antidepressant-like properties (Köhler et al., 2014). Indeed, positive results from preclinical animal models have been reported for a diverse array of anti-inflammatory agents, including several COX-2 selective and non-selective non-steroidal anti-inflammatory drugs (Maciel et al., 2013; Kurhe et al., 2014), the tetracycline antibiotics minocycline and doxycycline (Molina-Hernández et al., 2008a; Mello et al., 2013; Zheng et al., 2015), plant-based compounds such as curcumin and Perilla leaf essential oil (Li et al., 2014), and various cytokine-specific antagonists (Koo and Duman, 2008; Karson et al., 2013). Certainly, for several of these compounds there has been at least some indication of their potential safety and utility in the clinical setting, either as monotherapy or antidepressant-augmentation therapy; e.g., Miyaoka and colleagues' (2012) open label study of minocycline as adjunctive therapy for unipolar depression. However, there is limited

supporting evidence from randomized clinical trials, reflecting not only the inherent difficulty and cost of conducting these studies, but also the existence of null and conflicting results (e.g., Bergman et al., 2013; Andrade, 2014). Nonetheless, positive randomized clinical trial findings have recently been described for the COX-2 inhibitor celecoxib (Nery et al., 2008; Abassi et al., 2012; Köhler et al., 2014) and the diarylheptanoid derivative curcumin (Lopresti et al., 2014, 2015). Overall, then, it would appear that immunomodulation, and specifically antagonism of pro-inflammatory cytokines and agonism of anti-inflammatory ones, likely contributes to the therapeutic action of antidepressant drugs.

1.2.3 Mechanisms of cytokine action in depression

Cytokines primarily signal through one of three molecular pathways, involving the activation of 1) nuclear factor-kappa B (NF- κ B), 2) the mitogen-activated protein (MAP) kinases p38 and c-Jun N terminal kinase (JNK), or 3) various janus family tyrosine kinases (Jaks) and signal transducers and activators of transcription (Stats). These signal transduction pathways involve the sequential recruitment, phosphorylation and/or ubiquitination of a series of intracellular proteins, ultimately leading to the induction of inflammatory transcriptional programs (Leonard and Lin, 2000; Li and Lin, 2008). For instance, binding of TNF- α to the ubiquitously expressed cell surface TNF- α receptor 1 (TNF- α receptor 2 is primarily expressed in immune cells) triggers the phosphorylation and degradation of the inhibitory factor I κ B, which normally sequesters and inhibits NF- κ B in the cytosol. Upon liberation, NF- κ B translocates to the nucleus where it regulates the transcription of myriad immune, inflammatory and apoptotic genes (e.g., iNOS, COX-2, various cytokines/chemokines) (Li and Lin, 2008).

Substantial evidence supports the involvement of cytokine-associated signalling factors in the development of depression and related affective disorders (Litteljohn et al., 2010a). For instance, pharmacological inactivation of the NF- κ B pathway in mice abrogated both the depressive-like behavioural effects (reduced food intake, social withdrawal) and brain regional transcriptional activation (i.e., induction of the immediate early gene *c-fos*) induced by systemically administered IL-1 β (Nadjar et al., 2005). Moreover, mice genetically lacking the p50 subunit of NF- κ B displayed a pronounced anxiolytic-like phenotype (in the open field and elevated plus maze) (Kassed and Herkenham, 2004). Similarly, recent work has shown that inhibition of JNK, which causes glucocorticoid receptor (GR) function to become enhanced in vitro (Wang et al., 2005), mitigated the behavioural and neurochemical effects of an acute stressor and promoted antidepressant-like responses in rodent behavioural despair paradigms (Galeotti and Ghelardini, 2012) (although intriguingly our group found that JNK inhibition itself provoked stressor-like changes: Clarke et al., 2012). An analogous antidepressant-like phenotype was noted upon p38 MAP kinase blockade (Galeotti and Ghelardini, 2012).

Mediated largely through the above described signalling pathways, cytokines are thought to contribute to the development of depressive pathology by influencing: 1) central neurotransmitter systems, 2) neuroendocrine function, 3) processes aligned with neuroplasticity (e.g., neurogenesis and neurotrophin signalling), and 4) neurotoxic and neurodegenerative processes.

1.2.3.1 Central neurotransmitter systems

Perturbations of brain regional monoamine neurotransmission involving 5-HT, NE and DA are thought to underlie many of the motivational, cognitive and emotional

symptoms of depression (Undurraga and Baldessarini, 2012; Hamon and Blier, 2013; Gryglewski et al., 2014). This position is supported by a wealth of evidence drawn from human neurotransmitter depletion, neuroimaging and postmortem studies, together with preclinical animal models (Delgado and Moreno, 2000; Ruhé et al., 2007). Moreover, all monoamine-targeting antidepressants, whether reuptake inhibitor, receptor antagonist or monoamine oxidase (MAO) inhibitor, produce the same pharmacological effect: enhanced synaptic concentrations of monoamines and the consequent restoration of functional tone in depression-relevant brain circuits (critically involving parts of the frontal cortex, amygdala, and hippocampus) (Delgado and Moreno, 2000; Nutt, 2002; Hamon and Blier, 2013). Reductionist explanations of depression as being caused simply by 5-HT, NE and DA dysfunction are, however, clearly misguided; one need only look to the unsettlingly low response and remission rates achieved with conventional monoamine-acting antidepressants (Moncrieff and Kirsch, 2005). Nevertheless, that virtually all of the new players in the pathophysiology and treatment of depression, e.g., the non-monoamine neurotransmitters glutamate and gamma-aminobutyric acid (GABA) as well as neuroplastic and neurodegenerative processes, affect monoaminergic signalling in one way or another speaks to the functional relevance of 5-HT, NE and DA in depression and the antidepressant response (Gigliucci et al., 2013; Fukumoto et al., 2014; El Iskandrani et al., 2015).

Consistently, a considerable amount of evidence has accumulated indicating that cytokines are capable of influencing multiple aspects of monoamine neurotransmitter function (i.e., release, reuptake, synthesis) (Anisman et al., 2005; Hayley et al., 2005; Zunszain et al., 2013). Generally, while acute cytokine treatment tends to stimulate

central monoaminergic activity, the opposite is seen with chronic cytokine exposures (Hayley et al., 2005; Miller et al., 2009). Thus, a number of groups have reported that acute systemic or central administration of IL-1 β , IFN- α and, to a lesser extent, TNF- α increases 5-HT and NE release and/or utilization within stressor-sensitive brain regions, such as the PFC, nucleus accumbens (NAcc), hippocampus, and various hypothalamic and amygdaloid nuclei (Linthorst et al., 1995; Kamikawa et al., 1998; Ando and Dunn, 1999; Hayley et al., 1999; 2001a; Kamata et al., 2000). Consistently, acute IFN- α treatment decreased expression of the 5-HT_{1A} autoreceptor (which upon ligand binding inhibits further 5-HT release) (Cai et al., 2005) whereas chronic cytokine exposure increased 5-HT_{1A} receptor densities and activity/ligand affinity (Abe et al., 1999). And more recently, our group showed that repeated central administration of murine IFN- α induced anhedonic-like behaviour together with altered hippocampal 5-HT levels and brain regional 5-HT receptor mRNA expression (Hayley et al., 2013).

Similar findings have been reported for cytokine influences on dopaminergic neurotransmission (Shuto et al., 1997; Felger et al., 2007). For instance, systemic administration of IL-1 β enhanced DA metabolite accumulation in the PFC (Merali et al., 1997) while intra-hypothalamic infusion of the cytokine stimulated local DA release and utilization (Mohankumar et al., 1991; Shintani et al., 1993). Moreover, it has recently been shown that basal ganglia DA function is impaired among IFN- α -treated hepatitis C patients (i.e., reduced amine synthesis and/or release upon chronic cytokine treatment) (Capuron et al., 2012). With respect to mechanisms underlying cytokine-induced alterations of neurotransmitter release, in the acute setting these probably involve rapid changes in ionic conductances and the mobilization of second messenger signalling

cascades (Tancredi et al., 1992; Galic et al., 2012; see Chapter 4). In the chronic setting cytokines are probably having a greater influence on monoamine synthesis and re-uptake (see below); however, there have been indications that cytokines may impair vesicular monoamine transporter-2 (VMAT2) function or reduce its expression in neurons (Kazumori et al., 2004; Felger and Lotrich, 2013). As VMAT2 is responsible for the loading of monoamines into synaptic vesicles, a decreased VMAT2 function can be expected to diminish neurotransmitter release at presynaptic terminals (i.e., reduced quantal size).

The inducible enzyme IDO is an important molecular player linking pro-inflammatory cytokines to diminished 5-HT concentrations (Dantzer et al., 2008; Maes et al., 2011b; McCusker et al., 2013). Briefly, upon induction by pro-inflammatory cytokines (in particular the IFNs but also TNF- α , IL-1 β , IL-6 and others) IDO catabolizes the extra-hepatic conversion of tryptophan (TRP) to kynurenine (KYN), at once shunting metabolism away from 5-HT synthesis and towards the production of neuroactive KYN pathway metabolites, most notably 3-hydroxykynurenine (3-HK), quinolinic acid (QUIN) and kynurenic acid (KYNA) (Fig. 1.1). As TRP is the sole precursor of 5-HT, it is clear to see how cytokine-induced IDO activation could promote 5-HT deficiency and depressed mood (Miller et al., 2009; Maes et al., 2011b). Induction of the KYN pathway could also perturb monoamine homeostasis via deactivation of the highly redox-sensitive 5,6,7,8-tetrahydrobiopterin (BH₄), which is an obligatory cofactor of the monoamine synthetic enzymes (Miller et al., 2009; Sperner-Unterweger et al., 2014). Interestingly, although the NMDA receptor antagonist KYNA is generally considered to be neuroprotective, several reports have indicated that DA release is impaired secondary to

KYNA-mediated blockade of the $\alpha 7$ subunit of the nicotinic acetylcholine receptor (Rassoulpour et al., 2005; Wu et al., 2007). We pick up the discussion of potential IDO-related mechanisms in depression in a later section (§1.3.3.2).

Beyond their effects on neurotransmitter synthesis and release, pro-inflammatory cytokines can markedly influence the reuptake of monoamines from the synaptic cleft. The evidence in this regard is strongest for the 5-HT system, with several reports indicating that TNF- α , IL-1 β and others increase the expression, membrane trafficking and/or catalytic activation (via p38 MAP kinase) of the 5-HT transporter SERT (Ramamoorthy et al., 1995; Morikawa et al., 1998; Mössner et al., 1998; Zhu et al., 2006; Malynn et al., 2013). While fewer studies have implicated cytokines in the alteration of NE and DA transporters (i.e, NET and DAT), this remains a distinct possibility (Morón et al., 2003; Hozumi et al., 2008; van Heesch et al., 2013). Recent evidence also suggests that cytokines may act to increase the enzymatic metabolism of monoamines and other neurotransmitters, e.g, via a p38 MAP kinase-dependent upregulation of MAO (Cao et al., 2009; Ming et al., 2015).

1.2.3.2 Neuroendocrine function

Depression is associated with pronounced alterations of neuroendocrine function. In particular, there is a marked hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis, with the attendant chronic elevation of glucocorticoid hormones, most notably cortisol (corticosterone in rodents), and the eventual development of physiological resistance to them (i.e., glucocorticoid resistance, which reflects the down-regulated expression and/or impaired function/sensitivity of the GR) (Anacker et al., 2011; Laryea et al., 2015). Chronically elevated glucocorticoids and/or GR dysfunction

have been linked to a number of other depression-relevant pathophysiological domains, including impaired hippocampal neurogenesis and altered neurotrophin and monoamine neurotransmitter signalling (Pariante and Lightman, 2008). Moreover, seeing as how glucocorticoids normally help terminate the inflammatory response (via GR-mediated anti-inflammatory transcription), glucocorticoid resistance provides a permissive environment for inflammatory cytokine signalling (Wang et al., 2011; Horowitz and Zunszain, 2015). Interestingly, recent evidence also indicates that glucocorticoids may themselves exert pro-inflammatory effects under certain circumstances (depending on temporal and contextual factors) (Sorrells et al., 2009). This provides yet another route to the paradoxical co-existence, and possible synergism, of elevated glucocorticoids and inflammatory cytokines in depression (Horowitz and Zunszain, 2015).

Indeed, communication between the neuroendocrine and immune systems is bidirectional, and pro-inflammatory cytokines such as IL-1 β and IFN- α are capable of markedly increasing HPA axis activity (Pace et al., 2007; Pariante and Lightman, 2008). This occurs in at least two distinct ways. Firstly, cytokines can activate the HPA axis directly at the level of the brain, stimulating the release of CRH from the hypothalamus (Berkenbosch et al., 1987; Sapolsky et al., 1987; van der Meer et al., 1996). Acting on cells in the pituitary, CRH induces the synthesis and secretion of adrenocorticotrophic hormone (ACTH), which, in turn, induces adrenocortical cells to produce and secrete glucocorticoids (Berkenbosch et al., 1987). Secondly, cytokines can engender glucocorticoid resistance by impairing GR function or sensitivity (Pariante et al., 1999), an effect that is likely dependent on p38 MAP kinase and possibly JNK (Szatmáry et al., 2004; Wang et al., 2004). Since glucocorticoids normally exert negative (autoregulatory)

feedback on the HPA axis, a resistance to them at the receptor level can be seen to promote persistent HPA axis activity (Pace et al., 2007). Taken together, the available evidence strongly implicates cytokines in HPA axis dysfunction (with actions recalling those of various processive and neurogenic stressors) and suggests that cytokine-neuroendocrine interactions are of pathogenic relevance to depression.

1.2.3.3 Neuroplastic mechanisms: neurogenesis and neurotrophin signalling

In addition to neurotransmitter and neuroendocrine dysfunction, mounting evidence suggests that alterations of neuroplasticity contribute to depression (Pittenger and Duman, 2008; Pandey et al., 2010). In particular, impaired hippocampal cell proliferation and neurogenesis have been observed in several preclinical animal models (Hayley et al., 2005) and it has been suggested that such reductions, and their correction with antidepressant treatment, might at least partially account for hippocampal volume variations in depression (Neumeister et al., 2005; Kronmüller et al., 2008a, b; Boldrini et al., 2009; Snyder et al., 2011; Malykhin and Coupland, 2015; Schmaal et al., 2015). Importantly, however, recent reviews do not lend strong support to the position that impaired neurogenesis itself leads to depression, suggesting rather that such changes may facilitate or “set the stage” for stressor-induced pathology (i.e., altering stress resilience) (Petrik et al., 2012; Levone et al., 2015). However, it would appear that the antidepressant response is at least partially dependent on adult hippocampal neurogenesis (Czeh et al., 2001; Petrik et al., 2012; Peng et al., 2013; Levone et al., 2015).

Keeping this recent paradigm shift in mind, it is still informative to point out that numerous animal studies have revealed a capacity of pro-inflammatory cytokines, and inflammation more generally, to influence hippocampal neurogenesis and other aspects

of neuroplasticity (Ek Dahl et al., 2003; Monje et al., 2003; Hayley et al., 2005; Seguin et al., 2009; Borsini et al., 2015). For instance, hippocampal neurogenesis was markedly diminished among IL-6 over-expressing transgenic mice (Vallières et al., 2002) whereas IL-6 KO abrogated the LPS-induced inhibition of neurogenesis (Ji et al., 2013). Similarly, exogenous administration of IFN- α or IL-1 β reduced hippocampal neurogenesis and this effect was attenuated by inhibition of either cytokine (Kaneko et al., 2006; Koo and Duman, 2008; Zheng et al., 2014).

Although precise mechanisms are still being mapped out, there appear to be several intersecting routes by which cytokines could come to influence neuroplasticity in depression (Yirmiya and Goshen, 2011). Included here is the modulation of inflammatory signalling cascades (e.g., COX-2/prostaglandins, NO, p38 MAP kinase, JNK, NF- κ B) as well as plasticity-related immediate early genes and synaptic proteins (e.g., glutamate receptors, the activity-regulated cytoskeleton-associated protein Arc) (Mattson, 2005; Iosif et al., 2006; Widera et al., 2006; Yirmiya and Goshen, 2011). In this regard, our group and others has argued recently that the prototypical inflammatory transcription factor NF- κ B may be particularly well positioned to mediate impaired neurogenesis in inflammation, much as it mediates the detrimental effects of stress (Anisman et al., 2008b; Koo et al., 2010). Recent and emerging evidence appears to support this position (Ji et al., 2013; Pugazhenti et al., 2013).

Another possibility is that cytokines could negatively impact hippocampal cell proliferation and neurogenesis, not to mention other depression-relevant pathophysiological domains (e.g., monoamine neurotransmission: Haase and Brown, 2015), via actions on BDNF and related neurotrophins (in particular IGF-1 and TGF- β)

(Kenis et al., 2011; Audet and Anisman, 2013; Calabrese et al., 2014). Indeed, several reports have indicated that TNF- α , IL-1 β and IFN- α are capable of reducing BDNF expression and antagonizing aspects of BDNF-dependent neuronal signalling [(e.g., protein kinase B (PKB)/Akt, extracellular signal-regulated kinase (ERK), LTP] (Lapchak et al., 1993; Tong et al., 2008, 2012; Alboni et al., 2013; Song et al., 2013). Moreover, depressive behaviours provoked by IFN- α and IL-1 β were associated with reduced BDNF levels and reductions of hippocampal neurogenesis, and these effects normalized upon administration of the IL-1 β receptor antagonist, IL-1ra (Anisman et al., 2008b; Dedoni et al., 2012). Similarly, treatment with LPS or the viral mimic polyinosinic-polycytidylic acid (poly I:C) augmented hippocampal, prefrontal and peripheral pro-inflammatory cytokine concentrations whilst reducing BDNF levels, and these effects coincided with pronounced memory deficits and depression-like behaviours (Kranjac et al., 2012a, b; Gibney et al., 2013).

It is also important to underscore that pro-inflammatory cytokine influences on hippocampal neurogenesis are not uniformly deleterious. Indeed, numerous cytokines appear to be capable of exerting both stimulatory and inhibitory effects on structural and functional neuroplasticity (e.g., neural cell proliferation and differentiation, LTP). For instance, even as we highlight a potential critical role of NF- κ B in the inflammatory suppression of neurogenesis, the transcription factor is readily activated in response to excitatory neurotransmission and is believed to contribute to LTP, learning and memory, and neuronal survival following injury (via the induction of anti-apoptotic proteins, antioxidant enzymes and neurotrophin-associated signalling cascades) (Freudenthal et al., 2004; Kassed et al., 2004; Mattson, 2005; Widera et al., 2006). Similarly, pro-

inflammatory cytokines have not always been linked to diminished neurotrophin levels; e.g., TNF- α -induced upregulation of BDNF in cultured astrocytes (Saha et al., 2006). As discussed in an earlier section (§1.2.1), it is anticipated that cytokine concentration and chronicity of exposure will be critical in determining whether inflammatory signalling has detrimental or salutary effects on neuroplasticity (Yirmiya and Goshen, 2011).

1.2.3.4 Neurotoxicity and neurodegeneration

Neuroinflammation has long been implicated in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) and multiple sclerosis (Frank-Cannon et al., 2009; Chen et al., 2016). It appears increasingly likely that inflammation-mediated degenerative, oxidative and neurotoxic processes are also relevant to depression (and especially in certain subtypes of the disease e.g., late-life depression); this has given rise to recent conceptualizations of depression as a neuroprogressive illness (Moylan et al., 2013; Bakunina et al., 2015). Indeed, while hippocampal volume reductions among depressed patients seem to be linked to impaired neurogenesis (Malykhin and Coupland, 2015), neurodegenerative processes are probably also involved (Kronmüller et al., 2009; Gerhard et al., 2016). Moreover, depression-associated volumetric changes in the paraventricular nucleus of the hypothalamus (PVN), amygdala and anterior cingulate cortex are almost certainly reflective of neuronal and/or glial cell atrophy and degeneration (Sheline et al., 1998; Cotter et al., 2001; Manaye et al., 2005).

In keeping with the notion that inflammation may be a primary mover of the putative neurodegenerative process in depression, cytokines have been implicated in a plethora of oxidative and excitotoxic pathways to neuronal damage and demise. Included here is the induction of the pro-oxidant/inflammatory enzymes COX-2, inducible nitric

oxide synthase (iNOS) and IDO; the resultant production of free radicals, such as superoxide and peroxynitrate; oxidative damage to DNA, proteins and lipids; activation of caspases and other apoptotic proteins; and NMDA- and- α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor-mediated excitotoxicity (Moylan et al., 2013; Bakunina et al., 2015). Indeed, IFN- α treatment of cultured human neuroblastoma cells reduced cell density and BDNF gene expression whilst increasing mitochondrial activity, reactive oxygen species (ROS) and the Bax-to-Bcl-2 mRNA ratio (indicative of apoptotic activation) (Alboni et al., 2013). Moreover, co-treatment with the antioxidant and mitochondrial modulator N-acetyl-cysteine reversed these effects (Alboni et al., 2013). Also, both TNF- α and IFN- γ were shown to induce or otherwise potentiate glutamatergic neurotoxicity (Zou and Crews, 2005; Mizuno et al., 2008), and IL-1 β and IL-12 upregulated iNOS expression within microglia and astrocytes (Hartlage-Rübsamen et al., 1999; Pahan et al., 2001). Once again, NF- κ B can be expected to play an important role here as its activity is both regulated by and implicated in the further elaboration of ROS and inflammatory cytokines (Bakunina et al., 2015).

As previously mentioned, IDO-mediated depletion of 5-HT synthesis is considered a leading mechanism subserving cytokine influences in depression (§1.2.3.1). However, it will be recalled that TRP degradation along the KYN pathway also leads to the generation of several neuroactive KYN metabolites, including KYNA in astrocytes and 3-HK and QUIN in microglia (Fig. 1.1). While KYNA is generally considered to be neuroprotective (having anti-excitotoxic and anti-oxidant properties), the latter two are demonstrably neurotoxic (Wichers et al., 2005; Schwarcz et al., 2012). QUIN, itself a 3-HK derivative, is a potent NMDA receptor agonist and can stimulate NMDA receptor-

mediated excitotoxicity whereas 3-HK is an endogenous oxidative stress generator capable of inducing apoptotic cell death (and as we saw earlier the oxidative destruction of BH4) (Okuda et al., 1998; Wichers et al., 2005). It follows, then, that an immune-based imbalance in the levels of neurotoxic and neuroprotective KYN metabolites could promote neuronal atrophy and death, which might then influence the development or maintenance of depressive states (Maes et al., 2011a, b; Young et al., 2016). This position derives support from recent and emerging research findings (Gabbay et al., 2010; Steiner et al., 2011; Zunszain et al., 2012; Bay-Richter et al., 2015; Savitz et al., 2015a, b).

1.3 The growing case for IFN- γ in depression

As reviewed here, an extensive body of evidence implicates cytokines in depression. And while the research enterprise has largely focused on IL-1 β , IL-6, TNF- α , and IFN- α , we argue that a convincing case can likewise be made for IFN- γ . In the following sections we cover the evidence linking IFN- γ with depression and discuss the various mechanisms by which the cytokine could come to influence affective states. We begin with a brief discussion of the neurobiology of IFN- γ signalling.

1.3.1 Neurobiology of IFN- γ signalling: an overview

Interferons (IFNs) are broadly divided into either type I IFNs, including the IFN- α and IFN- β isoforms, or the structurally unrelated type II IFN, of which IFN- γ (formerly called macrophage activating factor) is the sole member. IFN- γ is biologically active as a noncovalent homodimer and binds to IFN- γ receptor-1 in a 2:2 stoichiometry (Schroder et al., 2004). Joining these two ligand-binding IFN- γ receptor-1 chains in the functional IFN- γ receptor complex are two signal-transducing IFN- γ receptor-2 chains as well as various intracellular signalling factors (Jaks and Stats, see below) (Schroder et al., 2004).

Although initially it was believed that IFN- γ is secreted exclusively from NK cells and Th1 and cytotoxic lymphocytes, it has become increasingly clear that B lymphocytes and other non-lymphoid professional antigen-presenting cells, such as dendritic cells and macrophages, also contribute to the IFN- γ response (Gessani and Belardelli, 1998; Schroder et al., 2004; Darwich et al., 2009). Additionally, low levels of the cytokine are synthesized de novo within the brain by activated microglia (Kawanokuchi et al., 2006), astrocytes (Lau and Yu, 2001) and probably even neurons (Ljungdahl et al., 1989; Neumann et al., 1997).

The IFNs are crucial mediators of both early innate and adaptive immune responses to microbial infection (e.g., antigen processing and presentation, immunoproteasome induction, inhibition of protein synthesis, activation of macrophages and NK cell effector functions, production of antigen-specific cytotoxic T cells), a fact reflected in the multiple immune cell types that have been found to secrete these cytokines (Shtrichman and Samuel, 2001; Chesler and Reiss, 2002). In addition to their antiviral actions, the IFNs play an important role in host defense against certain bacterial (especially mycobacteria in the case of IFN- γ), fungal and parasitic pathogens (Shtrichman and Samuel, 2001; Stevens et al., 2006). As well, IFN- γ has potent immunomodulatory, anti-proliferative and anti-tumor properties (Ikeda et al., 2002; Schroder et al., 2004; Dunn et al., 2005b).

As is true of the type I IFNs and many other cytokines and growth factors, IFN- γ mainly signals through the canonical Jak/Stat signal transduction pathway (Bach et al., 1997). This pathway involves the sequential recruitment of cytokine receptors and the activation of intracellular signalling components, namely various Jaks and Stats. As it

relates specifically to IFN- γ , ligand binding induces a conformational change in the IFN- γ receptor-1/2 chains, which leads to the sequential activation of Jak2 and Jak1 (the former via autophosphorylation and the latter via transphosphorylation by Jak2) and the recruitment, phosphorylation and homodimerization of Stat1 (Schroder et al., 2004). These Stat1 homodimers, otherwise called γ -IFN activation factors, translocate to the nucleus and bind to IFN- γ -activation sites (GAS) in the promoter region of IFN- γ -responsive genes; in this way, IFN- γ -Stat1 signalling can initiate or suppress the transcription of myriad immunologically-relevant target genes (Bach et al., 1997; Schroder et al., 2004). Additionally, IFN- γ signalling produces Stat1 heterodimers and heterotrimers (i.e., Stat1:Stat1/Stat2:IFN regulatory factor-9 complexes), which, along with various IFN regulatory transcription factors generated as part of the first wave of IFN- γ -induced transcription (e.g., IRF-1), can bind to IFN-stimulated response element (ISRE) promoter regions to regulate further rounds of transcription (Briscoe et al., 1996; Schroder et al., 2004) (Fig. 1.2). Interestingly, the Stat1:Stat2:IFN regulatory factor-9 complex, otherwise known as IFN-stimulated gene factor-3, is considered an important transcription factor in type I IFN-mediated antiviral responses and its activation by IFN- γ thus provides a mechanism for cross-talk between the IFN signalling pathways (Matsumoto et al., 1999).

Negative regulation of IFN- γ signalling occurs through several mechanisms. These include: a) internalization, degradation and/or recycling of the IFN- γ :IFN- γ receptor-1 complex; b) disruption of Jak1/2 phospho-activity and/or targeting of Jaks for proteasomal degradation by suppressors of cytokine signalling-1 (SOCS-1) and SOCS-3, which are highly inducible gene targets of IFN- γ (i.e., a form of negative feedback); c)

dephosphorylation of IFN- γ receptors and Jaks by the protein tyrosine phosphatases Src homology region 2 domain-containing phosphatase-1/2; and d) nuclear dephosphorylation of Stat1 (Schreiber et al., 1992; Haspel et al., 1996; You et al., 1999; Schroder et al., 2004; Starr et al., 2009). IL-12 and IL-18 released by antigen-presenting cells are the major positive regulators of IFN- γ production whereas glucocorticoids and the anti-inflammatory cytokines IL-4, IL-10 and TGF- β mediate the opposite effect (Ito et al., 1999; Hu et al., 2003; Schroder et al., 2004; Lin et al., 2005).

1.3.2 Evidence supporting a role of IFN- γ in depression

Many of the same lines of evidence implicating IL-1 β , TNF- α , IL-6, and IFN- α in depression are relevant to the case of IFN- γ . For instance, circulating IFN- γ mRNA or protein levels (or the IFN- γ /IL-4 ratio) are elevated in depression (Maes et al., 1994, Simon et al., 2008; Gabbay et al., 2009; Dahl et al., 2014) and antidepressant treatment tends to at least partially normalize these changes (Myint et al., 2005; Tsao et al., 2006). The latter findings are consistent with in vitro reports indicating that antidepressant drugs suppress IFN- γ and/or the IFN- γ /IL-10 (or IFN- γ /IL-4) production ratio (Maes et al., 1999; Kubera et al., 2001; Diamond et al., 2006). In addition, variation in the IFN- γ gene was reported to modify both depression risk and TRP metabolism among hepatitis C patients receiving IFN- α immunotherapy (Oxenkrug et al., 2011; Myint et al., 2013). Specifically, Oxenkrug et al. (2011) observed that carriers of at least one high producer T allele of the IFN- γ +874 T/A polymorphism were more likely to develop depression whereas Myint et al. (2013) noted that presence of the high producing IFN- γ CA repeat allele-2 (a microsatellite polymorphism that correlates with the +874 T allele: Pravica et al., 2000) predicted increased TRP breakdown over time.

Corroborating and expanding upon the findings from human studies, a growing number of basic and preclinical research studies appear to support a role for IFN- γ in depression and related stressor-associated conditions. Firstly, elevated circulating and brain levels of IFN- γ have been reported in various stressor- and- immune-based animal models of depression; e.g., chronic stress, LPS, infection with *Toxoplasma gondii* or *Mycobacterium bovis* bacillus Calmette Guerin (BCG) (Lestage et al., 2002; Moreau et al., 2005, 2008; O'Connor et al., 2009a; Liu et al., 2013; Rho et al., 2014; Wrona et al., 2014; Fischer et al., 2015; Mahmoud et al., 2016). Secondly, genetic ablation of the IFN- γ receptor in mice attenuated the depressive-like behavioural consequences (forced swim and tail suspension tests) and central TNF- α mRNA and IDO elevations provoked by infection with BCG (O'Connor et al., 2009b). And thirdly, acute infection with an IFN- γ adenovector provoked long-lasting depression-like behaviour (Kwant and Sakic, 2004). In contrast, Kustova et al. (1998) reported that mice genetically lacking IFN- γ displayed increased basal emotionality on tests thought to reflect anxiety-like behaviours (elevated plus maze, open field test) while Campos et al. (2014) described a basal neurogenic deficit among these animals. The source of the divergent effects that have been reported for IFN- γ manipulations in animal models is uncertain but potentially speaks to a differential role of the cytokine in basal and challenge conditions (see Chapter 3).

1.3.3 Depression-relevant mechanisms of IFN- γ action

In §1.2.3 we saw how pro-inflammatory cytokines, such as IL-1 β , TNF- α , IL-6, and IFN- α , could come to influence depressive-like states through their actions on monoamine neurotransmission, HPA axis activity, neurogenesis and BDNF, and potentially even neurodegenerative processes. As we briefly discuss in the following

sections, there is reason to believe that each of these processes or domains could also hold relevance for the behavioural effects of IFN- γ . As it currently stands, however, there is probably insufficient information to allow for any firm conclusions to be drawn. We should also be mindful of the important role played by IFN- γ in mobilizing immune cells and elaborating cytokine cascades (Schroder et al., 2004). Indeed, TNF- α and the IL family members IL-6, IL-7, IL-15, IL-12, IL-1 α , and IL-1 β , are all either directly or indirectly upregulated by IFN- γ (Rock et al., 2005; Moran et al., 2007; Vila-del Sol et al., 2008). Moreover, IFN- γ interacts synergistically with several different cytokines, most notably TNF- α , IL-1 β and IFN- α , to influence myriad immune-inflammatory processes; e.g., synergistic activation of NF- κ B and IDO by IFN- γ and TNF- α (Levy et al., 1990; Cheshire and Baldwin, 1997; Robinson et al., 2003). Thus, even as the evidence specifically implicating IFN- γ in the modulation of depression-relevant processes may be sparse in places, the possibility must be considered that IFN- γ could yet be influencing these pathways via downstream effects on or interactive effects with other cytokines (e.g., De Simoni et al., 1997; Maher et al., 2006). In a similar vein, cytokines that are upstream of IFN- γ , most notably IL-12 and IL-18, are increasingly being implicated in depression and related stressor-associated conditions (Lee and Kim, 2006; Haastrup et al., 2012; Prossin et al., 2016).

1.3.3.1 IDO-mediated 5-HT depletion

IFN- γ is a preferential activator of IDO (Wirleitner et al., 2003). As we saw previously, IDO activation accelerates the degradation of TRP along the KYN pathway, at once shunting metabolism away from 5-HT synthesis and toward the production of neuroactive and mostly deleterious KYN pathway metabolites (Dantzer et al., 2008). Not

surprisingly, both 5-HT depletion and neurotoxic KYN derivatives (e.g., QUIN and 3-HK) have been heralded as probable important mediators of the depressogenic effect of IFN- γ (Oxenkrug, 2011; Maes et al., 2011b). However, strong evidence has been somewhat difficult to come by, particularly in regards to the involvement of IDO-mediated 5-HT depletion. For instance, while Hughes et al. (2012) confirmed that depressed patients had a mild inflammatory profile (including increased IFN- γ) that was accompanied by reduced circulating TRP concentrations, the latter did not obviously stem from an IFN- γ -induced stimulation of IDO as there was no difference between depressed patients and controls in the levels of either plasma IDO or KYN. Moreover, while Zoga et al. (2014) showed that depressed patients had elevated levels of IFN- γ , CRP, TNF- α , and IDO, only the latter two were responsive to antidepressant treatment, with symptom improvement coinciding with reductions in IDO. One possible reading of these data is that IDO activation occurred independently of the influence of IFN- γ (Zoga et al., 2014). Consistently, the results of several animal studies have indicated that IFN- γ is not a critical mediator of LPS-induced IDO activation and depressive-like behaviour (Fujigaki et al., 2001; Connor et al., 2008; O'Connor et al., 2009c; Wang et al., 2010).

Yet, non-depressed female carriers of the high producer T allele of the IFN- γ +874 gene displayed increased IDO activity relative to carriers of the low producer A allele (i.e., elevated plasma KYN and KYN:TRP levels) (Raitala et al., 2005), and it will be recalled that this same high IFN- γ -producing genotype is associated with a heightened risk of developing IFN- α -induced depression (which is tied to 5-HT depletion: Bonaccorso et al., 2002; Raison et al., 2009). Also, depressed and non-depressed carriers of the high IFN- γ -producing CA repeat allele-2 had elevated serum KYN concentrations;

however, in this study the presence of depression and not IFN- γ genotype per se predicted diminished circulating concentrations of both TRP and the 5-HT metabolite 5-hydroxyindole acetic acid (5-HIAA) (Myint et al., 2013). Consistently, while O'Connor et al. (2009b) demonstrated the required nature of IFN- γ in the IDO-enhancing and depressive-like behavioural effects of BCG, 5-HT depletion was not implicated in this regard. Overall, the available evidence suggests that: a) the extent to which IFN- γ -mediated IDO activation contributes to depressive-like pathology likely hinges on characteristics of the initial immune-activating stimulus [e.g., Toll-like receptor-2 (TLR2)-activating agents such as BCG vs. TLR4 agonists such as LPS]; and b) even in the case where IFN- γ can be seen to influence depressive-like outcomes via IDO activation, it may be that reduced 5-HT bioavailability is not as important as once believed.

1.3.3.2 Monoamine and neuroendocrine alterations: IDO and beyond

Several possible routes exist by which IFN- γ could influence central monoamine systems independently of an IDO-mediated reduction in 5-HT synthesis. We have already seen how QUIN, 3-HK and other neurotoxic metabolites generated along the IDO-KYN pathway could induce atrophy or degeneration of monoaminergic as well as non-monoaminergic neurons (Myint and Kim, 2014). In this regard, chronic daily systemic administration of IFN- γ in mice caused a sustained increase in brain and circulating QUIN concentrations, together with a persistent activation of IDO (Saito et al., 1991). Also, stimulation of primary human macrophages with IFN- γ alone or in combination with IFN- α or TNF- α produced extremely high levels of QUIN (Pemberton et al., 1997). More recently, Hochstrasser et al. (2011) showed that IFN- γ reduced the survival of, and

upregulated IDO expression in, serotonergic neurons of the dorsal raphe nucleus (using organotypic slice cultures), a brain region that displayed significant 5-HIAA alterations in response to exogenous IFN- γ (Clement et al., 1997). However, these events did not appear to be causally linked as NMDA receptor blockade with MK-801 failed to reverse the IFN- γ -induced 5-HT cell loss, as would be expected if QUIN-mediated excitotoxicity were involved. Rather, the authors point to IFN- γ -associated inflammation and oxidative stress more generally, presumably mediated by microglia in the organotypic slice cultures, as the likely cause of 5-HT neuron demise (Hochstrasser et al., 2011).

Beyond frank degeneration, it's been suggested that IFN- γ -induced oxidative stress (whether via the aforementioned generation of 3-HK or other means) could lead to a reduction in monoamine biosynthesis via destruction of the amine synthetic enzyme cofactor BH4 (Sperner-Unterweger et al., 2014). In this regard, oxidative destruction of BH4 has been linked to disturbed adrenergic neurotransmission in a range of chronic inflammatory conditions that are highly co-morbid with depression [e.g., human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS), cancer, pancreatitis] (Miller et al., 2013; Sperner-Unterweger et al., 2014). Also, as was described earlier for other pro-inflammatory cytokines, there has been some evidence linking IFN- γ to increased SERT transcription in neurons (Morikawa et al., 1998); this would be expected to reduce the synaptic availability of 5-HT. Nevertheless, it should be underscored that very few studies, one of which is the above-cited report of Clement et al. (1997), have expressly investigated the central monoaminergic effects of IFN- γ .

As discussed in an earlier section (§1.2.3.2), cytokine-HPA axis interactions are considered germane to depression; these may be especially pertinent to depression co-morbid with neurodegenerative disease or other inflammatory-based medical illnesses (Anisman and Hayley, 2012; Du and Pang, 2015). Given the important role ascribed to IFN- γ in many of these medical conditions (e.g., AD and PD) (Barcia et al., 2011; Browne et al., 2013; Belkhefha et al., 2014), one might reasonably speculate that IFN- γ likewise contributes to (co-morbid) depression via modulation of HPA axis signalling. However, surprisingly little evidence exists on this matter, and that which does is at least somewhat contradictory (see Chapter 4). Aside from a possible direct modulatory action of IFN- γ on canonical HPA axis structures (e.g., IFN- γ acting directly on hypothalamic neurons or pituitary corticotrophs to stimulate the release of CRH or ACTH), an interesting possibility to consider is that a QUIN/3-HK-mediated atrophy or degeneration of GR-expressing hippocampal neurons could contribute to a loss of negative autoregulatory feedback on the HPA axis (Allison and Ditor, 2014).

1.3.3.3 IFN- γ , neurogenesis and BDNF

Accumulating evidence indicates that IFN- γ contributes to the regulation of hippocampal neurogenesis and other aspects of structural and functional neuroplasticity (Yirmiya and Goshen, 2011; Donzis and Tronson et al., 2014); it is certainly conceivable that such processes could hold relevance for the cytokine's putative depressogenic effect. Several groups have reported that IFN- γ can positively regulate neurogenesis and facilitate recovery from neural injury (often with concomitantly improved memory function) while others have ascribed to the cytokine more detrimental actions (Kim et al., 2007; Baron et al., 2008; Mastrangelo et al., 2009; Li et al., 2010a; Walter et al., 2011;

Campos et al., 2014). Much like what's been reported for IL-1 β (Yirmiya and Goshen, 2011), an inverted U-shaped curve may ultimately come to best describe the relationship between IFN- γ and neurogenesis: whereas limited concentrations of the cytokine may foster a pro-neurogenic environment (Shaked et al., 2005; Baron et al., 2008), IFN- γ levels that are either substantially above or below the physiological range would conceivably promote the opposite.

We saw earlier how cytokine-mediated IDO induction could possibly contribute to the generation of an anti-neurogenic environment by tipping the balance of neuroprotective-to-neurotoxic KYN metabolites in favour of the latter (e.g., in response to IL-1 β : Zunszain et al., 2012); as a preferential inducer of IDO, one can reasonably speculate that IFN- γ may also act through such a pathway. More generally, IFN- γ could negatively affect hippocampal neurogenesis and related neuroplastic processes by ramping up local neuroinflammation; this will likely involve IFN- γ -induced Jak-Stat signalling in local glial cells (Takeuchi et al., 2006; Hashioka et al., 2010, 2015). Most relevant here is the IFN- γ -induced activation of pro-inflammatory cytokine cascades and various inducible enzyme systems, in particular iNOS, COX-2 and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (in addition to IDO) (Matsuoko et al., 1999; Horikawa et al., 2010; Zhou et al., 2015). Although these inflammatory enzymes confer protection against pathogenic invasion and low levels of NO, ROS and prostanoids (i.e., the enzymatic products) may actually have salutary cognitive effects (Cowley et al., 2008; Puzzo et al., 2012), chronic activation of these enzyme systems has been linked to behavioural deficits and impaired structural and functional neuroplasticity (Hoffmann et al., 2007; Carreira et al., 2014; Mouihate, 2014; Zhang et al., 2014; Johansson et al.,

2015). Of note, IFN- γ signalling may also drive the downregulation of neuroprotective species in glial cells, and this could increase progenitor cell and neuronal vulnerability to oxidative and inflammatory damage (Litteljohn et al., 2011). For example, IFN- γ was reported to suppress microglial expression of the anti-inflammatory cytokine IL-10 (Rock et al., 2005) and the trophic factor insulin-like growth factor (IGF)-1 (Moran et al., 2004), both of which exert pro-neurogenic, neuroprotective and antidepressant-like effects (Hoshaw et al., 2008; Miltiadous et al., 2011; Kiyota et al., 2012; Voorhees et al., 2013).

Finally, while accumulating evidence implicates IFN- γ in the regulation of neuronal birth and survival, very little research has investigated the influence of the cytokine on neurotrophic factors such as BDNF. Work from our own group suggested that IFN- γ might contribute to paraquat-induced neurodegeneration in part by mediating an early-occurring reduction in central BDNF (Mangano et al., 2012). More directly, IFN- γ down-regulated BDNF mRNA expression in human bronchial smooth muscle cells (Kemi et al., 2006) and reversed stem cell factor-induced potentiation of neurotrophin levels in microglia (Zhang and Fedoroff, 1998). However, Campos et al. (2014) only recently reported that mice genetically lacking IFN- γ displayed unaltered hippocampal and PFC BDNF protein expression. Echoing our discussion of the possible hormetic-like influence of IFN- γ and other pro-inflammatory cytokines on hippocampal neurogenesis, it is conceivable that IFN- γ could differentially affect BDNF under physiological and pathological conditions.

1.4 Linking IFN- γ to co-morbid depression in PD: a possible role for LRRK2

Another untested and intriguing possibility is that IFN- γ could influence depression-relevant processes via the PD-linked kinase and recently identified IFN- γ

target gene leucine-rich repeat kinase 2 (LRRK2). Indeed, we have recently argued that the high degree of depressive co-morbidity observed in PD patients is at least partially related to neuroinflammatory processes regulated by IFN- γ (Litteljohn et al., 2010a; Hayley, 2011; Anisman and Hayley, 2012). In this final section we briefly survey the current state of knowledge regarding the structural and functional biology of LRRK2, focusing especially on the emerging role of LRRK2 as a primary inflammatory response gene. We also highlight recent evidence linking LRRK2 with depressive illness and entertain a preliminary discussion of the possible mechanisms subserving this link.

1.4.1 LRRK2, PD and other inflammatory disorders

Variation within and around the LRRK2 gene has been implicated in both familial and sporadic forms of PD (Cookson, 2015; Wallings et al., 2015). While LRRK2 mutations are the most frequent known genetic cause of PD, common LRRK2 polymorphisms (and mutations in fact) are associated with a heightened risk of developing sporadic disease (Tan et al., 2010; Ross et al., 2011; Cai et al., 2013; Wallings et al., 2015). LRRK2 mutations are inherited in an autosomal dominant manner and display reduced penetrance and variable expressivity; i.e., not all carriers of a given LRRK2 mutation will go on to develop PD and among those who do there is considerable clinical and neuropathological variability (thus matching the clinical and pathological heterogeneity seen in idiopathic PD) (Zimprich et al., 2004; Healy et al., 2008; Latourelle et al., 2008; Chen-Plotkin et al., 2008; Marder et al., 2015). These findings have led our group and others to suggest that environmental events, such as exposure to toxins and chronic stressors, as well as other genetic factors (e.g., other PD-associated genes or loci) may be important triggers or modifiers of LRRK2-related PD (Latourelle et al., 2008; Lin

et al., 2011; Wang et al., 2012; Hayley and Litteljohn, 2013a; Karuppagounder et al., 2016).

Although LRRK2-related parkinsonism has been considered to closely resemble sporadic PD (Healy et al., 2008; Lin and Farrer, 2014), there have been indications that LRRK2 mutations might actually cause an altered neuropsychiatric phenotype (Hayley and Litteljohn, 2013a). In particular, several clinical studies found that PD patients and asymptomatic carriers harboring the LRRK2 G2019S point mutation (glycine-to-serine substitution at position 2019, which is the most commonly encountered LRRK2 mutation) have higher rates of depressive and cognitive symptoms, which tend also to be more severe (Goldwurm et al., 2006; Marongiu et al., 2006; Belarbi et al., 2010; Brockmann et al., 2011; Marras et al., 2011; Shanker et al., 2011; Thaler et al., 2012; Mirelman et al., 2015; Dzamko et al., 2016). However, the evidence appears to be split on this issue, with a considerable number of studies reporting a lack of phenotypic differences (e.g., Ben Sassi et al., 2012; Gaig et al., 2014; Trinh et al., 2014; Alcalay et al., 2015). It would thus appear that any real difference in the prevalence, severity and/or course of non-motor symptoms between LRRK2 G2019S PD and sporadic disease is likely to be small.

Interestingly, however, the possibility that LRRK2 G2019S may influence non-motor PD symptoms appears to have a parallel in the burgeoning basic and preclinical animal research into LRRK2-related PD. For instance, mice and/or rats genetically engineered to overexpress LRRK2 G2019S displayed anxiety-like behaviours and cognitive deficits, coupled with impaired hippocampal neurogenesis and perturbations of glutamatergic and dopaminergic neurotransmission (Dächsel et al., 2010; Melrose et al.,

2010; Winner et al., 2011; Beccano-Kelly et al., 2014; Volta et al., 2015; Sloan et al., 2016). As well, treatment of cultured LRRK2 G2019S hippocampal neurons with a combination of pro-oxidant pesticides (maneb and paraquat) dramatically altered the expression of genes linked to neurogenesis, above and beyond those changes seen with toxin challenge or G2019S overexpression alone (Desplats et al., 2012).

In addition to PD, LRRK2 has been linked to Crohn's disease (an inflammatory bowel disease) and leprosy (an infectious disease also known as Hansen's disease) (Lewis and Manzoni, 2012). Preliminary evidence from preclinical animal studies also points to a role of LRRK2 in diabetic neuropathy (Yang et al., 2014), uveitis (Wandu et al., 2015) and HIV-1 associated neurocognitive disorders (Puccini et al., 2015). An in-depth discussion of the proposed role of LRRK2 in these various conditions exceeds the scope of the present work and the interested reader is directed elsewhere (Greggio et al., 2012; Bae and Lee, 2015). However, it bears mentioning that all of these LRRK2-associated conditions have in common with PD a prominent immuno-inflammatory component (Greggio et al., 2012; Russo et al., 2014) and conspicuously high rates of depressive co-morbidity (Del Guerra et al., 2013; Jindal et al., 2013; Maca et al., 2013; Szigethy et al., 2014; Moulton et al., 2015).

1.4.2 LRRK2: a novel regulator of CNS-immune system interactions

LRRK2 is a large multimeric protein (286 kDa) whose catalytic core comprises Ras of complex (Roc), c-terminal of Roc (COR) and kinase domains (Cookson, 2015; Wallings et al., 2015) (Fig. 1.3). While the former are implicated in guanine triphosphate hydrolysis (GTPase function), the latter MAP kinase kinase kinase-type domain mediates serine-threonine phosphorylation events (Gloeckner et al., 2009; Cookson, 2015).

Surrounding this enzymatic core, in which all of the dominant LRRK2 mutations have so far been localized, are several protein-protein interaction domains: the armadillo (ARM), ankyrin (ANK) and leucine-rich repeat (LRR) motifs at the amino end (N-terminus) of the LRRK2 protein, and the WD40 domain at the carboxyl end (C-terminus) (Gilsbach and Kortholt, 2014). A preponderance of the evidence suggests that an enhanced kinase activity is a major contributor to the pathogenesis of LRRK2-related PD; e.g., the LRRK2 G2019S mutation increases kinase activity ~ 2-3 fold and kinase knockdown attenuates LRRK2 toxicity (West et al., 2005, 2007; Gloeckner et al., 2006; Greggio et al., 2006; Smith et al., 2006; Sheng et al., 2012). However, not all LRRK2 variants are associated with increased kinase activity (Rudenko et al., 2012; Cookson, 2015).

Despite the clear importance of LRRK2 in PD and the ongoing intensive research focus on elucidating underlying pathomechanisms of LRRK2-related neurotoxicity, the precise normal role(s) of LRRK2 remains elusive (Russo et al., 2014; Wallings et al., 2015). However, in keeping with the principle that protein structure informs function, it is not surprising that LRRK2 should at least provisionally be implicated in a diverse range of cellular functions, including autophagy, cytoskeletal dynamics, intracellular membrane trafficking, synaptic vesicle cycling/neurotransmission, and the inflammatory response (Caesar et al., 2013; Cirnaru et al., 2014; Manzoni et al., 2015; Wallings et al., 2015). As it relates to LRRK2 kinase function specifically, the growing list of suspected or validated LRRK2 kinase substrates includes: LRRK2 itself (autophosphorylation) (Greggio et al., 2008), MAP kinase kinases (which are immediately upstream of JNK and p38 MAP kinase) (Gloeckner et al., 2009), the NF- κ B super-repressor I κ B α (Hongge et al., 2015), the early endosome regulator Rab5a (itself a small GTPase protein) (Yun et

al., 2015), and the key presynaptic protein N-ethylmaleimide-sensitive factor (NSF) (Belluzzi et al., 2016; see Chapter 5).

In addition to being expressed in a variety of peripheral tissues, most notably the kidney, lung, liver, heart, and spleen (Westerlund et al., 2008; Baptista et al., 2013), LRRK2 is readily detectable in neurons over a wide range of brain regions. Included here are the SNc and striatum (surprisingly very low in the former but high in the latter), as well as the hippocampus, PFC, locus coeruleus, and various hypothalamic and amygdaloid nuclei (Melrose et al., 2006, 2007; Biskup et al., 2007; Simón-Sánchez et al., 2006; Taymans et al., 2006; Higashi et al., 2007; Westerlund et al., 2008; Giesert et al., 2013). Subcellular localization studies indicate that LRRK2 associates with membranous and vesicular structures, including mitochondria, lysosomes, endosomes, lipid rafts, and transport/synaptic vesicles (Biskup et al., 2006, Hatano et al., 2007; Higashi et al., 2007; Mandemakers et al., 2012; Trancikova et al., 2012; Schreij et al., 2015); the latter three are highly represented in presynaptic neuron terminals (Biskup et al., 2006; Wang, 2014). Such findings have led to the suggestion that LRRK2, beyond its preeminent role in nigrostriatal motor function, may contribute to the regulation of emotional and cognitive processes (Simón-Sánchez et al., 2006; Hinkle et al., 2012). Moreover, it would appear that the regulation of synaptic vesicle cycling/neurotransmission might factor importantly in this regard (Piccoli et al., 2011, 2014; Migheli et al., 2013).

Intriguingly, LRRK2 is also highly expressed in circulating and tissue immune cells such as monocytes, B lymphocytes, dendritic cells, microglia, and astrocytes (Miklossy et al., 2006; Gardet et al., 2010; Maekawa et al., 2010; Hakimi et al., 2011; Thévenet et al., 2011; Gillardon et al., 2012; Kubo et al., 2016). Perhaps not surprisingly,

then, recent and emerging evidence has highlighted a prominent role for LRRK2 and its mutations in the inflammatory response (Dzamko and Halliday, 2012; Russo et al., 2014). For instance, LRRK2 silencing in macrophages and/or microglia attenuated LPS-induced NF- κ B transcriptional activity, iNOS and COX-2 expression, and TNF- α , IL-1 β and IL-6 release (Kim et al., 2012; Gillardon et al., 2012; Moehle et al., 2012; Russo et al., 2015) whereas overexpression of the PD-linked LRRK2 R1441G mutation (arginine-to-glycine substitution at position 1441) exacerbated many of these endotoxin-induced effects (Gillardon et al., 2012). Similarly, even as WT LRRK2 enhanced IL-1 β -mediated induction of NF- κ B transcriptional activity and vascular cell adhesion molecule-1 in cultured human endothelial cells, these effects were exaggerated upon LRRK2 G2019S overexpression (Hongge et al., 2015).

Beyond LPS-mediated phospho-activation and upregulation (i.e., non-genomic and genomic mechanisms), it would appear that LRRK2 is phosphorylated more generally in the course of canonical TLR/myeloid differentiation primary response protein (MyD88)/NF- κ B signaling; this encompasses all TLRs save for TLR3, which recognizes double-stranded viral RNA (Dzamko and Halliday, 2012; Schapansky et al., 2014). LRRK2 has also been found to interact with the death adaptor Fas-associated protein with death domain (FADD), which links TNF- α (among other stimuli) to caspase 8-dependent apoptosis (Ho et al., 2009). Intriguingly, among the pro-inflammatory cytokines, IFN- γ stands out as a clear preferential inducer of LRRK2. Thus, IFN- γ has been found to markedly increase LRRK2 expression in human peripheral blood mononuclear cells (Gardet et al., 2010), particularly the CD14⁺CD16⁺ or nonclassical subset of monocytes (typical of inflammatory conditions) (Thévenet et al., 2011), as well

as in circulating macrophages (Kuss et al., 2014) and brain-resident microglia (Gillardot et al., 2012). However, it is currently unknown whether IFN- γ can activate LRRK2 via a rapid non-genomic route (e.g., via PKA-mediated phosphorylation: Muda et al., 2014).

Incredibly, the recent paper by Park et al. (2013) raised the possibility that LRRK2 might also be inducible by glucocorticoids. However, this study used cultured DA neurons and it is unclear whether glucocorticoids exert a similar influence on LRRK2 expression in microglia and other immune cells (and it will be recalled that glucocorticoids are generally anti-inflammatory). In any case, we can begin to piece together a tentative model wherein LRRK2 potentially acts as a neuro-endocrine-immune interface, integrating disparate inputs (e.g., IFN- γ , LPS, and possibly even stress-related glucocorticoids) to influence common neurobiological processes underlying chronic inflammatory diseases.

1.5 Hypothetical statement

Several lines of evidence support a role for the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 and IFN- α in depression and related stressor-associated conditions. While there is growing reason to suspect that IFN- γ may also be involved, the cytokine's contributions in ethologically relevant stressor-based depression models have yet to be studied. Addressing this matter constitutes the first of three major aims of the present thesis. In Chapters 2 and 3 we describe a set of experiments designed to test the hypothesis that IFN- γ deficiency confers protection against the depressive-like effects of chronic stress. While the former (Chapter 2) surveys the contributions of IFN- γ to stress-induced perturbations of HPA axis and brain regional monoamine activity, peripheral cytokine networks and anxiety-and-depression-like behaviours, the latter (Chapter 3)

looks expressly at the cytokine's role in some of the memory-related effects of chronic stress; i.e., hippocampal neurotransmission, BDNF expression and spatial memory function.

The second major aim of this thesis is to provide direct evidence linking systemically administered IFN- γ to alterations of depression-relevant pathophysiological domains, namely central monoamine neurotransmission, HPA axis activity and circulating cytokine networks. We tackle this issue in the studies described in Chapters 4 and 5, and in the former we also explore potential sex-specific and stress-synergizing effects of IFN- γ .

The third main aim of the present thesis is to begin to address novel signalling pathways that could potentially underlie the depressogenic effect of IFN- γ . Specifically, in Chapter 5 we test the hypothesis that IFN- γ /LPS administration in LRRK2 G2019S overexpressing mice will induce exaggerated plasma corticosterone, cytokine and central monoamine responses, and thus provide proof-of-concept for an IFN- γ /inflammatory-LRRK2 signalling pathway in PD-related depression. Our long-term goals include targeting novel endogenous neuroimmune pathways for the alleviation of co-morbid and treatment-resistant depression. Indeed, we believe that such cases stem in part from faulty neuroimmune communication that is not adequately addressed by traditional antidepressants (Hayley and Litteljohn, 2013b).

1.6 Overview of methodological approach

In the present thesis we used genetic mouse models to study the depression-relevant neuroendocrine, immunological, central monoaminergic, and behavioural effects of IFN- γ and LRRK2. It is therefore implicitly assumed that mice and humans share

certain genotype-phenotype relations and that mice make good albeit imperfect animal models of human disease; there is strong evidence to suggest this is the case (Takao and Miyakawa, 2015). The studies described in Chapters 2-5 are also linked by common methodological and statistical techniques; e.g., various processive and systemic stressors, behavioural tests, and specific blood/brain-based assays. To avoid redundancies, where these techniques and procedures are repeated the reader is directed to the first occurrence.

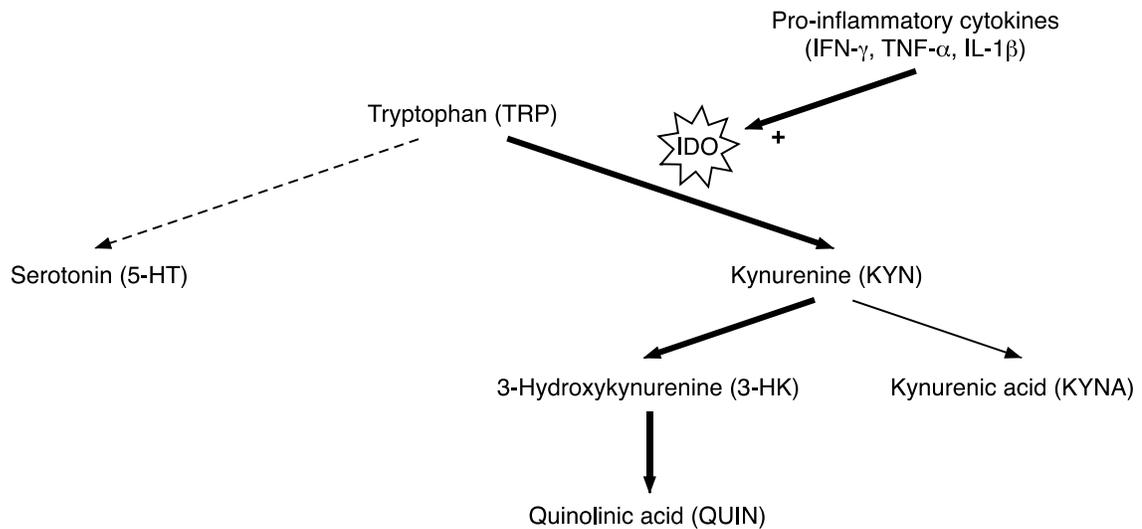


Figure 1.1. Simplified overview of the tryptophan catabolic pathway and the role of cytokine-induced IDO activity. IDO catalyzes the conversion of the essential amino acid tryptophan (TRP) to kynurenine (KYN). While KYN is inactive in neurons, it is further metabolized into neuroactive KYN intermediates by cell type-specific enzymes: 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) in microglial cells and kynurenic acid (KYNA) in astrocytes. The former are considered neurotoxic whereas the latter is generally thought of as being neuroprotective. IDO is readily inducible by pro-inflammatory cytokines, most notably IFN- γ , and this has the dual depression-relevant effect of 1) shunting TRP metabolism away from 5-HT synthesis (dashed arrow) and 2) increasing the production of neuroactive and potentially deleterious KYN pathway metabolites (bolded arrows).

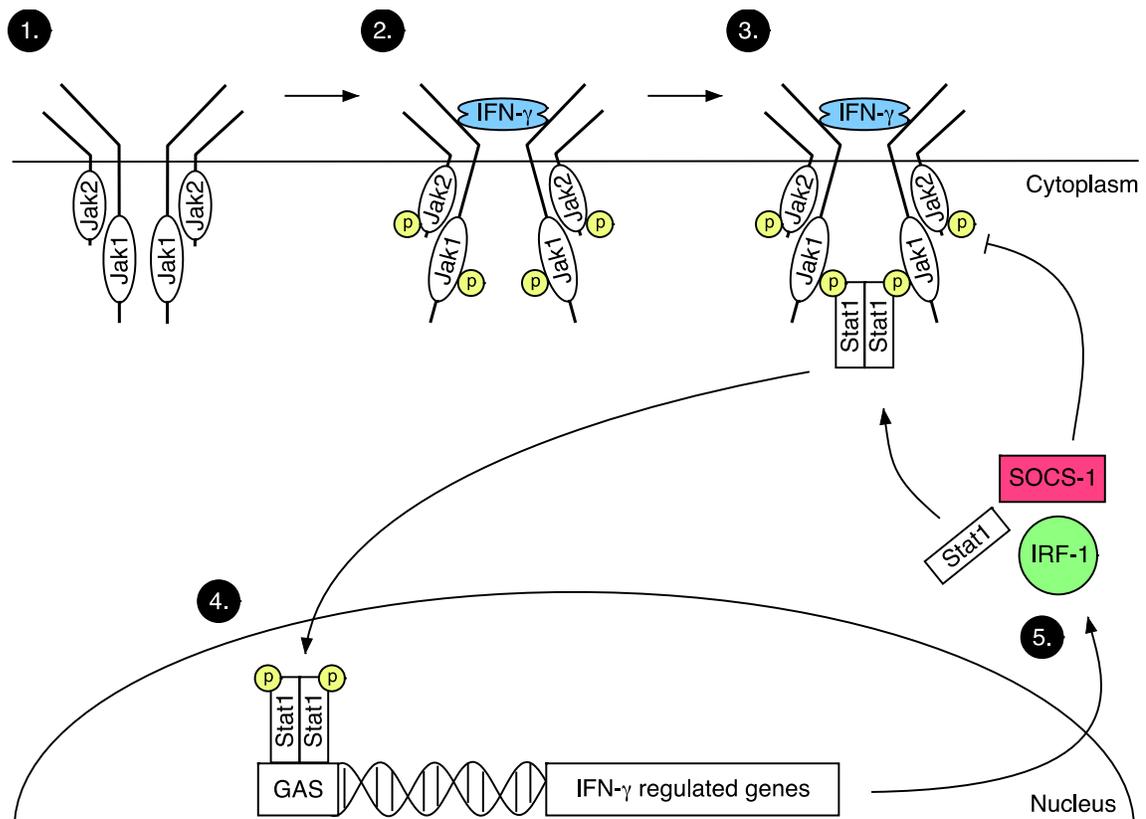


Figure 1.2. Simplified overview of canonical IFN- γ signal transduction. (1) Functional IFN- γ receptor comprises two ligand-binding chains (IFN- γ receptor-1) and two signal-transducing chains (IFN- γ receptor-2), together with receptor-associated Jaks (Jak1 and Jak2). (2, 3) Ligand binding induces a conformational change in the IFN- γ receptor, leading to the sequential activation of Jak2 and Jak1, and the recruitment, phosphorylation and homodimerization of Stat1. (4) Stat1 homodimers translocate to the nucleus and regulate the transcription of genes containing promoter IFN- γ -activation sites (GAS). IFN- γ signalling also produces Stat1 heterodimers and heterotrimers, which along with various IFN regulatory transcription factors (e.g., IRF-1) can bind to IFN-stimulated response element promoter regions to regulate further waves of transcription (not shown here). (5) In this way, IFN- γ signalling mediates both positive (e.g., upregulation of Stat1) and negative autoregulatory feedback loops [e.g., upregulation of suppressor of cytokine signalling-1 (SOCS1)]. Arrows indicate positive/stimulatory relationships or movement across time and/or space. Solid lines with blunted ends indicate negative/inhibitory relationships.

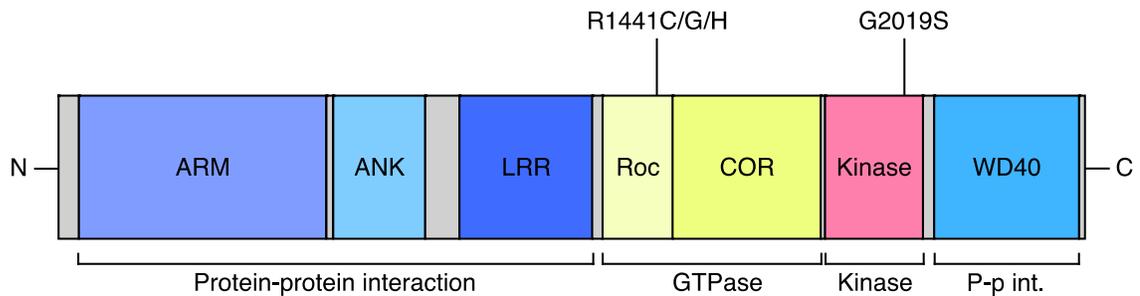


Figure 1.3. Stylized representation of LRRK2 domain structure and select common PD-related mutations. The ras of complex (Roc) and c-terminal of Roc (COR) domains are implicated in LRRK2 GTPase function; these are shown in yellow. The LRRK2 serine/threonine kinase domain, which exhibits MAP kinase kinase kinase-type activity (Gloeckner et al., 2009), is depicted in pink. Flanking this enzymatic core are several protein-protein interaction domains: the armadillo (ARM), ankyrin (ANK) and leucine-rich repeat (LRR) motifs at the amino end (N-terminus) of the LRRK2 protein, and the WD40 domain at the carboxyl end (C-terminus). Although LRRK2 exists as a monomer (shown here) and in higher molecular weight macro-molecular complexes (Cirnar et al., 2014), it is most active in dimeric form (Berger et al. 2010; Liu et al., 2010). Mounting evidence implicates the COR domain as the critical dimerization device (Gotthardt et al., 2008), although the WD40 domain probably also plays a role (Jorgensen et al., 2009). Also depicted here are the common PD-linked LRRK2 G2019S and R1441C/G/H substitution mutations; the former occurs in the kinase domain whereas the latter localizes to the Roc domain. Adapted from Berwick and Harvey (2013) under the Creative Commons Attribution 3.0 license: © 2013 Berwick and Harvey.

**2 Chapter. IFN- γ deficiency modifies the effects of a chronic stressor
in mice: implications for psychological pathology**

2.1 Abstract

Pro-inflammatory cytokines promote behavioural and neurochemical variations similar to those evident following stressor exposure and have been implicated in promoting depressive illness. Indeed, immunotherapeutic application of the cytokine IFN- α promoted depressive-like illness in cancer and hepatitis C patients. We assessed the possibility that another IFN cytokine family member, IFN- γ , might contribute to the behavioural and biochemical alterations provoked by a chronic stressor regimen that has been used to model neuropsychiatric pathology in rodents. As predicted, IFN- γ -deficient mice displayed basal differences in behaviour (e.g., reduced open field exploration) and altered neurochemical activity [e.g., increased noradrenergic and serotonergic activity within the central amygdala (CeA)] relative to their wild-type (WT) counterparts. Moreover, stressor-induced elevations of corticosterone and the pro-inflammatory cytokine, TNF- α , were attenuated in IFN- γ -deficient mice. Similarly, the IFN- γ null mice were refractory to the chronic stressor-induced alterations of dopamine metabolism (within the PFC, PVN and CeA) evident in WT mice. Yet, the chronic stressor provoked signs of anxiety (e.g., reduced open field exploration) and depression-like behaviour (e.g., increased forced swim immobility, reduced consumption of a palatable solution) in the WT and IFN- γ KO mice alike, suggesting a dissociation of behavioural functioning from the stressor-induced alterations of immunological, hormonal and dopaminergic activity. Together, these data suggest a complex neurobehavioural phenotype wherein IFN- γ deletion engenders a state of heightened basal emotionality coupled with increased monoaminergic activity in the amygdala. At the same time, IFN- γ deficiency appears to

blunt some of the neurochemical, corticoid and cytokine alterations ordinarily associated with chronic stressor exposure.

2.2 Introduction

Stressful life events, particularly those of a chronic and unpredictable nature, are important risk factors for the development of neuropsychiatric disturbances, including anxiety and major depressive disorder (Anisman et al., 2008a; Wang et al., 2009). In this regard, in rodents, chronic stressors typically promote many of the same (or analogous) behavioural features of these disorders in humans, as well as neurochemical alterations thought to be associated with them (Henn and Vollmayr, 2005). For instance, variations in central monoamine activity and altered neuroendocrine functioning (e.g., elevated corticosterone and corticotropin releasing hormone) have been associated with both clinical depression and stressor effects (Anisman et al., 2008b). As well, chronic stressor exposure has been associated with impaired hippocampal neuroplasticity (e.g., reductions of neurogenesis and dendritic branching) (Duman, 2004), just as reduced hippocampal volume and metabolic activity was reported among depressed patients (Bremner et al., 2000; Sheline, 2003).

Although chronic stress is associated with a neurobehavioural syndrome reminiscent of depression, numerous processes have been proposed as mechanisms underlying this relationship. One mechanism by which stressors might come to affect the multiple processes aligned with depressive- and anxiety-like disorders is through activation of the inflammatory immune system (Irwin and Miller, 2007; Anisman et al., 2008b). For instance, among cancer and hepatitis C patients, immunotherapeutic application of the pro-inflammatory cytokine IFN- α provoked a depression-like syndrome amenable to antidepressant treatment (Capuron et al., 2001, Musselman et al., 2001; Raison et al., 2005). Additionally, members of the interleukin family of cytokines,

including IL-1 β , IL-6 and IL-2 (and their respective soluble receptors), as well as the pro-inflammatory cytokine TNF- α , promote anxiety- and depressive-like pathology in rodents (Hayley et al., 2001b, Hayley et al., 2005; Anisman et al., 2008c). Importantly, stressors can elevate peripheral and central cytokine expression (Anisman et al., 2005; Buchanan et al., 2008) and pro-inflammatory cytokines, such as IL-1 β , contribute to or exacerbate the behavioural and neurochemical effects of psychological stressors (Gibb et al., 2008; Goshen et al., 2008). Conversely, inhibition of cytokine activity attenuated many of the central consequences of stressors (Koo and Duman, 2008; Wu and Lin, 2008) and a cyclooxygenase inhibitor augmented the therapeutic effects of antidepressants (Müller et al., 2006).

Although less well studied, the Th1 pro-inflammatory cytokine IFN- γ , a type II IFN that has been implicated in early immune-mediated viricidal and tumoricidal processes as well as later-occurring adaptive immune responses (Billiau, 1996; Ikeda et al., 2002), may also contribute to depressive- and anxiety-like pathology. In this regard, circulating IFN- γ levels are elevated in major depression and a number of anxiety-related conditions (Maes et al., 1994, Woods et al., 2005, Simon et al., 2008; Gabbay et al., 2009), and treatment with the selective serotonin reuptake inhibitor, fluoxetine, normalized the enhanced IFN- γ mRNA expression apparent in the serum of depressed patients (Tsao et al., 2006). The latter findings are consistent with reports indicating that antidepressant treatment suppresses IFN- γ production as well as the IFN- γ :IL-10 (or IFN- γ :IL-4) production ratio (Kubera et al., 2001, Brustolim et al., 2006; Diamond et al., 2006). In addition to findings in clinical studies, genetic ablation of the IFN- γ receptor in mice attenuated the depressive-like behavioural consequences (forced swim and tail

suspension tests) and TNF- α mRNA elevations provoked by *M. bovis* BCG infection (O'Connor et al., 2009b); and conversely, acute infection with an IFN- γ adenovector provoked long-lasting depressive-like changes in motivated responding (Kwant and Sakic, 2004). In contrast, Kustova et al. (1998) reported that genetic ablation of IFN- γ actually increased basal emotionality on tests thought to reflect anxiety-like behaviours (elevated plus-maze, open field test).

The source for the divergent effects that have been reported concerning IFN- γ manipulation is uncertain. However, it has been suggested that the actions of other cytokines (e.g., IFN- α) and endotoxin treatments (e.g., LPS) may depend on the stressor context in which they were administered (Anisman et al., 2007, Gandhi et al., 2007; Gibb et al., 2008). Thus, in the present investigation, we assessed the impact of IFN- γ deficiency on anxiety- and depressive-like behavioural changes associated with an unpredictable chronic stressor regimen. In addition, we sought to determine whether the behavioural effects of these treatments would be paralleled by variations of circulating cytokine levels, corticosterone and central monoamine activity within several stressor-sensitive brain regions that have been implicated in depression and anxiety.

2.3 Methods

2.3.1 Animals

Development of the IFN- γ KO mouse has been described previously (Dalton et al., 1993). IFN- γ KO and WT mice raised on a C57BL/6J genetic background were obtained from The Jackson Laboratory (Bar Harbor, ME). Thereafter, IFN- γ KO and WT mice were generated in-house from heterozygous (IFN- γ +/-) breeding pairs, as determined via PCR analyses. At 6–7 weeks of age, animals were singly housed in

standard (27 × 21 × 14 cm) polypropylene cages and maintained on a 24 h light/dark cycle with lights on at 0700 h. A diet of Ralston Purina (St. Louis, MO) mouse chow and water was provided ad libitum and room temperature maintained at approximately 21 °C. After a further 4–5 weeks (i.e., at 10–12 weeks of age), mice were subjected to the experimental manipulations. All experimental procedures were approved by the Carleton University Committee for Animal Care and complied with the guidelines set out by the Canadian Council for the Use and Care of Animals in Research.

2.3.2 Chronic stressor regimen

Animals of either genotype were randomly assigned to a chronic stressor condition or a non-stressed control group. Of these mice, independent cohorts were used for: 1) brain monoamine and plasma corticosterone determinations ($n = 8-10$), 2) serum cytokine determinations ($n = 6$), and 3) behavioural testing ($n = 18-20$). Within each cohort, groups of mice were either not stressed and remained in their home cages or were exposed to a 42-day chronic stressor regimen that comprised the application of two stressor sessions per day (different stressors in the morning and afternoon) on a variable and unpredictable schedule. The chronic stressor procedure was adapted from previous studies that used the presentation of chronic mild stressors to model affective-like disturbances in rodents (D'Aquila et al., 1994; Froger et al., 2004). However, because outcomes observed with very mild chronic stressor preparations are not particularly robust (Harris et al., 1997, Anisman and Matheson, 2005; Schweizer et al., 2009), the intensity of the stressors used in the present investigation, as in our recent studies (e.g., Tannenbaum et al., 2002; Wann et al., 2009), were more extensive (i.e., up to 42 rather than 21 days) and somewhat more intense (i.e., mild and moderate stressors).

The chronic stressor regimen included the following stressors: restraint in semicircular Plexiglas tubes (4 × 12 cm) with tails taped to prevent mice from turning (15 min); restraint in tight-fitting triangular bags with a nose-hole for breathing (15 min); exposure to social stressors (interactions but not fighting) by placing mice in a large cage (40 × 25 × 15 cm) divided into separate quadrants with three non-experimental mice (60 min); exposure to social stress by placing mice in a conspecific's soiled cage (with the latter having been removed, 60 min); predator (fox) odor by exposure to 250 cc of fox urine-infested air (Foxpert, St. Benjamin, QC) while in a novel cage (5 min); exposure to predator (rat) odor/stimuli by placing mice in a cage containing rat feces-soiled bedding (60 min); footshock (15 shocks, 500 ms duration at 30 s intervals, 0.3 mA, 60 Hz, AC.) administered in individual shock chambers (30 × 14 × 15 cm) as described previously (Tannenbaum et al., 2002); intraperitoneal injection of 0.2 ml sterile physiological saline (Sigma Aldrich, USA); damp bedding (60 ml of water/l of sawdust bedding; 60 min); tail hanging (30 s); placement in an empty cage without sawdust or nesting (60 min); lights on during dark phase; intermittent background noise in an isolated chamber (30 × 14 × 15 cm, 40 dB, 10 min); and 30° cage tilt (60 min). Following the 1st (morning) daily stressor, mice were returned to their home-cage until application of the 2nd (afternoon) stressor. Due to the nature of the stressor paradigm, animals that received the chronic stressor regimen were housed in holding rooms separate from but otherwise identical to their non-stressed counterparts.

2.3.3 Behavioural assessments

Behavioural testing was conducted between experimental days 30-42, occurring in the final 2 weeks of the 6-week stressor regimen. The sequence of tests was organized

in such a way as to minimize the effects of prior test experience. Specifically, mice ($n = 8-10/\text{group}$) were assessed in the open field test (day 30), a novel object test (day 37), and a forced swim test that is frequently used as a screen for antidepressant treatments (i.e., behavioural despair) (day 41). In addition, a separate cohort of mice ($n = 8-10/\text{group}$) was evaluated for anhedonia-like deficits in a chocolate milk consumption test (day 38). All behavioural testing was conducted under dim ambient lighting between 0800 and 1200 h. Mice were acclimated to the relevant testing room/apparatus for 30 min prior to testing and all apparatus were thoroughly cleaned between trials/mice with a dilute ethanol solution to minimize the effects of lingering olfactory cues. Also, in order to minimize the acute effects of the experimental stressors mice were tested on the morning after the previous day's afternoon stressor session. Moreover, on testing days mice received only a single afternoon stressor.

2.3.3.1 Open field test

A 40-cm³ open transparent Plexiglas arena was used to assess exploratory performance in the open field test, a measure that has also been used as a reflection of anxiety (Belzung and Griebel, 2001; Litteljohn et al., 2008). Animals were individually placed in a corner of the arena and permitted free exploration of the apparatus for 15 min, during which their movements were tracked through a video camera connected to an automated video tracking system (EthoVision, Noldus, The Netherlands). Relevant indices of anxiety and exploratory behaviour were recorded, including the frequency of entries into and total duration of time spent in a pre-defined center zone of the open field. In addition, the total number of fecal boli produced during the open field test was recorded for each mouse. Increased defecation in a novel environment has been taken to

reflect emotional reactivity or anxiety in rodents (Kustova et al., 1998; Lalonde and Qian, 2007).

2.3.3.2 Novel object test

The novel object test, described previously by Kwant and Sakic (2004), was used to assess the reaction of mice to a novel object encountered in a familiar environment. Briefly, mice were individually placed in the same Plexiglas apparatus that was used one week earlier for the open field test and permitted free exploration of the arena for 15 min. A small piece of white plastic tubing (5 cm diameter \times 8 cm long) was subsequently introduced into the center of the arena for 5 min and the total contact duration with the novel object was recorded (time spent sniffing, touching, or biting the tubing).

2.3.3.3 Forced swim test

Forced swim was assessed using a slightly modified version of the Porsolt et al. (1977) method. Mice were individually placed in a glass cylinder (20 cm diameter \times 25 cm high) containing cold water (22 ± 1 °C; 15 cm deep) for 6 min, during which time their behaviour was recorded. Immobility time (i.e., floating, with only minimal paw movements necessary to keep the head above water), latency to first immobility and time spent in vigorous struggle (i.e., attempting to climb/jump up sides of the apparatus) were subsequently determined for each mouse. Following the test session, mice were dried (in a standard polypropylene cage layered with clean paper towels and placed on top of a heating pad) and returned to their home-cage. In general, forced swim might not be a reflection of depression but is used as a screen for antidepressant drugs. Nevertheless, stressor treatments that promote depressive-like behaviours in other paradigms also elicit greater floating in this test. As the forced swim was part of the chronic stressor protocol,

we took advantage of this to assess depressive-like effects associated with the preceding stressor experiences.

2.3.3.4 Chocolate milk consumption test

Mice were presented with a palatable substance [1:3; cream (10% milk fat, Parmalat)-to-chocolate milk (1% milk fat, Parmalat) solution] for 30 min on each of three consecutive training days (days 35–37). The milk solution was delivered by metal sippers affixed to 10 ml serological pipettes positioned exterior to the home-cage; mice were permitted free access to water. Previous results from our laboratory have indicated that a 3 d period is sufficient for mice raised on a C57/BL6 background to learn to drink the chocolate milk solution (Litteljohn and Hayley, unpublished findings). Indeed, this was true for the present study wherein untreated IFN- γ KO mice and WT animals consumed commensurate volumes of the solution during a 30 min session. Following the 3 days of training (i.e., day 38), mice were again given unlimited access to the palatable solution for 30 min, during which time the total volume consumed was recorded.

2.3.4 Brain dissection technique

Between 0900 and 1100 h on the day following completion of the chronic stressor regimen (i.e., 17–19 h following application of the final stressor), chronically stressed mice and control animals alike were rapidly decapitated under identical experimental conditions. Trunk blood was collected and brains excised and sectioned into sequential coronal slices using razor blades and a chilled stainless steel microdissecting block with adjacent slots arranged approximately 0.5 mm apart. The PVN and CeA were obtained by micropunch using a hollow 1.0 mm diameter biopsy needle; the medial prefrontal cortex (PFC) was microdissected in its entirety using chilled razor blades. All brain punches

were taken with reference to the mouse brain atlas of Franklin and Paxinos (1997). The tissue was maintained in a homogenizing solution containing 14.17 g monochloroacetic acid, 0.0186 g disodium ethylenediaminetetraacetate dihydrate (EDTA disodium salt), 5.0 ml methanol, and 500 ml H₂O, and stored at -80 °C until determination of central monoamine and metabolite levels using high-performance liquid chromatography (HPLC).

2.3.5 Plasma corticosterone determination

At the time of decapitation trunk blood of mice from each of the treatment groups was collected in tubes containing 10 µg EDTA. Samples were centrifuged (3000 g for 15 min) and the plasma removed and stored at -80 °C for later corticosterone determination with commercially available radioimmunoassay kits (ICN Biomedicals, CA, USA). As described previously, inter-assay variability was precluded via assaying all samples (in duplicate) within a single run (Anisman et al., 1997); the intra-assay variability was less than 10%.

2.3.6 Serum cytokine determinations

Levels of circulating cytokines were determined by multiplex analysis using a custom multiple cytokine detection kit (Beadlyte Mouse Multi-Cytokine Detection System, Upstate Cell Signalling Solutions) and the Luminex 100 suspension-based bead array system (Luminex Corp., Austin, TX), as described previously (Mangano and Hayley, 2009). Briefly, following decapitation, trunk blood was collected in centrifuge tubes containing a serum diluent (Upstate Cell Signaling, Cat. #43-007) and centrifuged (6000 g for 10 min) at 4 °C, after which 50 µl of the supernatant was collected for use in the Luminex analysis. To prepare standards for Luminex analysis, 5000 pg of Multi-

Cytokine 2 standard was re-suspended in 1 ml serum diluent and vortexed at a medium speed for 15 s, after which serial dilutions were prepared. After 25 μ l of Beadlyte Cytokine Assay Buffer was added to the wells, plates were vortexed and a vacuum manifold applied to remove excess liquid. Subsequently, 25 μ l of serum diluent and 25 μ l of sample were added to each well. Following 20 min incubation on a shaker, the anti-mouse multi-cytokine beads were vortexed, sonicated and 25 μ l of the bead solution added to the wells. After a brief vortex plates were incubated overnight at 4 °C. Thereafter, samples were re-suspended in 50 μ l of Beadlyte Cytokine Assay Buffer and the vortex and washing procedures repeated. Finally, 25 μ l of biotin conjugated cytokine beads were added for 90 min incubation in the dark. Just prior to the end of the incubation period the Beadlyte Streptavidin-PE was diluted (1:25) and 25 μ l added to each well for the final 30 min incubation. The assay was then halted using 25 μ l of Beadlyte Stop Solution. Filter plates were read in a Luminex 100 instrument and the data fitted with a five-parameter logistic regression curve using QT Masterplex software (MiraiBio, Hitachi, CA) (Hulse et al., 2004).

2.3.7 HPLC determination of central amine and metabolite concentrations

Levels of NE, 5-HT and DA, and their respective primary metabolites, 3-methoxy-4-hydroxyphenylglycol (MHPG), 5-HIAA, and 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA, were determined by HPLC within relevant brain punches according to a modified version of previously reported methods (Chiueh et al., 1983; Hayley et al., 1999). The PFC, PVN and CeA were selected for monoamine analyses because each brain region is considered to be relatively stressor-sensitive, and hence, potentially relevant for psychological disturbances such as anxiety and depression.

Moreover, the PFC is thought to play a critical role in processing the cognitive aspects of stressors whereas the amygdala and PVN are important for anxiety and neuroendocrine aspects of stressor exposure, respectively (Anisman et al., 2008b). Indeed, our group has previously reported substantial neurochemical changes within each of these brain regions in response to acute or chronic stressors (e.g., Tannenbaum et al., 2002; Sudom et al., 2004).

Briefly, tissue punches were homogenized by ultrasonic disruption (Sonic Dismembrator Model 100, Fisher Scientific) in the homogenizing solution in which they were initially frozen (with 2,3-dihydroxybenzoic acid as an internal standard). The level of protein was determined with the Pierce bicinchoninic acid assay (BCA) Protein Assay Kit (Thermo Scientific 23225). Homogenized samples were centrifuged (12000 rpm for 3 min at 4°C), after which 50µl of supernatant was injected, at a flow rate of 1 ml/min, into the automated HPLC system (Agilent 1100) with electrochemical detector (DECADE II SDC, Antec) and ZORBAX Eclipse XDB-C8 columns (Agilent: 4.6 mm inner diameter, 150 mm length, 5µm particle size; thermostated at 40°C); the oxidation potential was maintained at 0.60 V. The mobile phase comprised: 90 mM sodium phosphate monobasic, 1.7 mM 1-Octanesulfonic acid, 50 mM EDTA, 10% acetonitrile, 50 mM citric acid (monohydrate), 5 mM KCL, and HPLC-grade water. Monoamine and metabolite concentrations were expressed relative to the protein content of the samples, and final results presented as ng/mg protein.

2.3.8 Statistical analyses

Data were analyzed by 2 (Genotype; WT vs. IFN- γ KO) \times 2 (Treatment; non-stressed vs. stressed conditions) analysis of variance (ANOVA) followed by Fisher's

least significant difference (LSD) where appropriate ($p < 0.05$). Data were evaluated using a StatView (version 6.0) statistical software package (SAS Institute, Inc.).

2.4 Results

2.4.1 IFN- γ deficiency attenuated circulating corticosterone and cytokine variations following chronic stressor treatment

As shown in Fig. 2.1, plasma corticosterone levels varied as a function of the interaction between the chronic stressor treatment and IFN- γ deletion, ($F_{1, 74} = 7.53, p < 0.01$). The follow-up analyses revealed that concentrations of corticosterone in the non-stressed IFN- γ KOs were elevated relative to their WT counterparts ($p < 0.05$), and that the chronic stressor provoked a rise in corticosterone among mice of both genotypes. However, the degree to which corticosterone levels rose in response to the chronic stressor differed markedly between the KO and WT animals. Indeed, the chronic stressor robustly elevated corticosterone levels among WT mice ($\sim 220\%$ increase relative to non-stressed WT mice) ($p < 0.001$) whereas the increase in chronically stressed KO mice was relatively modest ($\sim 45\%$ increase relative to non-stressed KOs) ($p < 0.05$), suggesting that IFN- γ deletion attenuated the chronic stressor-induced variations of corticosterone.

With respect to circulating cytokine levels, the ANOVA revealed that serum concentration of the pro-inflammatory cytokine, TNF- α , varied as a function of the interaction between IFN- γ deletion and stress ($F_{1, 20} = 5.19, p < 0.05$). As shown in Fig. 2.2a and confirmed by the follow-up comparisons, the chronic stressor provoked a marked increase in serum TNF- α concentrations among WT animals ($p < 0.01$), but had no effect on the levels of this cytokine among IFN- γ -deficient mice. A separate ANOVA revealed a significant main effect of the chronic stressor on circulating IL-2 levels ($F_{1, 20}$

= 4.91, $p < 0.05$), indicating that levels of this cytokine were increased in chronically stressed mice. However, as shown in Fig. 2.2b, the stressor-induced increase of serum IL-2 concentrations appeared to be largely restricted to WT animals. In contrast to the effects of the stressor and IFN- γ KO on TNF- α and IL-2 concentrations, circulating levels of the anti-inflammatory cytokines, IL-10 and IL-4, were not significantly affected by these treatments (data not shown). Moreover, peripheral IL-1 β was not reliably detected by the assay (either the cytokine was not sufficiently present to allow detection or procedural issues related to our assay precluded its quantification) and these data were therefore not analyzed.

2.4.2 IFN- γ deletion modified the effects of chronic stress on regional brain DA utilization

The chronic stressor induced marked changes of DA metabolism within several brain regions implicated in anxiety- and depressive-like disorders, and some of these effects were attenuated in the IFN- γ -deficient mice. Specifically, although the chronic stressor increased DA levels within the PFC of both the WT and KO mice ($F_{1,34} = 6.40$, $p < 0.05$) (Fig. 2.3a), accumulation of the primary DA metabolite, DOPAC, varied as a function of the Genotype \times Stress interaction ($F_{1,34} = 5.52$, $p < 0.05$). As shown in Fig. 2.3b, the follow-up tests indicated that the stressor only increased DOPAC accumulation within the PFC of WT animals ($p < 0.05$).

Similarly, the chronic stressor significantly increased DA levels within the PVN among mice of either genotype ($F_{1,32} = 10.57$, $p < 0.01$) (Fig. 2.3c). The ANOVA also revealed a significant main effect of Genotype on PVN DA concentration, as IFN- γ null mice displayed elevated DA levels compared to WT mice, irrespective of chronic stressor

exposure ($F_{1, 32} = 12.94, p < 0.01$) (Fig. 2.3c). Once again, PVN DOPAC levels varied as a function of the significant interaction between Genotype and Stress ($F_{1, 31} = 5.20, p < 0.05$). As shown in Fig. 2.3d and confirmed by the follow-up analyses, the chronic stressor robustly enhanced DOPAC accumulation within the PVN of WT mice ($p < 0.05$) whereas this outcome was not apparent among IFN- γ -deficient animals.

Lastly, DA levels within the CeA were not significantly influenced by the chronic stressor or by interactions between Stress and Genotype (Fig. 3.3e). However, DOPAC concentrations once again varied as a function of the significant Genotype \times Stress interaction ($F_{1, 30} = 5.04, p < 0.05$). The follow-up comparisons revealed that, as in the case of the PFC and PVN, DOPAC accumulation within the CeA was markedly enhanced in WT mice exposed to the chronic stressor relative to non-stressed WT controls ($p < 0.05$). Moreover, as shown in Fig. 2.3f, IFN- γ KO abolished the stressor-induced rise in DOPAC levels.

2.4.3 IFN- γ null mice displayed augmented NE and 5-HT activity within the CeA

Whereas neither NE nor 5-HT utilization within the PFC or PVN was significantly influenced by Stress or Genotype (data not shown), the utilization of these amines within the amygdala varied as a function of Genotype. As shown in Fig. 2.4a, CeA NE levels were not significantly altered by Stress or IFN- γ deletion ($F_s < 1$). However, levels of the primary NE metabolite, MHPG, were markedly elevated among IFN- γ null mice relative to WT animals ($F_{1, 31} = 15.16, p < 0.001$) and this effect was not further altered by Stress (Fig. 2.4b). Likewise, neither Genotype nor Stress significantly affected 5-HT concentrations within the CeA ($F_s < 1$) (Fig. 2.4c). However, as depicted in Fig. 2.4d, accumulation of the 5-HT metabolite, 5-HIAA, was markedly enhanced

within this brain region among the IFN- γ KO mice relative to their WT counterparts, irrespective of chronic stressor exposure ($F_{1,31} = 5.61, p < 0.05$).

2.4.4 Chronic stressor exposure provoked anxiety- and depressive-like behaviours independent of Genotype

In order to ascertain the joint effects of the chronic stressor and IFN- γ deficiency on a range of anxiety- and depressive-like behaviours, mice were assessed in the open field and novel object tests, as well as a forced swim test and a chocolate milk consumption test. It appeared that the behavioural effects of the chronic stressor were task- and parameter-specific, and that IFN- γ deficiency modified very few of these effects. Interestingly, however, IFN- γ deletion itself provoked behavioural alterations that are consistent with anxiety and depressive-like symptoms (Kustova et al., 1998; Litteljohn et al., 2009).

In the open field test, the chronic stressor treatment significantly reduced the duration of time spent exploring the center zone of the open field arena among both the WT and IFN- γ null mice ($F_{1,35} = 8.71, p < 0.01$) (Fig. 2.5a); however, the frequency of entries was unaffected by Stress (Fig. 2.5b). As well, IFN- γ KO mice made fewer entries into the center zone of the arena than did their WT counterparts, regardless of chronic stressor exposure ($F_{1,35} = 5.38, p < 0.05$) (Fig. 2.5b). Yet, the chronic stressor differentially affected defecation in the WT and IFN- γ KO mice, as the ANOVA revealed a significant interaction between Genotype and Stress ($F_{1,35} = 9.64, p < 0.01$) for defecation scores. As shown in Fig. 2.5c and confirmed by the follow-up comparisons, the mean number of fecal boli produced during the open field test was elevated among non-stressed IFN- γ null mice relative to WT controls ($p < 0.001$). In addition, the chronic

stressor significantly enhanced defecation among WT mice ($p < 0.05$ relative to WT controls) but exerted the opposite effect among IFN- γ null animals ($p < 0.05$ relative to non-stressed KOs).

In the novel object test, contact with the object (time spent biting, sniffing or touching the object) did not differ significantly between WT and IFN- γ KO mice. Moreover, as shown in Fig. 2.6, the chronic stressor did not significantly affect contact times among mice of either genotype, although the interaction between these treatments did approach significance ($F_{1, 31} = 3.23, p = 0.083$). Yet, as shown in Fig. 2.6, there was a clear trend towards reduced contact with the object in the chronically stressed WT mice whereas the IFN- γ KOs appeared to display less contact overall irrespective of stressor treatment (but this was not significant).

Immobility time in a forced swim test was significantly elevated among chronically stressed mice of either genotype compared to non-stressed control animals ($F_{1, 35} = 4.85, p < 0.05$) (Fig. 2.7a). In addition, the chronic stressor markedly reduced the latency to becoming immobile ($F_{1, 35} = 14.39, p < 0.001$) and, as was the case for immobility time, IFN- γ deletion did not further influence this effect (Fig. 2.7b). Likewise, a separate ANOVA revealed that the time spent in vigorous struggle (attempting to climb/jump up sides of the apparatus) was affected by the stressor treatment ($F_{1, 33} = 34.88, p < 0.001$). As shown in Fig. 2.7c, the chronic stressor greatly reduced struggling time in both the WT and IFN- γ KO animals.

Lastly, the chronic stressor affected the consumption of a palatable substance (chocolate milk solution) in the WT and IFN- γ KO mice alike (Fig. 2.8). Specifically, the ANOVA uncovered a significant main effect of Stress ($F_{1, 35} = 10.43, p < 0.01$) such that

consumption of the chocolate milk solution was markedly reduced among chronically stressed mice of either genotype. Consistent with the other behavioural tests, the Genotype \times Stress ANOVA interaction term was not significant, indicating that the stressor reduced consumption independently of IFN- γ .

2.5 Discussion

Chronic stressors have been shown to promote behavioural (e.g., anhedonia, behavioural despair, deficits in exploration) and neurochemical changes (e.g., altered monoamine and neuroendocrine activity) reminiscent of those associated with anxiety and depression (Anisman et al., 2008b; Wang et al., 2009). It has likewise been shown that cytokines induce many of these same behavioural and neurochemical changes (Anisman et al., 2008c, Hayley et al., 2005; Simen et al., 2006), and mediate at least some of the central effects of psychological stressors (Shintani et al., 1995; Goshen et al., 2008). Moreover, depressed patients display increased levels of pro-inflammatory cytokines, including IL-1 β , IL-2, IL-6, IFN- α , and TNF- α (Maes et al., 1997, Mikova et al., 2001; Alesci et al., 2005), and depression symptom severity has been noted to correlate positively with several of these cytokines or their soluble receptors (Levine et al., 1999, Brietzke et al., 2009, Grassi-Oliveira et al., 2009; Lindqvist et al., 2009).

There is reason to expect that the pro-inflammatory Th1 cytokine, IFN- γ , which is a potent microglial activator and inducer of central neuroinflammatory responses (e.g., COX-2) (Mir et al., 2009), may also be relevant to the pathogenesis of major depression. In support of this contention, elevated blood and mRNA levels of IFN- γ were apparent in depressed patients (Maes et al., 1994, Tsao et al., 2006; Simon et al., 2008), and the cytokine was associated with depression-like disturbances in response to immune

challenge (Kwant and Sakic, 2004; O'Connor et al., 2009b). Yet, very little is known concerning the role that IFN- γ may play in stress models of depression and related psychological disturbances.

There are several means through which IFN- γ could contribute to the central effects of stressors. Since it has been reported that psychological stress in humans increased IFN- γ whilst down-regulating the Th2 anti-inflammatory cytokines, IL-4 and IL-10, the involvement of IFN- γ in stressor-induced pathology might be related to a shift in the Th1/Th2 balance in favor of a Th1 response (Maes et al., 1998). Alternatively, as stressors may increase BBB permeability (Esposito et al., 2002), stressor exposure could conceivably enhance the infiltration of peripheral IFN- γ -producing immune cells, such as activated T lymphocytes and NK cells (Thäle and Kiderlen, 2005), into the brain parenchyma. Furthermore, stressor-induced hormonal and mast cell alterations were reported to promote intestinal inflammation and affect gut mucosal permeability (Taché and Perdue, 2004), which could augment circulating cytokines and potentially mobilize immune cell trafficking to the brain. Indeed, IFN- γ , much like other cytokines, has specific transporter mechanisms at the BBB (Pan et al., 1997). Finally, given that microglial cells also express IFN- γ locally within the brain (Kawanokuchi et al., 2006), stressor exposure could promote central IFN- γ activity by acting directly on glial cells.

Clinical studies have shown that other pro-inflammatory cytokines, such as IFN- α and IL-2, induce depression in humans (Capuron et al., 2001, Musselman et al., 2001; Raison et al., 2005). Yet, it should be emphasized that the human studies have been conducted with cancer or hepatitis C patients who likely are experiencing considerable distress. To this end, we assessed whether IFN- γ deficiency would blunt the central

impact of a chronic stressor regimen. As summarized in Table 2.1, although a lack of IFN- γ attenuated many of the stressor-induced central monoamine, corticoid and cytokine alterations, the behavioural changes engendered by the chronic psychological stressors were generally not affected. However, the non-stressed IFN- γ KOs did show basal differences (relative to non-stressed WT controls) in open field exploration (reduced) and novelty-induced defecation (increased), as well as augmented baseline plasma corticosterone levels and elevated NE and 5-HT activity within the CeA.

The elevated basal corticosterone levels coupled with differences in open field exploration observed in the IFN- γ KOs is in agreement with the one other study that assessed the behavioural profile associated with IFN- γ deficiency in mice. Specifically, Kustova et al. (1998) found that IFN- γ KO mice displayed augmented basal emotionality, as reflected by reduced open field exploration and increased novelty-induced defecation. The fact that in the absence of any stressor challenge both HPA axis and amygdaloid differences were evident in mice lacking IFN- γ from birth raises the possibility that the cytokine normally has important neurodevelopmental functions with regards to maturation of these systems. Alternatively, it is conceivable that IFN- γ might contribute to the normal regulation of HPA and amygdala monoamine activity during adulthood, possibly by affecting peripheral immune cell or metabolic processes, which, in turn, influence CNS functions.

As already mentioned, IFN- γ deficiency attenuated many of the neurobiological effects of chronic stressor exposure. Specifically, IFN- γ deletion limited the stressor-induced increase in the accumulation of the DA metabolite, DOPAC, within the PFC, PVN and CeA. Stressors ordinarily provoke increased utilization of DA (reflected by

enhanced DOPAC accumulation), which may, in turn, lead to a reduction of DA stores (Anisman and Zacharko, 1986, Pani et al., 2000; Stein, 2008). Hence, by attenuating the stressor-induced changes in DA utilization, IFN- γ KO may have precluded potential changes of DA or DA receptor activity that might otherwise have been sustained (over time). Similarly, IFN- γ deletion had a blunting effect on the stressor-induced elevations of circulating corticosterone concentrations, such that the magnitude of the stressor-induced corticoid response (i.e., degree of change from baseline) was reduced among the IFN- γ KO mice relative to WT animals. This finding suggests that the cytokine contributes to hypothalamic-regulated neuroendocrine changes and as such might have important implications for metabolic processes that are altered in the face of stressors.

In addition to the neurochemical alterations evident in the KO mice, it may be particularly significant that lack of endogenous IFN- γ completely abrogated the chronic stressor-induced elevation of TNF- α . Inasmuch as TNF- α may contribute to the development of depressive symptoms (Anisman et al., 2005; Himmerich et al., 2008), it is possible that treatments that limit the activity of IFN- γ or TNF- α would attenuate these outcomes. In line with this view, antidepressant drugs (e.g., bupropion, imipramine) suppressed the production of IFN- γ (Brustolim et al., 2006), and it was reported that IFN- γ -induced microglial generation of several pro-inflammatory factors (e.g., IL-6 and nitric oxide) was inhibited by these same antidepressants (Hashioka et al., 2007). Such findings are consistent with the possibility that attenuation of stressor-induced increases of circulating TNF- α may have contributed to the blunting action of IFN- γ deficiency on chronic stressor-induced hormonal and neurotransmitter effects.

Although a lack of IFN- γ protected against several of the stressor-induced biochemical variations, the KO and WT mice displayed similar behavioural alterations following chronic stress. As summarized in Table 2.1, the chronic stressor provoked signs of anxiety (i.e., reduced open field exploration) and depression-like behaviour (e.g., increased forced swim immobility and reduced consumption of a palatable solution), but deficiency of IFN- γ failed to modify these effects. In contrast, one other recent study did show that infection with *M. bovis* BCG increased IFN- γ levels and provoked depressive-like behaviours among WT mice (increased immobility in the forced swim and tail suspension tests), whereas these behavioural alterations were completely absent in BCG-treated IFN- γ receptor KOs (O'Connor et al., 2009b). In effect, the stressor-induced behavioural deficits observed in the present investigation appear to be independent of the alterations of immunological, hormonal and dopaminergic activity, suggesting that stressor exposure was not affecting behaviour through IFN- γ -dependent responses, as would be expected with BCG inoculation.

2.6 Conclusion

Overall, it seems that IFN- γ may affect TNF- α levels, as well as the neurochemical and corticoid variations provoked by chronic stressor exposure. These findings are consistent with several reports indicating that cytokines provoke HPA axis activation and inflammatory responses implicated in stressor-associated disturbances (Dunn, 2000; Hayley et al., 2004). Furthermore, IFN- γ appears to differentially affect neurochemical and behavioural processes under basal vs. chronic stressor conditions. In effect, IFN- γ deficiency may play a dual role by promoting an anxious or depressive-like basal phenotype, possibly coupled to increased monoamine activity at the amygdala, but

at the same time acting to attenuate the immunological (TNF- α elevations), hormonal (corticoid variations) and monoaminergic (regional brain DA activity) effects of psychologically relevant chronic stressors. That said, we cannot rule out the possibility that compensatory processes stemming from the loss of IFN- γ during key neurodevelopmental times might have been responsible for at least some of the observed differences in the present study.

Table 2.1. Summary of significant experimental findings

Experimental endpoint	Type of significant effect		
	Main effect of Stress	Main effect of Genotype	Genotype × Treatment interaction
Blood			
Corticosterone			×
TNF- α			×
IL-2	×		
DA concentration			
PFC	×		
PVN	×	×	
DOPAC concentration			
PFC			×
PVN			×
CeA			×
MHPG concentration			
CeA		×	
5-HIAA concentration			
CeA		×	
Open field test			
Duration in center	×		
Frequency into center		×	
# Of fecal boli			×
Forced swim test			
Immobility time	×		
Latency to immobility	×		
Vigorous struggle	×		
Chocolate milk test			
Consumption	×		

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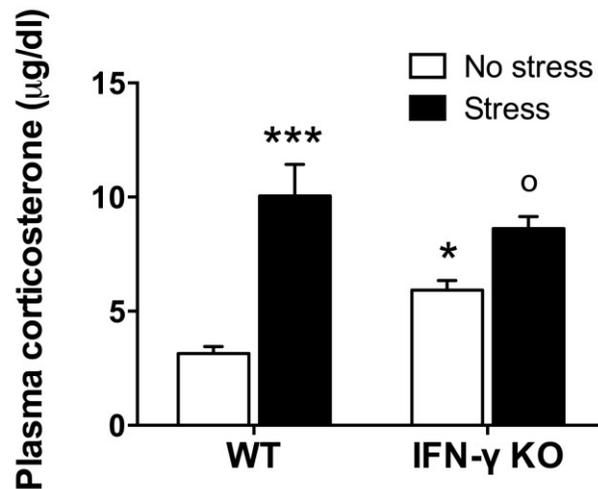


Figure 2.1. Chronic stress differentially affected plasma corticosterone concentration among WT and IFN- γ KO mice. Specifically, although the chronic stressor provoked elevations in corticosterone levels in both the WT and KO mice, this effect was moderately attenuated by IFN- γ deletion. Yet, basal corticosterone levels were also significantly higher among the KO mice relative to WT controls. All data are expressed as mean \pm SEM. * $p < 0.05$ and *** $p < 0.001$ relative to non-stressed WT mice; ° $p < 0.05$ relative to non-stressed KO control animals. Reprinted from Brain, Behavior, and Immunity, vol. 24(3), Litteljohn et al., Interferon-gamma deficiency modifies the effects of a chronic stressor in mice: Implications for psychological pathology, pp. 462-473, © 2009, with permission from Elsevier.

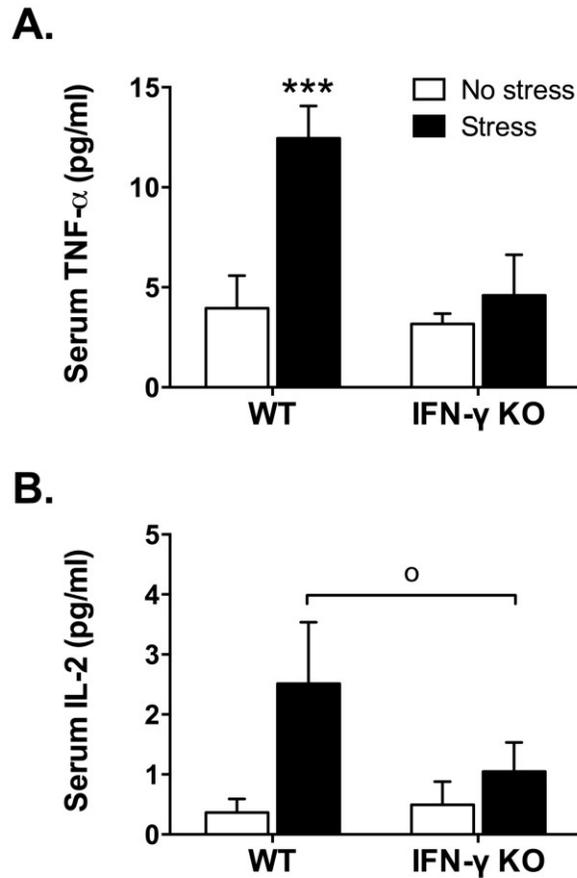


Figure 2.2. IFN- γ deletion and chronic stress jointly influenced circulating (serum) cytokine concentrations. Indeed, TNF- α (a) levels were robustly elevated among WT mice following chronic stressor exposure, as compared to non-stressed WT mice. However, the chronic stressor had no effect on the levels of this cytokine among the IFN- γ KOs. Chronically stressed mice also displayed increased serum IL-2 levels relative to non-stressed control animals; however, IFN- γ deletion did not further influence this effect (b). All data are expressed as mean \pm SEM. *** $p < 0.001$ relative to non-stressed WT mice; ° $p < 0.05$ relative to non-stressed control animals (collapsed over genotype). Reprinted with permission from Elsevier © 2009.

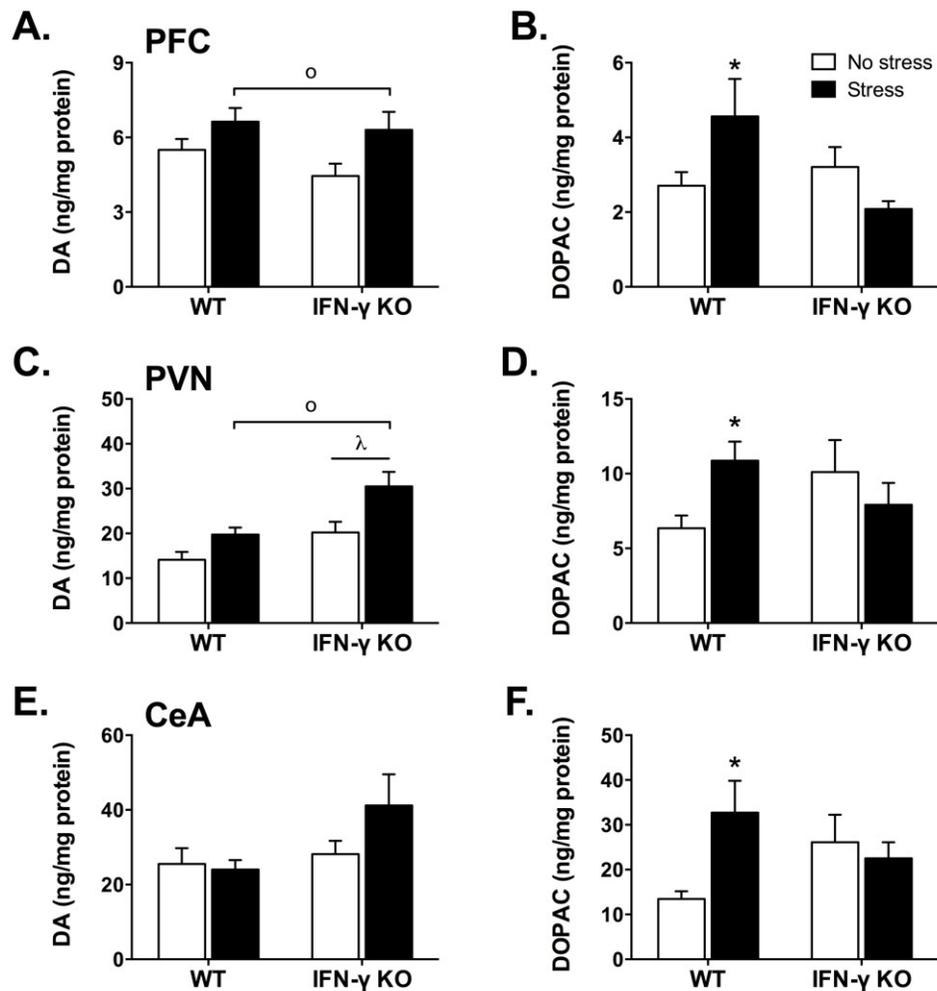


Figure 2.3. Chronic stress and IFN- γ deficiency influenced DA activity within the PFC, PVN and CeA. In this regard, both WT and IFN- γ KO animals displayed augmented DA levels within the PFC (a) and PVN (c) following chronic stressor exposure, relative to non-stressed control mice. Also, the KOs had higher levels of DA overall within the PVN compared to WT animals. In contrast, DA concentration within the CeA was not affected by either of the treatments (e). Yet, levels of the primary DA metabolite, DOPAC, varied within all three of the brain regions as a function of the joint effects of chronic stress and IFN- γ deletion. Specifically, DOPAC accumulation was significantly enhanced within the PFC (b), PVN (d) and CeA (f) among WT mice following chronic stressor exposure. However, IFN- γ deletion abrogated the chronic stress-induced increase of DOPAC concentration within all three of the brain regions assayed. All data are expressed as mean \pm SEM. * $p < 0.05$ relative to non-stressed WT mice; $^{\circ}p < 0.05$ relative to non-stressed mice; $^{\lambda}p < 0.01$ relative to WT mice. Reprinted with permission from Elsevier © 2009.

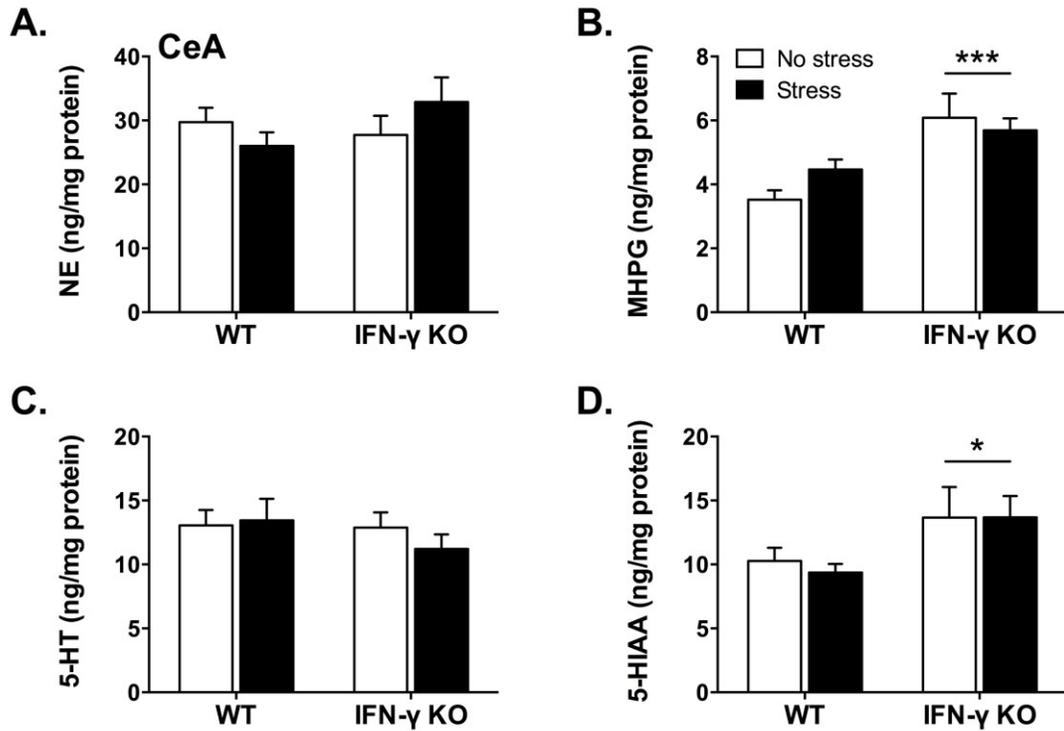


Figure 2.4. IFN- γ deficiency alone promoted variations in noradrenergic and serotonergic activity within the CeA. Although neither chronic stress nor IFN- γ deletion significantly influenced NE (a) or 5-HT (c) levels within the CeA, concentration of either amine's primary metabolite did vary as a function of the KO alone. Specifically, CeA levels of the NE metabolite, MHPG (b), and the 5-HT metabolite, 5-HIAA (d), were both significantly elevated among the IFN- γ KO mice compared to WT littermate mice (irrespective of chronic stressor exposure). All data are expressed as mean \pm SEM. * p < 0.05 and *** p < 0.001 relative to WT mice. Reprinted with permission from Elsevier © 2009.

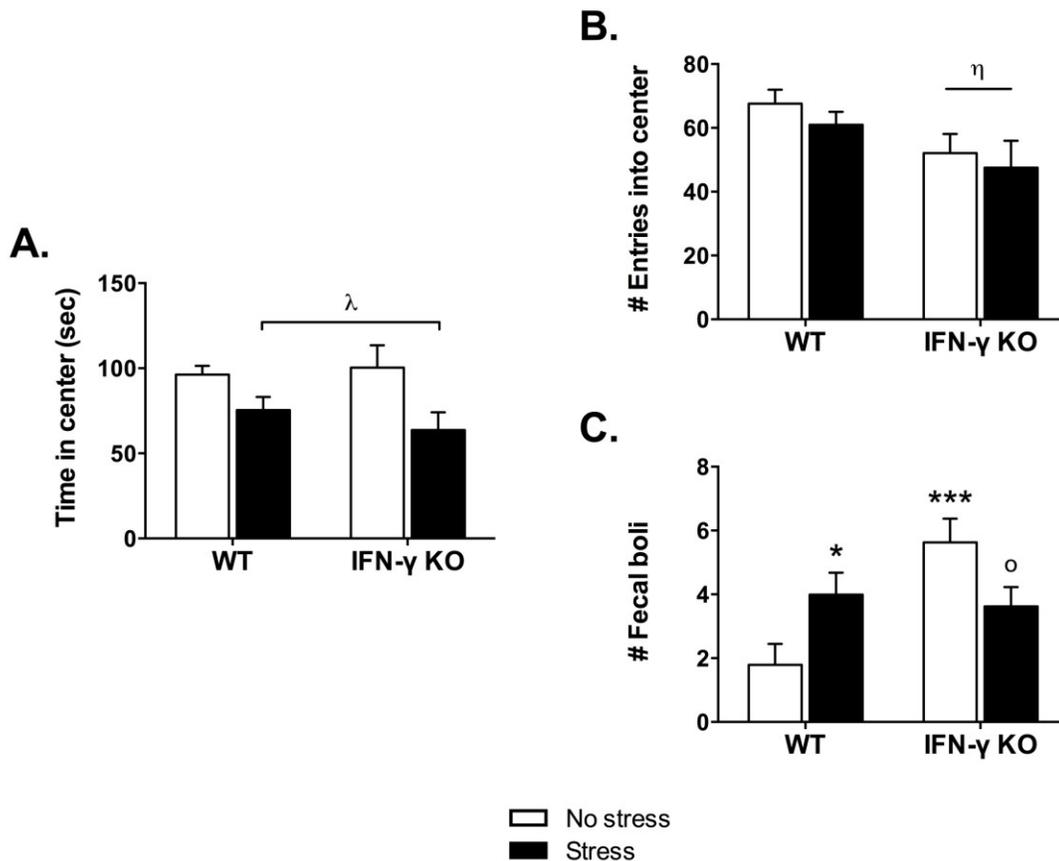


Figure 2.5. Effects of chronic stress and IFN- γ deficiency on exploratory behaviour in the open field test. Chronically stressed WT and IFN- γ KO mice alike spent less time exploring the center zone of the open field arena (a). In contrast, the chronic stressor had no effect on the number of entries into the center zone of the arena in either the WT or KO animals (b). Yet, chronic stress and IFN- γ deletion jointly influenced total defecation scores (# of fecal boli) (c). Specifically, WT mice produced a greater number of fecal boli following the chronic stressor compared to non-stressed WT controls. Moreover, although non-stressed KO mice produced more fecal boli than their non-stressed WT counterparts (i.e., basal difference), the chronically stressed KO animals actually defecated less than the KO controls. All data are expressed as mean \pm SEM. * p < 0.05 and *** p < 0.001 relative to non-stressed WT mice; ^o p < 0.05 relative to non-stressed KO controls; $^{\lambda}$ p < 0.01 relative to non-stressed mice (collapsed over genotype); $^{\eta}$ p < 0.05 relative to WT mice (collapsed over the chronic stressor treatment). Reprinted with permission from Elsevier © 2009.

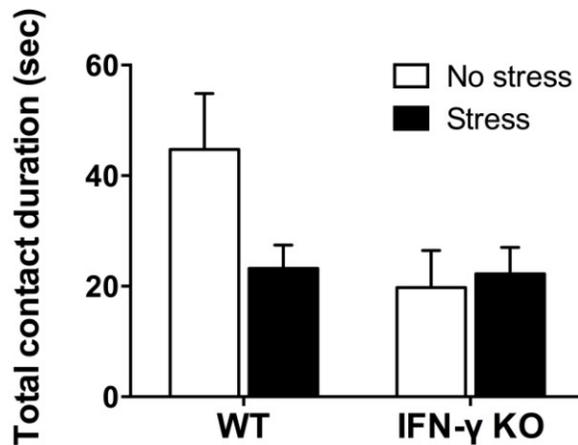


Figure 2.6. The response to a novel object (a piece of tubing presented in a familiar environment), as measured by time spent in contact with the object (biting, touching, sniffing), was not significantly affected by chronic stressor exposure or IFN- γ deletion. Indeed, as mentioned in the text, the omnibus ANOVA failed to reach significance ($p = 0.083$). However, it is clear that the stressor exposure (black bars) resulted in a marked reduction in time spent exploring the novel object among WT mice, whereas IFN- γ KO animals had quite reduced contact with the object irrespective of stressor exposure. All data are expressed as mean \pm SEM. Reprinted with permission from Elsevier © 2009.

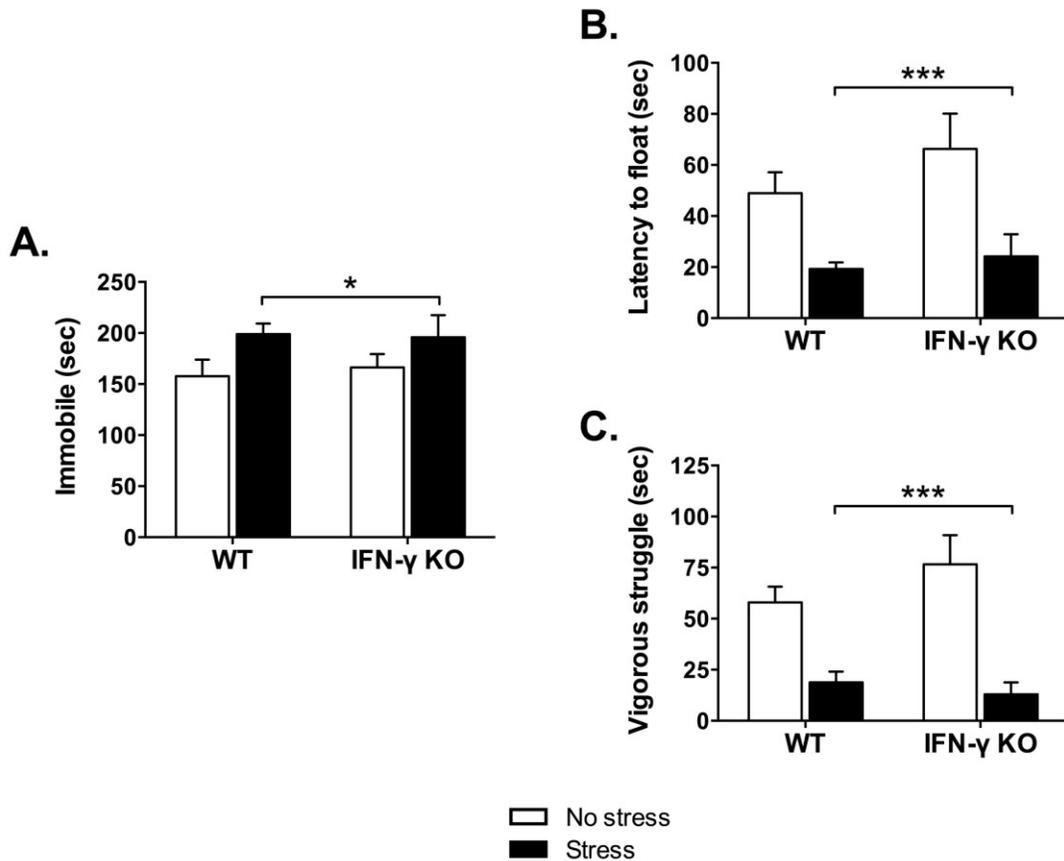


Figure 2.7. The chronic stressor provoked depressive-like behaviour in a forced swim test in both WT and IFN- γ KO mice. Specifically, the chronic stressor increased immobility time in the WT and IFN- γ KO mice alike (a). Similarly, both WT and KO mice displayed decreased latencies to 1st immobility as a function of the chronic stressor (relative to non-stressed animals) (b). Lastly, chronically stressed mice, irrespective of IFN- γ deletion, struggled for less time than the non-stressed control mice (c). All data are expressed as mean \pm SEM. * p < 0.05 and ** p < 0.001 relative to non-stressed mice (collapsed over genotype). Reprinted with permission from Elsevier © 2009.

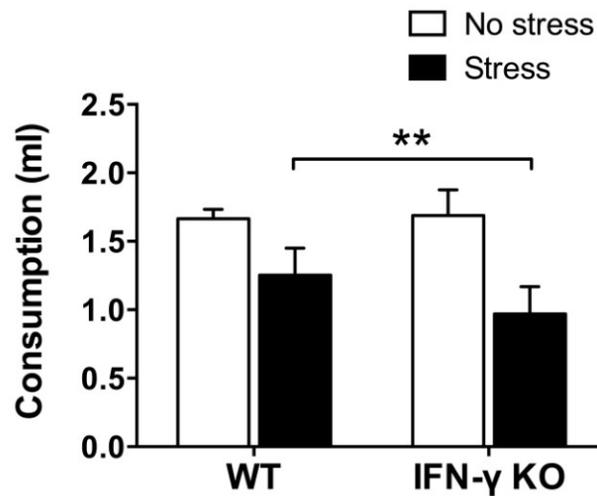


Figure 2.8. Following 3 days of chocolate milk exposure to establish reliable levels of drinking, the total volume of chocolate milk consumed was measured over a 30 min test session on day 38 of the chronic stressor regimen. The chronic stressor (black bars) markedly reduced chocolate milk consumption in the WT and IFN- γ KO mice alike. All data are expressed as mean \pm SEM. $**p < 0.01$ relative to non-stressed mice (collapsed over genotype). Reprinted with permission from Elsevier \textcopyright 2009.

3 Chapter. IFN- γ differentially modulates spatial memory and brain noradrenergic activity under normal and chronic stressor conditions

3.1 Abstract

Cytokines are inflammatory messengers that orchestrate the brain's response to immunological challenges, as well as possibly even toxic and psychological insults. In the preceding chapter (Litteljohn et al., 2010) we reported that genetic ablation of the pro-inflammatory cytokine IFN- γ attenuated some of the corticosteroid, cytokine and limbic dopaminergic variations induced by 6 weeks of exposure to an unpredictable psychologically relevant stressor. Presently, we sought to determine whether a lack of IFN- γ would likewise modify the impact of chronic stress on hippocampus-dependent memory function and related neurotransmitter and neurotrophin signalling systems. As predicted, chronic stress impaired spatial recognition memory (Y-maze task) in the WT animals. In contrast, although the IFN- γ KOs showed memory disturbances in the basal state, under conditions of chronic stress these mice actually exhibited facilitated memory performance. Paralleling these findings, while overall the KOs displayed altered noradrenergic and/or serotonergic activity in the hippocampus and LC, NE utilization in both of these memory-related brain regions was selectively increased in the chronically stressed KOs. However, contrary to our expectations, neither IFN- γ deletion nor chronic stressor exposure significantly affected nucleus accumbens dopaminergic neurotransmission or hippocampal BDNF protein expression. These findings add to a growing body of evidence implicating cytokines in the differential regulation of neurobehavioural processes in health and disease. Whereas in the basal state IFN- γ appears to be involved in sustaining memory function and the activity of related brain monoamine systems, in the face of ongoing psychologically relevant stress the cytokine

may, in fact, act to restrict potentially adaptive central noradrenergic and spatial memory responses.

3.2 Introduction

It is widely accepted that the likelihood of developing depression, anxiety and other psychological disorders is greatly influenced by exposure to stressors, particularly those of a chronic, unpredictable and/or psychosocial nature (Hill et al., 2012). The prevailing view over many years has been that stressor-induced alterations of brain monoamine activity were largely responsible for the emotional and cognitive symptoms seen to predominate in these conditions (Schildkraut, 1965). While evidence continues to implicate monoaminergic neurotransmitter processes (Fava, 2003; Hamon and Blier, 2013), deficits in trophic growth factors, such as brain-derived neurotrophic factor (BDNF), and even structural brain changes (e.g., impaired neurogenesis) have emerged as important players too in this regard (Pittenger and Duman, 2008; Calabrese et al., 2009; Mahar et al., 2014).

It's also become increasingly clear that cytokines and other elements of the inflammatory immune system contribute importantly to depression and other stress-related psychological disturbances (Miller et al., 2009; Anisman and Hayley, 2012). For instance, numerous studies have reported that pro-inflammatory cytokines, most notably IL-1 β , IL-6, IFN- α , and TNF- α , are altered in major depression and stressor-based animal models (Dowlati et al., 2010; Liu et al., 2012; Dahl et al., 2014). Moreover, administration of these cytokines to rodents induced behavioural, hormonal, monoamine, and neuroplastic changes that are reminiscent of at least some depressive-like clinical changes (Anisman et al., 2008b, c; Myint et al., 2007; Kaster et al., 2012; Sukoff Rizzo et al., 2012). The fact that anti-inflammatory and anti-cytokine treatments (e.g., minocycline, curcumin, cytokine-specific antagonists) were reported to lessen the neural

and behavioural impact of stressor exposure further supports a link between cytokines and stressor pathology (Koo and Duman, 2008; Hinwood et al., 2012; Jiang et al., 2013; Krügel et al., 2013).

Interferon- γ , which is a crucial mediator of both innate and adaptive immune responses, is another cytokine that has recently been posited to play a role in stressor-related psychological pathology. Several studies have reported elevated circulating levels of IFN- γ among depressed patients (Simon et al., 2008; Gabbay et al., 2009; Dahl et al., 2014; Schmidt et al., 2014), and many of the most commonly used antidepressants were found to antagonize IFN- γ signalling (Maes et al., 1999; Kubera et al., 2001; Brustolim et al., 2006). Moreover, variation in the IFN- γ gene was recently reported to modify both depression risk (in the context of IFN- α treatment; Oxenkrug et al., 2011) and antidepressant medication effectiveness (Myint et al., 2013). Consistent with these findings, Kwant and Sakic (2004) reported that mice infected with IFN- γ adenovector displayed persistent anhedonic-like symptoms, and O'Connor and colleagues (2009b) showed that IFN- γ is a major driver of the IDO-enhancing and depressive-like behavioural effects of *M. bovis* BCG. Yet, compared with many of the other cytokines that have been linked to depressive illness, far fewer studies have actually set out to specifically test the influence of endogenous IFN- γ in ecologically inspired chronic stressor animal models; this is especially true in regards to the cognitive aspects of depressive-like pathology.

As one of the most potent activators of microglial cells and a key regulator of the anti-viral response (Chesler and Reiss, 2002), IFN- γ is likely to be especially important for conditions in which infection overlaps with stressor exposure or in genetically

vulnerable individuals (Litteljohn et al., 2010b). In this regard, we previously found that IFN- γ -deficient mice had attenuated hormonal, cytokine and brain regional dopaminergic responses to chronic stress, despite showing several conspicuous behavioural and physiological differences in the basal state (i.e., increased anxiety-like behaviour, elevated circulating corticosterone levels and central amygdala monoamine utilization) (Litteljohn et al., 2010b; Chapter 2). This complex pattern of effects led us to theorize that IFN- γ contributes to a range of affective and perhaps cognitive processes, albeit probably in very different ways and to markedly different ends under basal and chronic stress conditions. Working under this theoretical framework, in the present investigation we sought to assess the largely unexplored role of IFN- γ in the spatial memory, psychomotor, and hippocampal BDNF and monoamine changes that are often evident following protracted exposure to psychologically relevant stressors.

3.3 Methods

3.3.1 Experimental animals

For this experiment we used 10-12 week old male IFN- γ KO and WT littermate mice from our in-house breeding colony (described in §2.3.1). All experimental procedures were approved by the Carleton University Committee for Animal Care and complied with the guidelines set out by the Canadian Council for the Use and Care of Animals in Research.

3.3.2 Chronic stressor regimen

Figure 3.1 presents a schematic of the experimental design and timeline. Animals of either genotype ($n = 46$) were randomly assigned to a 6-week chronic stressor condition or a non-stress control group ($n = 23$). Mice of each condition were further

divided into behavioural testing ($n = 10$) and behaviourally naïve cohorts ($n = 13$), the latter of which were used for end-of-study brain regional monoamine ($n = 10$) or hippocampal BDNF analyses ($n = 3$). The chronic stressor regimen comprised the application of two stressors per day (or a single stressor on behavioural testing days) on a variable and unpredictable schedule, as described in Chapter 2 (§2.3.2); a list of the various stressors used is provided in Table 3.1.

3.3.3 Behavioural assessments

3.3.3.1 Spontaneous home-cage activity

Measurements of horizontal motor activity were obtained during complete, uninterrupted 12 h light/dark cycles using a Micromax infrared beam-break apparatus (AccuScan Instruments, Columbus, OH) exterior to the home-cage. The same animals were tested on two separate occasions, corresponding to the midway point (Week 3: Day 21) and endpoint (Week 6: Day 42) of the stressor paradigm, and the data analyzed using ANOVA with repeated measures. Testing commenced 60 min after termination of the morning stressor.

3.3.3.2 Spatial memory in the two-trial Y-maze

During the final week of the experiment (Day 40), intermediate-term spatial recognition memory was assessed in a two-trial Y-maze task, in accordance with previously published methods (Dellu et al., 2000; Ferguson et al., 2000). The testing apparatus comprised three arms ($30 \times 8 \times 15$ cm) fashioned from black Plexiglas with an outer wood shell. The testing room was dimly lit and had on its walls various cardboard cutouts of basic geometric shapes. During the first trial (acquisition phase) one arm of the maze was blocked with an opaque, removable panel. Mice were then placed individually

in one of the two remaining accessible arms (i.e., the ‘start’ arm, which remained so in the second trial), with head directed away from the center of the maze. Animals were allowed to explore the open arms of the Y-maze for 5 min, after which they were returned to the home-cage. After 30 min, the second trial (retrieval phase) was conducted under identical experimental conditions to the first, excepting that the mice were now permitted free exploration for 5 min of all three arms of the maze (start, familiar and novel). The blocked arm (i.e., novel arm in the retrieval phase) varied between mice in a predetermined, pseudo-random manner, and the maze was cleaned with a dilute (2%) ethanol solution after each trial. Total arm entries (for each trial, defined as all four legs having entered a given arm) and % duration in the novel, start and familiar arms (Trial 2) were determined for each mouse. A discrimination index (DI) for novelty was calculated as follows: $\text{novel arm duration} - (\text{start arm duration} + \text{familiar arm duration})/2$. A DI value not significantly different than zero (0) is understood to reflect a deficit in novelty discrimination (e.g., see Leconte et al., 2011). Y-maze testing occurred between 09:00 and 13:00 (i.e., 17-21 h after the previous day’s afternoon stressor), after which mice in the stressor groups received a single afternoon stressor.

3.3.4 Brain dissection method and HPLC determination of central amine and metabolite concentrations

Animals were rapidly decapitated between 09:00 and 11:00 on the day following the completion of the 6-week chronic stressor regimen (i.e., 17-19 h after the final stressor treatment, which for all animals was a 15-min flat-bottom restraint). Brains were excised and sectioned and tissue samples maintained according to the methods described in Chapter 2 (§2.3.4). In this study we collected the LC, NAcc and dorsal hippocampus.

A subset of the hippocampal samples was flash-frozen and stored at -80°C for later determination of BDNF content by Western blot ($n = 3$).

3.3.5 Hippocampal BDNF protein determination

Western immunoblotting was performed largely in accordance with our previously published methods (Mangano et al., 2011). Briefly, brain tissues ($n = 3$) were homogenized on ice in radio-immunoprecipitation assay (RIPA) lysis buffer containing 50 mM Tris-base (pH 8.0), 150 mM NaCl, 1% Triton-X, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, and cOmplete Mini EDTA-free protease inhibitor (Roche, Basel, Switzerland). Lysates were centrifuged for 5 min (5000 rpm at 4°C) and supernatants collected. Total protein was then determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA). Protein from the hippocampus (50 $\mu\text{g}/\text{well}$) was diluted to a final volume of 35 μl in RIPA lysis buffer and 1X loading buffer (5% glycerol, 5% β -mercaptoethanol, 3% SDS and 0.05% bromophenol blue), and samples heated in boiling water for 5 min. Proteins were separated by electrophoresis (120 V) on 12.5% sodium dodecyl sulphate-polyacrylamide gels and transferred overnight at 4°C (180 mA) onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Membranes were then blocked for 1-h with gentle agitation at room temperature in a Tris-buffered saline (TBS-T: 10 mM Tris-base (pH 8.0), 150 mM NaCl, 0.5% Tween-20) solution containing non-fat dry milk (5% w/v). Anti-BDNF primary antibody (1:500, sc-546, Santa Cruz Biotechnology, Dallas, TX) was applied for 1.5-h at room temperature. After 4 successive 10-min washes in TBS-T, membranes were incubated with secondary antibody for 1-h at room temperature and with gentle shaking (goat anti-rabbit IgG peroxidase, 1:1000, A6154, Sigma). After another series of TBS-T washes, bands were

visualized by exposing Kodak X-OMAT film (10 min for BDNF, 10 sec for β -actin) to membranes treated with ECL substrate (Perkin Elmer, Waltham, MA; for 1 min). The immunoblots were imaged using a Konica Minolta SRX-101A processor (Konica Minolta, Marunouchi, Chiyoda-ku, Tokyo), and band density quantified using AlphaEaseFC v.3.1.2 densitometry software (Alpha Innotech, San Leandro, CA). After normalizing against β -actin (anti- β -actin; 1:5000, sc-47778, Santa Cruz), the BDNF/actin ratios were averaged across blots and the standard error of the mean determined for each treatment group.

3.3.6 Statistical analyses

The monoamine and Western immunoblot data were analyzed by 2 (Genotype; WT vs. IFN- γ KO) \times 2 (Treatment; non-stressed vs. stressed) ANOVA followed where appropriate by Student-Newman-Keuls pairwise multiple comparisons ($p < 0.05$). The home-cage activity data were analyzed by repeated measures ANOVA with Genotype and Treatment as the between-subject variables and Time (Week 3 vs. Week 6) as the repeated measures variable. A mixed model ANOVA was also used for analyzing % duration in the 3 arms of the Y-maze; here, Genotype and Treatment were the between-subject variables and Arm (novel vs. start vs. other) served as the within-subject variable. Planned univariate t-tests facilitated comparisons of spatial memory performance in each of the experimental groups (% novel arm duration, Discrimination index) with that of a theoretical group performing at chance level (33.33% and 0, respectively). In addition, Spearman's rank-order correlation coefficients (ρ) were calculated to assess the degree of association between locomotion (total arm entries) and memory performance (DI) in the Y-maze; separate analyses were conducted for the total sample (collapsing across IFN- γ

KO and stress) and each of the 4 treatment groups. On account of procedural error, 1 mouse was excluded from the home-cage activity assessments and two mice excluded from the Y-maze analyses. During the course of tissue dissection and monoamine determination, a few samples were lost due to error or variability (> 2.5 standard deviations from the mean); hence, the degrees of freedom for the statistical analyses varied within and across some brain regions and/or neurochemical substrates. Data were evaluated using a StatView (version 6.0) statistical software package and plotted with GraphPad Prism 6 (La Jolla, CA).

3.4 Results

3.4.1 Chronic stressor treatment time-dependently influenced home-cage activity in IFN- γ WT and KO mice

It is understood that psychologically relevant stressors can modulate motor functioning, and at times in diametrically opposed ways (Soblosky and Thurmond, 1986; Venzala et al., 2012). Indeed, psychomotor symptoms are a quite common occurrence in depression, as well as myriad other stressor-related psychiatric conditions (Buyukdura et al., 2011). Here, we report the outcomes of our time series analysis of home-cage locomotor activity. The repeated measures ANOVA revealed significant Genotype \times Treatment and Treatment \times Time interactions for home-cage activity ($F_{s1, 35} = 5.27$ and 14.66, respectively, $p < 0.05$). As shown in Figure 3.2, at the midway point of the experiment (Week 3) motor activity was significantly reduced by the chronic stressor. However, by the end of the experiment (Week 6) animals that were exposed to the stressor actually displayed increased home-cage activity ($p < 0.05$). Despite finding a significant Genotype \times Treatment interaction for home-cage activity, follow-up analyses

failed to reveal any statistically significant simple main effects. The existence of such a “crossover” interaction suggests that the early-occurring hypolocomotive effect of the chronic stressor predominated in the WT animals, whereas the later-occurring, activity-boosting effect of the stressor was most pronounced in the KOs; this interpretation is borne out by visual inspection of the data (see Fig. 3.2). Moreover, the multiple Bonferonni-corrected pairwise comparisons revealed that, at Week 6, only the stressed IFN- γ KO mice displayed significantly higher levels of activity compared to their non-stressed counterparts ($p < 0.0018$).

3.4.2 Chronic stress facilitated spatial recognition memory in the IFN- γ KO mice

Table 3.2 presents the total number of arm entries during the acquisition (Trial 1) and retention phases (Trial 2) of the Y-maze test. During the acquisition phase, the IFN- γ KOs made fewer total arm entries than the WT animals, regardless of stressor treatment ($F_{1, 34} = 9.05, p < 0.01$). In contrast, during the retention phase the total number of arm entries did not differ significantly between groups ($F_s < 2.8$, see Table 3.2). Since the acquisition phase of the Y-maze task is comparatively anxiety-laden, it is not surprising that the IFN- γ KOs, for which our group and others have previously described an anxious phenotype (Kustova et al., 2004; Litteljohn et al., 2010b; Campos et al., 2014), should display reduced activity in this context.

Time spent exploring the novel arm of the Y-maze is considered a reliable index of spatial memory functioning in rodents (Dellu et al., 2000; Leconte et al., 2011). The initial mixed model ANOVA revealed a significant main effect of Arm ($F_{2, 68} = 6.88, p < 0.01$), such that overall the mice spent significantly more time exploring the novel vs. start or familiar arms ($p < 0.05$). Yet, as shown in Figure 3.3a and confirmed by the

planned univariate t-tests, of the four treatment groups, only the non-stressed WT controls and the chronically stressed IFN- γ KO mice performed significantly above chance level (33.33%, $p < 0.05$). Equivalently, our analysis of DI scores revealed that whereas both the WT control and stressed KO animals discriminated the novel arm to a significant extent (DI scores greater than 0, $p < 0.05$, see Fig. 3.3b), neither the non-stressed IFN- γ KO controls nor the chronically stressed WT mice had DI values significantly different than 0 ($p > 0.1$). However, the corresponding ANOVA failed to uncover any statistically significant between-group differences ($F_s < 3.75$), and the within-group variability of DI scores was clearly quite considerable (see Fig. 3.3b).

In view of the stressor-induced and genotype-specific changes in home-cage activity, and despite the lack of significant treatment effects on total arm entries in the retention phase of the Y-maze, it was of interest to determine whether Y-maze locomotion correlated with spatial memory performance. In this regard, there was an utter lack of association between DI scores and total Y-maze arm entries when collapsing across the treatment groups ($\rho = -.05$, $p > 0.70$, see Fig. 3.3c). This was similarly the case when separate analyses were performed for the WT control, WT stressed and IFN- γ KO control groups ($\rho = 0.43$, 0.14 , -0.14 , respectively, $p > 0.25$). Yet, in the case of the chronically stressed KOs, though the negative correlation between DI scores and Y-maze activity was not statistically significant at the $\alpha = 0.05$ level ($\rho = -0.60$, $p = 0.07$), the p value was considerably less than 0.10. While acknowledging the need for caution in interpreting this marginally significant trend, as seen in Figure 3.3d it would appear that, in the chronically stressed KOs only, reduced Y-maze locomotion tended to correspond with better spatial memory performance.

3.4.3 Brain regional monoaminergic effects of chronic stress and IFN- γ KO

Within the dorsal hippocampus, neither chronic stress nor IFN- γ deletion significantly affected the levels of 5-HT or its primary metabolite, 5-HIAA ($F_s < 2.7$; see Fig. 3.4a, b). However, as shown in Figure 3.4c, 5-HT turnover (i.e., the ratio of metabolite to parent amine) was significantly diminished overall among mice genetically lacking IFN- γ ($F_{1, 34} = 5.75, p < 0.05$). A separate ANOVA revealed that the KOs also had diminished hippocampal NE levels relative to the WT mice, regardless of stressor history ($F_{1, 33} = 7.15, p < 0.05$; see Fig. 3.4d). In addition, concentrations of the primary NE metabolite, MHPG, varied according to the significant interaction of Genotype with Stress ($F_{1, 33} = 5.05, p < 0.05$). As shown in Figure 3.4e and confirmed by the post-hoc comparisons, whereas chronic stress had no effect on hippocampal MHPG levels in the WT animals, accumulation of the metabolite was robustly enhanced in the stressed KOs ($p < 0.05$, relative to KO controls). A significant Genotype \times Stress interaction was likewise uncovered for NE turnover ($F_{1, 32} = 6.24, p < 0.05$) such that the ratio of hippocampal MHPG to NE was markedly elevated in the stressed IFN- γ KOs compared to all other groups ($p < 0.05$; see Fig. 3.4f).

Within the LC, NE concentrations were significantly higher in the IFN- γ -deficient animals compared to their WT littermates, irrespective of chronic stressor exposure ($F_{1, 35} = 8.48, p < 0.01$; see Fig. 3.5a). But akin to what was observed in the hippocampus, LC MHPG levels varied as a function of the interaction between Genotype and Stress ($F_{1, 34} = 6.18, p < 0.05$). As depicted in Figure 3.5b and confirmed by the follow-up tests, among the IFN- γ KOs chronic stress induced a marked rise in MHPG concentrations ($p < 0.05$ compared to all other groups). In WT mice, however, MHPG levels were completely

unaffected by the stressor. Notwithstanding these changes, the ANOVA for LC NE turnover did not reveal any significant main or interaction effects of IFN- γ deletion and chronic stress ($F_s < 1.4$; see Fig. 3.5c).

Dopaminergic gating of information through the stressor-sensitive nucleus accumbens is considered to play an important role in both motor and memory function (Costall et al., 1984; Mele et al., 2004; Baker and Kalivas, 2005). It was therefore of interest in the present study to characterize the accumbal dopaminergic effects of chronic stress and IFN- γ deficiency. As shown in Table 3.3, neither of the experimental treatments (nor their interaction) significantly affected indices of dopaminergic neurotransmission in this brain region (DA, DOPAC and HVA concentrations, as well as DA turnover; $F_s < 3.4$).

3.4.4 Hippocampal BDNF expression was unchanged following chronic stressor exposure

A large body of evidence demonstrates that the neurotrophic factor, BDNF, is essential for hippocampus-dependent memory function and adaptive neuroplastic responses to stressors (Schmidt and Duman, 2010; Taliáz et al., 2010). We were therefore somewhat surprised to find that neither chronic stress nor IFN- γ deletion significantly influenced hippocampal BDNF protein expression ($F_s < 1$, see Fig. 3.6a, b). The Western immunoblot analysis likewise failed to reveal any significant main or interaction effects of the experimental treatments on the hippocampal protein concentrations of a neuronally secreted immature form of BDNF (i.e., proBDNF; $F_s < 1$, see Fig. 3.6a, c).

3.5 Discussion

Accumulating evidence suggests a role for IFN- γ in depression and other stressor-associated psychological disturbances (O'Connor et al., 2009b; Dahl et al., 2014); however, the question of whether IFN- γ contributes to the pathological process in ecologically inspired chronic stressor models has gone largely untested. In what is to the best of our knowledge the only other study published to date on this subject, we previously reported that IFN- γ deficiency conferred protection in mice against some of the immune, stress hormone and limbic monoamine effects associated with chronic exposure to a psychologically relevant stressor (Litteljohn et al., 2010b). Intriguingly, our results also suggested that IFN- γ may have divergent emotion-relevant actions under normal and chronic stressor conditions, as the KO animals basally showed enhanced anxiety-like behaviour coupled with heightened corticosteroid levels and central amygdala NE and 5-HT usage. Such findings are actually consistent with the earlier results of Kustova et al. (1998), as well as the more recent ones of Campos et al. (2014), which indicated that mice genetically lacking IFN- γ are characterized basally by increased emotionality and anxiety. The results of the present investigation extend these findings by demonstrating that stressor context also appears to be crucial in determining the influence of IFN- γ on spatial memory function and related neurochemical systems. Indeed, under normal conditions mice genetically lacking IFN- γ exhibited impaired spatial memory, which we suggest might be related to altered hippocampal (and perhaps LC) monoaminergic neurotransmission but not BDNF signalling or nucleus accumbens dopaminergic activity. Contrastingly, under conditions of chronic stress IFN- γ deficiency

appeared actually to facilitate memory function, and this pro-mnemonic effect coincided with enhanced hippocampal and LC noradrenergic activity.

3.5.1 Impact of IFN- γ deficiency on memory and related brain processes under normal conditions

It is now well recognized that pro-inflammatory cytokines can modulate cognitive processes, with pathological consequences probably at the forefront of attention. Yet, there is increasing evidence to suggest that cytokines and other pro-inflammatory stimuli (e.g., prostaglandins, amyloid- β peptide: Cowley et al., 2008; Puzzo et al., 2012) may under certain circumstances actually be beneficial for learning and memory; the key determinant here appears to be cytokine level (McAfoose and Baune, 2009; Yirmiya and Goshen, 2011). While highly elevated concentrations of pro-inflammatory cytokines, as can occur for instance in depression and many of its comorbid conditions (Anisman and Hayley, 2012; Dahl et al., 2014), generally provoke anti-mnemonic effects, it would appear that a certain basal physiological level of pro-inflammatory cytokine signal is required for normal memory function (Yirmiya and Goshen, 2011). Evidencing the latter, mice genetically lacking TNF- α or TNF receptor 1 displayed impaired spatial memory (TNF-R2 KO mice, however, displayed intact memory) (Camara et al., 2013), and IL-1 β signalling blockade produced a similar functional effect (Yirmiya et al., 2002; Goshen et al., 2007).

Consistent with such a view, we presently report that mice lacking IFN- γ showed disturbed spatial recognition memory in the basal state. Interestingly, Baron et al. (2008) revealed that *limited* central overexpression of IFN- γ resulted in improved hippocampus-dependent memory, whereas pathologically elevated concentrations of the cytokine have

generally been associated with memory impairment (Lapter et al., 2009; Dutra et al., 2013; Too et al., 2014). Thus, when considered together, these data suggest that the hormetic-like dose-response pattern that was described elsewhere for memory and IL-1 β (and several other immune actors) (Yirmiya and Goshen, 2011) may very well be relevant too for IFN- γ . Further investigation is warranted to substantiate this possibility, and such efforts will do well to include a detailed time-and-dose-response analysis, as well as determinations of both circulating and brain regional IFN- γ concentrations in ecologically relevant animal disease models. Also, some caution should be exercised when interpreting the Y-maze behavioural data in the present study: while basally the KOs failed to perform significantly better than chance (consistent with impaired spatial memory), the ANOVA test did not reveal any significant between-group differences. As mentioned previously, there was considerable within-group variability in the behavioural data, and this likely reflects the critical but often overlooked influence of individual differences in chronic stress susceptibility (e.g., Bergström et al., 2008). Therefore, it is our suggestion that the present findings be viewed as proof-of-principle for more comprehensive and larger-scale investigation into the prospective learning and memory effects of IFN- γ .

With respect to the possible neural substrate(s) subserving these memory changes, we submit that it may be particularly telling that the IFN- γ KOs overall displayed altered NE content both within the LC and hippocampus (increased in the former brain region and reduced in the latter), as well as diminished hippocampal serotonin turnover. Each of these highly interconnected, stressor-sensitive brain regions plays a vital role in memory function, with the hippocampus considered especially critical for memory consolidation

and spatial navigation (McGaugh, 2000; Suzuki, 2006), and the noradrenergic LC mediating the potent cognition-modulatory effects of emotional arousal (Gibbs et al., 2010; Sara and Bouret, 2012). Moreover, human and animal studies alike have implicated both noradrenergic and serotonergic signalling in the memory process, and generally in a facilitative capacity (Lee and Ma, 1995; Murchison et al., 2004; Meeter et al., 2006). It seems reasonable therefore to suggest that dysregulated monoaminergic neurotransmission, particularly in the hippocampus, could have been at least partially responsible for the memory deficit seen in these animals. Notably, memory impairment may have occurred both in spite of the observed increase of LC NE (e.g., Gibbs et al., 2010) and independently of dopaminergic neurotransmission in the nucleus accumbens (which as will be recalled was unaffected by the experimental manipulations). Importantly, since the KO-specific hippocampal and LC monoamine changes occurred irrespective of stressor challenge, it would appear that IFN- γ normally plays a role in the homeostatic regulation of these neurotransmitter systems (but presumably not the accumbal DA system). Alternatively, as the KOs were without IFN- γ signal from birth, it is possible that physiological concentrations of the cytokine are required for the normal maturation of these monoamine systems across development (Litteljohn et al., 2010b).

Of course, such a reading cannot discount the possibility that other molecular and/or cellular processes might also have contributed to the presently described memory effects. Indeed, in our previous work (Litteljohn et al., 2010b) we showed that mice genetically lacking IFN- γ had increased basal corticosterone levels and central amygdala monoamine activity, and we argued that such changes were relevant to the anxious phenotype already on record for these animals (Kustova et al., 1998). Yet, hippocampus-

dependent memory is also subject to modulation by corticosterone and central amygdala monoaminergic neurotransmission – mainly retrieval in the case of the former and consolidation as regards the latter (de Quervain et al., 1998; Hermans et al., 2014) – and it is entirely plausible that one or both of these processes could have contributed to basal memory impairment among the IFN- γ KOs. Similarly, as a wealth of evidence has implicated hippocampal BDNF in memory function, with a reduction of neurotrophin levels generally being tied to poor memory outcomes and an increase in BDNF levels usually signalling the opposite (Mizuno et al., 2000; Shirayama et al., 2002), we had speculated that any IFN- γ KO-associated decline in memory might also be attended by a reduction in hippocampal BDNF. Contrary to our expectations, however, it will be recalled that neither mature nor proBDNF protein levels were affected by IFN- γ deletion. These null BDNF results are in agreement with the recent enzyme-linked immunosorbent assay (ELISA)-based findings of Campos et al. (2014), and together our studies provide good evidence that altered hippocampal BDNF does not underlie the neurobehavioural phenotype of IFN- γ KO mice. That said, our results do not preclude the involvement of BDNF in prospective IFN- γ -associated proactive memory effects; in fact, Baron et al. (2008) provide evidence that the memory enhancement seen in mice overexpressing IFN- γ may at least partially be attributable to an upregulation of central BDNF.

3.5.2 Influence of IFN- γ KO on memory and related neurochemical processes under chronic stressor conditions

If a lack of IFN- γ can under normal conditions be seen to predispose to memory dysfunction, under conditions of chronic stress the result seems to be memory facilitation. Indeed, whereas neither the basal-state KOs nor the chronically stressed WT mice

demonstrated Y-maze performance that was significantly better than chance, both the stressor-treated IFN- γ -deficient mice and the non-stressed WT controls displayed intact spatial memory. These data are aligned somewhat with the findings of other studies indicating that chronic variable stress can paradoxically (and akin to what's been reported for acute as well as predictable chronic stress: e.g., Parihar et al., 2011; Uysal et al., 2012) enhance memory and learning (Bartolomucci et al., 2002; McLaughlin et al., 2005; Hawley et al., 2012); here we provide evidence suggesting that IFN- γ , or rather a lack thereof, may be key. Interestingly, paralleling the behavioural data, our neurochemical analyses revealed that noradrenergic, but not serotonergic, metabolism was markedly and selectively augmented in the hippocampus and LC among IFN- γ null mice that were exposed to the chronic stressor. Given the aforementioned importance of these brain regions in learning and memory (Gibbs et al., 2010), and the generally facilitative role ascribed to noradrenergic signalling in this regard (Lee and Ma, 1995), it appears likely that IFN- γ acts to restrict brain noradrenergic and, *consequently*, spatial memory responses to chronic stress.

Recent reports have documented elevations of circulating and brain IFN- γ levels among rodents exposed to chronic stressors (Liu et al., 2013; Wrona et al., 2014). And while these findings contrast with those of several other animal (e.g., Palumbo et al., 2012) as well as human studies (Segerstrom and Miller, 2004), there is a growing recognition that stress hormones can under certain circumstances augment brain inflammation (Sorrells et al., 2009). A number of routes exist by which IFN- γ could come to be influenced by, and hence contribute to, the central actions of psychological stressors (Litteljohn et al., 2010b). One such potential mechanism involves a stressor-

induced shift in the Th1/Th2 cytokine balance in favour of the former (though stressor chronicity is a major influence here: Segerstrom and Miller, 2004) (Maes et al., 1998). In this way, depression related pro-inflammatory Th1 responses (e.g., IDO activation and 5-HT depletion), of which IFN- γ is the principal effector, could become accentuated at the expense of anti-inflammatory Th2 ones (e.g., those mediated by IL-4 and IL-10) (Najjar et al., 2013). Stressors can also provoke intestinal barrier dysfunction and mucosal inflammation (Vicario et al., 2010), which could lead to not only increased circulating and even central IFN- γ levels but also the potentiated trafficking of immune cells into the brain parenchyma (Tran et al., 2000; Schroder et al., 2004). Notably, the latter could also be realized through a stressor-induced disruption of blood–brain barrier integrity (Friedman et al., 1996; Northrop and Yamamoto, 2012). And finally, that brain-resident microglia cells are themselves capable of producing IFN- γ under the direction of endogenous cytokine signals (i.e., emanating from other glial cells and not necessarily brain-infiltrating leukocytes) (Kawanokuchi et al., 2006) raises the intriguing possibility that stressors could act directly on microglial cells to influence central IFN- γ signalling.

Yet, it should be noted that by the end of the 6-week stressor regimen the chronically stressed KOs also displayed increased spontaneous locomotor activity; this was evident too in the stressed WT animals, but seemingly to a lesser degree. Several studies have linked hyperactivity in rodents to increased brainstem and hippocampal noradrenergic activity (though the role of NE in regulating motor brain circuitry is not straightforward and almost certainly involves cross-talk with brain DA and 5-HT systems) (Suwabe et al., 2000; Ruocco et al., 2010; Lambertsen et al., 2012), and there is reason to think that a similar situation could be relevant to certain clinical contexts – for

instance ADHD, impulse control disorders and (atypical) depression (Viggiano et al., 2004; Fan et al., 2012). Possibly, then, in the face of ongoing stress a lack of IFN- γ and the consequent potentiation of noradrenergic signalling could function as a double-edged sword – at once serving to facilitate the memory process (Tully and Bolshakov, 2010) and predisposing to hyperactivity, with the latter perhaps best viewed as a harbinger of impending allostatic overload. All the same, it is important to recognize that in the present study the motor-modulatory effect of stress was clearly task-specific. Indeed, whereas by the end of the 6-week experiment locomotion in the home-cage was increased among the stressor-exposed mice (predominantly in the KOs), Y-maze testing only two days prior revealed no such effect of stress (i.e., on total arm entries). Moreover, it will be recalled that, in the chronically stressed KOs only, Y-maze performance actually tended to correlate *negatively* with total arm entries (retention phase) – a phenomenon that was revealed elsewhere to be associated with good spatial memory performance under chronic stressor conditions (Conrad et al., 2003). Thus, if it can be allowed that enhanced NE utilization did, in fact, contribute to both the cognitive and home-cage motor changes observed in the stressed KOs, then these animals were presumably yet able to harness said noradrenergic drive to their mnemonic advantage and when the peculiarity of the circumstances demanded it.

In addition to monoaminergic imbalances, depression and other stressor-related behavioural disorders may involve disturbances of neuroplasticity, including changes in brain structure and neurotrophin systems (Calabrese et al., 2009; Schmidt and Duman, 2010; Hayley and Litteljohn, 2013b). In particular, circulating levels of BDNF were found to be diminished in depressed subjects and to correlate with clinical recovery after

treatment initiation (Shimizu et al., 2003; Huang et al., 2008). Similarly, animal studies revealed that chronic stress often diminishes central BDNF levels (Murakami et al., 2005; Luo et al., 2013), whereas BDNF-augmenting strategies (e.g., direct infusions, stimulators) typically induce antidepressant-like behavioural consequences (Shirayama et al., 2002; Schmidt and Duman, 2010; Ye et al., 2011). We were therefore somewhat surprised to find that hippocampal BDNF remained unchanged in the present study upon exposure to chronic stress. Interestingly, though definitely in the minority, a number of studies have reported a lack of or even inverse relationship between hippocampal BDNF and chronic stress (Lucca et al., 2008; Larsen et al., 2010; Hanson et al., 2011). And while not contesting the crucial role of BDNF in memory function, it's worth noting that hippocampal BDNF has not always been found to positively correlate with memory performance (Muñoz et al., 2010; Bechara and Kelly, 2013). Conceivably, then, germline loss of IFN- γ may have altered the hippocampal neuroinflammatory milieu in such a way that, in the face of chronic stress, upregulated (compensatory) expression of alternative growth factors (e.g., GDNF, nerve growth factor, neurotrophin-3, IGF-1) and possibly even anti-inflammatory and/or anti-apoptotic messengers (e.g., IL-10 and bcl-2, respectively) could have positively affected memory-related processes independently of BDNF (Bian et al., 2012; Xuan et al., 2014).

Alternatively, since chronic stress has been shown to time-dependently regulate brain regional BDNF expression (Fanous et al., 2010; Xiao et al., 2011; Lakshminarasimhan and Chattarji, 2012; Capoccia et al., 2013), it is possible that our null BDNF findings might be related to the timing of mouse sacrifice relative to stressor initiation and/or termination. Considering that in the present study animals were exposed

to a rather lengthy 6-week stressor, a plausible scenario would have the stressor transiently suppressing BDNF levels, only for them to return to baseline by the time of sampling. This contention can be seen to derive at least some support from the home-cage activity data: whereas locomotor activity was significantly elevated after 6 weeks of exposure to stress (and predominantly in the KOs), at the mid-way point of the experiment the opposite was, in fact, noted. In effect, chronic stress time-dependently influenced home-cage motor activity and IFN- γ appeared to modulate the magnitude if not direction of such effects. Without time series data for the neurochemical endpoints we cannot exclude that hippocampal BDNF expression similarly changed across time; this is a definite weakness of the present study and a suggested worthwhile avenue of future research. In addition, we cannot speak to any potential effect of chronic stress or IFN- γ deficiency on BDNF expression at the mRNA level, nor can we rule out the possibility that a more sensitive assay such as ELISA could have detected presumably very subtle changes in BDNF protein expression. However, that Campos et al. (2014) failed to demonstrate an effect of IFN- γ deletion on ELISA-determined BDNF protein levels within the hippocampus and PFC would seem to lend credence to the present null findings, at least in regards to basal state BDNF expression. And finally, our use of genetically engineered mice on a C57BL/6J background might have been an issue, as this mouse strain is considered to be somewhat less stressor-sensitive than certain other strains (e.g., BALB/c) (Tannenbaum and Anisman, 2003; Pothion et al., 2004). In this regard, Bergström and colleagues (2008) showed that chronic mild stress differentially modulates hippocampal BDNF expression in stressor-resilient and stressor-sensitive rats.

3.6 Conclusion

To summarize, our data suggest that IFN- γ differentially modulates memory-related processes under normal and chronic stressor conditions. Specifically, in the basal state IFN- γ appears to facilitate hippocampus-dependent spatial memory, probably at least partially due to the cytokine's involvement in the homeostatic regulation of relevant brain monoamine systems. Under conditions of chronic stress, however, IFN- γ appears instead to restrict potentially adaptive brain noradrenergic and spatial memory responses. While our data do not support a role of hippocampal BDNF in this regard, they do not preclude the possible involvement of other structural and/or functional neuroplastic processes (e.g., IFN- γ -dependent alterations of hippocampal neurogenesis: Baron et al., 2008; Li et al., 2010a; Campos et al., 2014); we advocate here for further investigation of this intriguing possibility. More generally, the current findings are aligned with a growing body of work indicating that stressor context can greatly influence the behavioural, immune and neurochemical effects of other cytokine and immune challenges (e.g., IFN- α , LPS, poly I:C) (Anisman et al., 2007, Gandhi et al., 2007; Gibb et al., 2008). It should be noted, however, that compensatory neuronal or immunological changes stemming from a lack of IFN- γ during key developmental stages could have contributed to the present findings.

Table 3.1. List of stressors

Stressor	Duration	Specifications
Social interaction	60 min	Placement in a large cage (40 × 25 × 15 cm) divided into separate quadrants with 3 non-experimental male C57BL/6J mice (3-6 months old); this set-up allowed for interactions but not fighting
Soiled cage	60 min	Introduction into a congener's soiled cage
Fox urine	5 min	Exposure to 250 cc fox urine-infested air (Foxpert, St. Benjamin, QC) while in a novel, empty cage
Rat feces	60 min	Introduction into an unfamiliar cage with fresh rat feces
Flat bottom restraint	15 min	Restraint in semicircular Plexiglas tubes (4 × 12 cm) with tails taped to prevent mice from turning
Plastic bag restraint	15 min	Restraint in tight-fitting triangular plastic bags equipped with a nose-hole for breathing
Footshock	—	15 shocks, 500 ms duration at 30 s intervals, 0.3 mA, 60 Hz, a.c.), administered in individual shock chambers (30 × 14 × 15 cm)
Injection / handling	—	Intraperitoneal injection of 0.2 ml sterile physiological saline (Sigma Aldrich, USA)
Damp bedding	60 min	60 ml of water/l of sawdust bedding in novel cage
Tail hang	30 sec	—
Empty cage	60 min	Introduction into an empty cage without sawdust or nestlet
Noise	10 min	Intermittent background noise (40 dB) in isolated restraint chambers (30 × 14 × 15 cm)
Cage tilt	60 min	30° tilt of home-cage
Forced swim	3 min	Forced swim in a glass cylinder (20 cm diameter × 25 cm high) containing cold water (22 ± 1 °C, 15 cm deep)
Light/Dark cycle disruption	12 h	Lights on during dark phase

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Table 3.2. Total number of arm entries during the Y-maze acquisition and retention phases.

	Treatment condition			
	WT-No stress	WT-Stress	KO-No stress	KO-Stress
Acquisition phase	27.4 ± 1.1	25.1 ± 2.5	21.1 ± 1.4*	20.7 ± 1.5*
Retention phase	25.1 ± 1.8	20.1 ± 1.6	21.1 ± 1.7	20.6 ± 1.5

Data are presented as mean ± SEM ($n = 8-10$). * $p < 0.05$ relative to WT mice (collapsed across the stressor treatment). Reproduced under the Creative Commons Attribution 4.0 International license: © 2014 Litteljohn, Nelson and Hayley.

Table 3.3. Dopaminergic activity within the nucleus accumbens as a function of chronic stress and IFN- γ deletion.

	Concentration (ng/mg protein)			DA turnover
	DA	DOPAC	HVA	
WT-No stress	123.57 \pm 14.76	15.43 \pm 1.19	7.95 \pm .43	.216 \pm .035
WT-Stress	152.78 \pm 17.56	15.98 \pm 1.04	5.96 \pm .91	.173 \pm .039
KO-No stress	156.33 \pm 20.07	16.81 \pm 1.36	5.50 \pm .54	.155 \pm .017
KO-Stress	186.87 \pm 19.00	17.67 \pm 1.61	6.28 \pm .85	.145 \pm .022

Data are presented as mean \pm SEM. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; DA turnover = [(DOPAC+HVA)/DA]. Reproduced under the Creative Commons Attribution 4.0 International license: © 2014 Litteljohn, Nelson and Hayley.

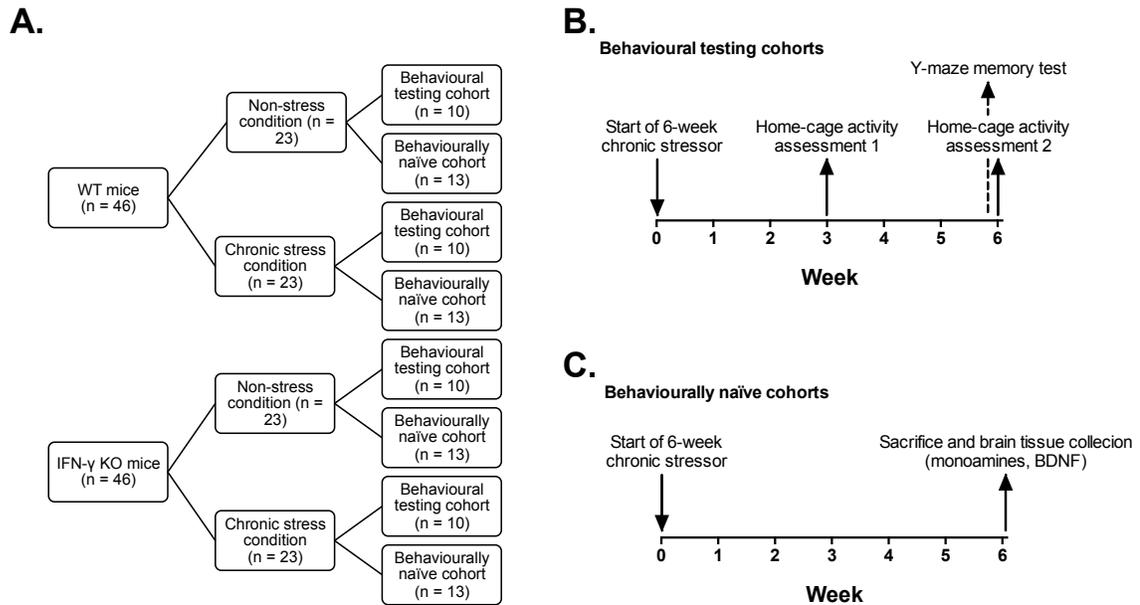


Figure 3.1. Schematic showing the experimental design and timeline. IFN- γ KO and WT littermate mice ($n = 46$ per genotype) were randomly assigned to a 6-week chronic stressor condition or a non-stress control group ($n = 23$) (A). Mice of each condition were further divided into behavioural testing ($n = 10$) (B) and behaviourally naïve cohorts ($n = 13$) (C), the latter of which were used for end-of-study brain regional monoamine ($n = 10$) or hippocampal BDNF analyses ($n = 3$). Reproduced under the Creative Commons Attribution 4.0 International license: © 2014 Litteljohn, Nelson and Hayley.

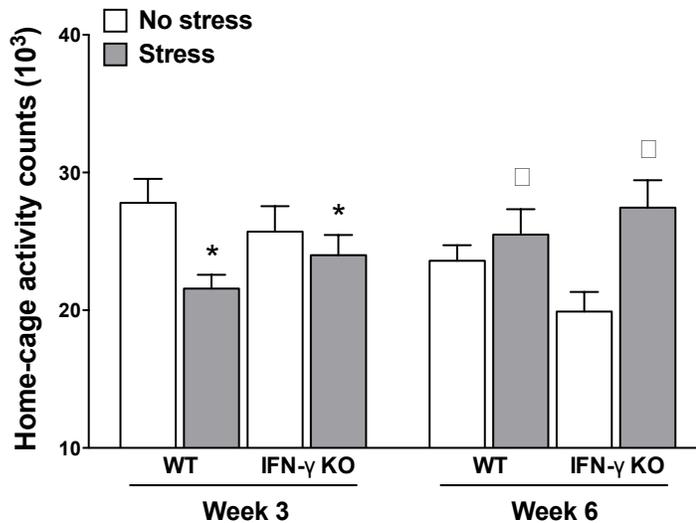


Figure 3.2. *Influence of chronic stress on home-cage locomotor activity among IFN- γ WT and KO mice.* Overall, chronic stressor exposure had the effect of reducing home-cage activity at Week 3 (mid-way through the experiment), but increasing locomotor activity at Week 6 (end of experiment). However, the early-occurring hypolocomotive effect of stress was clearly most prominent in the WT animals, whereas the stressor's later-occurring hyperlocomotive effect was most evident in the KO mice (see accompanying text). Data are presented as mean \pm SEM. * $p < 0.05$ relative to non-stressed mice (collapsed across Genotype) at Week 3, and $^{\wedge}p < 0.05$ relative to non-stressed mice (collapsed across Genotype) at Week 6 (Two-way repeated measures ANOVA). Reproduced under the Creative Commons Attribution 4.0 International license: © 2014 Litteljohn, Nelson and Hayley.

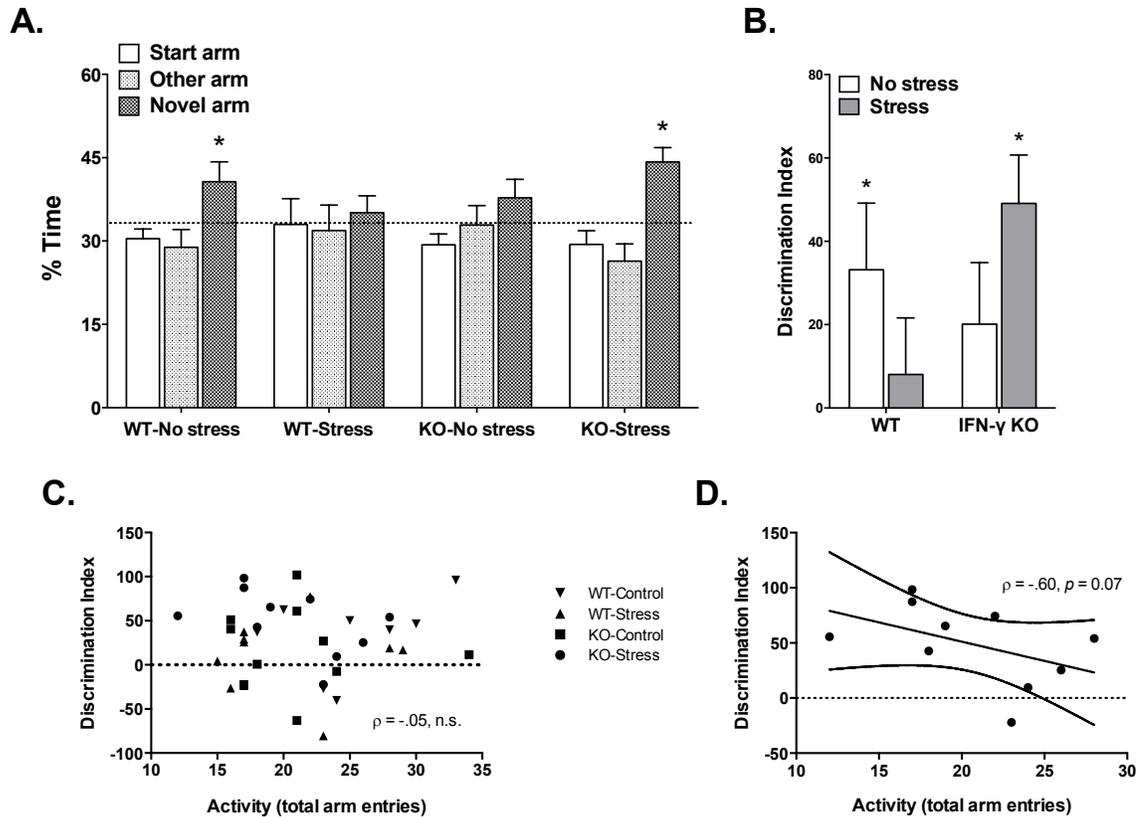


Figure 3.3. Impact of IFN- γ deletion on short-term spatial memory function under basal vs. chronic stressor conditions. As regards both the percentage (%) time spent exploring the novel arm of the Y-maze (A) and the Novelty Discrimination Index (DI) (B), of the four treatment groups, only the non-stressed WT controls and the chronically stressed IFN- γ KO mice performed at a level significantly above chance (33.3 % and 0, respectively). While overall there was no association between DI scores and total Y-maze arm entries (C), when the correlation analysis was restricted to the chronically stressed KOs, Y-maze locomotion tended to correlate negatively with short-term spatial memory performance (D) (see accompanying text). Data are presented as mean \pm SEM; horizontal dashed lines represent chance-level performance (33.3% or 0), the solid line represents the best-fit linear regression line, and the curved dashed lines show the 95% confidence interval. * $p < 0.05$ relative to chance (univariate t-test). Reproduced under the Creative Commons Attribution 4.0 International license: © 2014 Litteljohn, Nelson and Hayley.

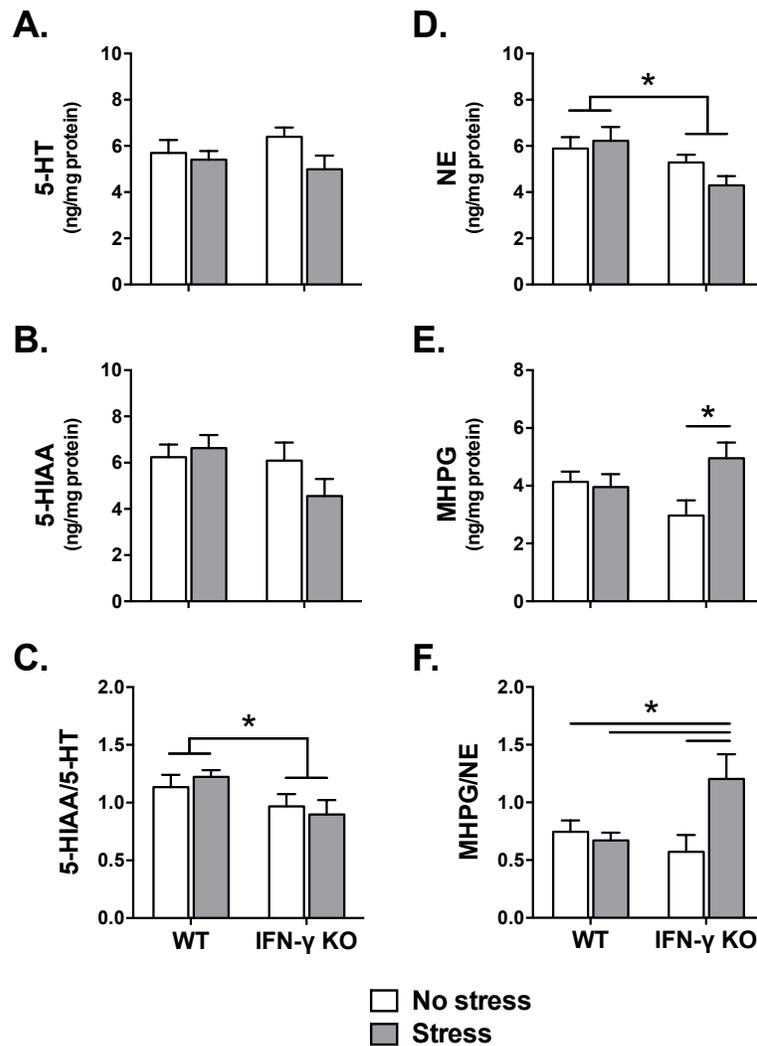


Figure 3.4. Hippocampal serotonin and noradrenergic activity as a function of *IFN- γ* KO and chronic stressor exposure. Within the hippocampus, neither 5-HT (A) nor 5-HIAA (B) was significantly affected by chronic stress or *IFN- γ* deletion. Yet, 5-HT turnover (i.e., the ratio of 5-HIAA to 5-HT) (C) was diminished overall in the *IFN- γ* KOs compared to the WT mice. With respect to noradrenergic neurotransmission in the hippocampus, NE levels (D) were significantly reduced among the *IFN- γ* KO mice, irrespective of chronic stress. In contrast, both MHPG accumulation (E) and NE turnover (MHPG-to-NE ratio) (F) were significantly enhanced following chronic stress, but only among the *IFN- γ* KOs. Data are presented as mean \pm SEM; * p < 0.05 (Two-way ANOVA followed by Student-Newman-Keuls test). Reproduced under the Creative Commons Attribution 4.0 International license: © 2014 Littelljohn, Nelson and Hayley.

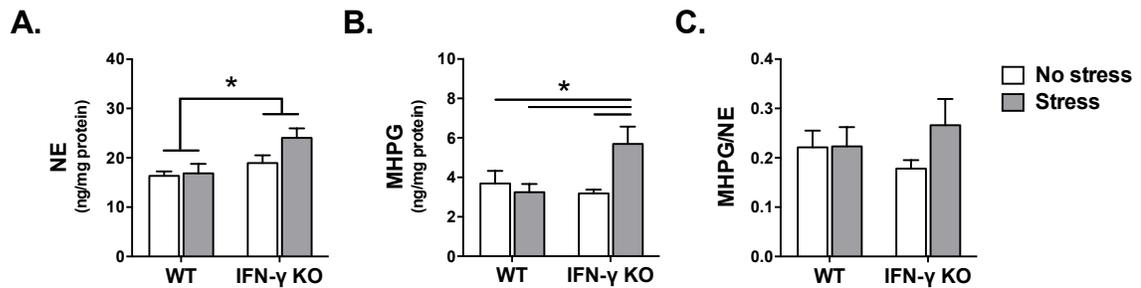


Figure 3.5. *Locus coeruleus noradrenergic activity as a function of IFN- γ KO and chronic stressor exposure.* Compared to the WT animals, concentrations of LC NE (A) were significantly higher among the IFN- γ -deficient mice, regardless of stressor exposure. As regards MHPG in this brain region (B), whereas metabolite levels were unaffected by chronic stress in the WT mice, in the stressed IFN- γ KOs MHPG accumulation was significantly enhanced. The experimental manipulations did not, however, significantly alter LC NE turnover (ratio of MHPG-to-NE) (C). Data are presented as mean \pm SEM; * $p < 0.05$ (Two-way ANOVA followed by Student-Newman-Keuls test). Reproduced under the Creative Commons Attribution 4.0 International license: © 2014 Litteljohn, Nelson and Hayley.

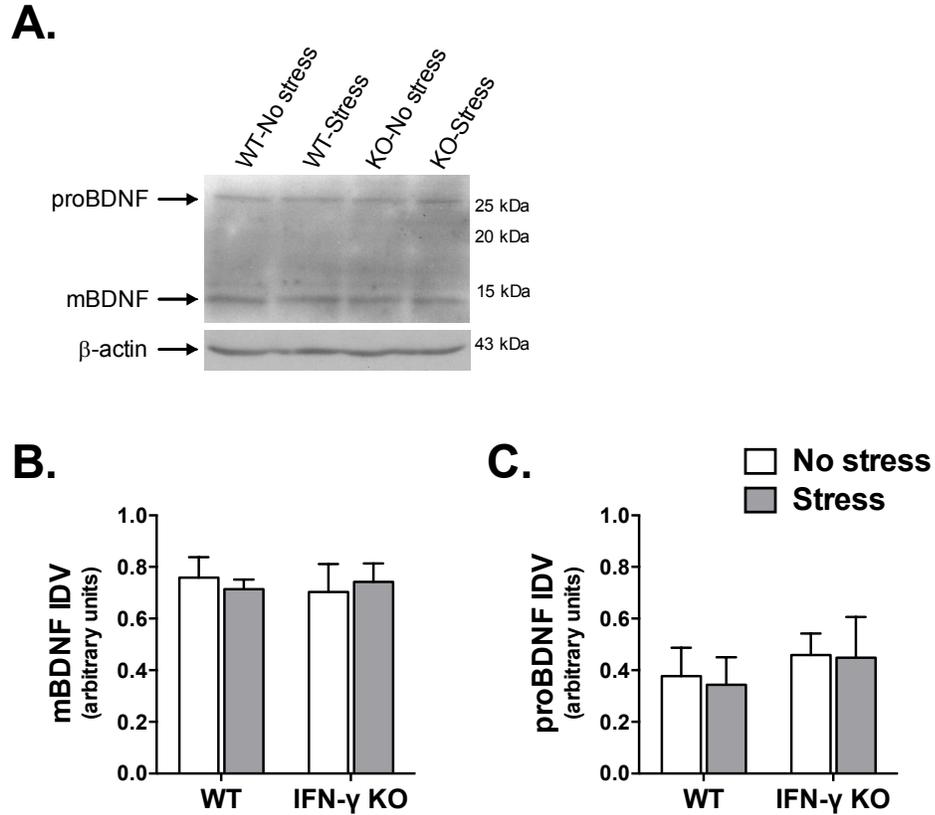


Figure 3.6. Influence of chronic stress and IFN- γ KO on BDNF protein expression within the hippocampus. Representative immunoblots (A) were cropped and adjusted for brightness and contrast using Fotor photo editor software (version 1.3.1). Neither chronic stress nor IFN- γ KO significantly affected mature BDNF (B) or proBDNF (C) protein expression within the hippocampus. Actin-normalized integrated density values (IDV) are presented as mean \pm SEM ($n = 3$). Reproduced under the Creative Commons Attribution 4.0 International license: © 2014 Litteljohn, Nelson and Hayley.

4 Chapter. Individual and interactive effects of systemic IFN- γ and acute restraint stress: a focus on HPA axis and brain regional monoamine activity

4.1 Abstract

Compelling evidence supports the involvement of the pro-inflammatory cytokines IL-6, IFN- α and TNF- α in depression and related stress-associated pathologies. A role has also been suggested for IFN- γ , with most mechanistic accounts focusing on the cytokine's marked capacity to induce IDO, leading to diminished TRP bioavailability and the generation of neurotoxic KYN metabolites. Beyond these IDO-dependent routes, there is surprisingly little evidence directly linking IFN- γ to alterations of brain regional monoamine activity and HPA axis functioning. Our specific aims in the present study were twofold: 1) assess the behavioural, plasma corticosterone and brain regional monoamine effects of acute systemic IFN- γ , with or without short duration restraint stress (15 min), and 2) determine the sex-specific nature of these effects. As predicted, IFN- γ stimulated monoaminergic activity within a number of stressor-sensitive limbic brain regions, most notably the PVN, CeA and PFC. While several of these effects were sex-specific, there was little in the way of synergism between the cytokine and stressor treatments (despite trends in the data). Nonetheless, IFN- γ did interact synergistically with acute restraint stress to increase plasma corticosterone concentrations, and this effect was most pronounced in the male mice. These data are among the first to show that systemically administered IFN- γ , much like other depression-linked cytokines, can alone or in conjunction with psychologically relevant stress modify brain regional monoamine activity and the plasma corticosterone stress response. We discuss possible mechanisms subserving the cytokine's repertoire of action and suggest future research directions.

4.2 Introduction

Multiple lines of evidence point to a role for inflammatory factors, including acute phase proteins (e.g., complement components, CRP) and the pro-inflammatory cytokines IL-6, IFN- α and TNF- α , in depression and related stress-associated psychiatric conditions (Sluzewsk et al., 1996; Berk et al., 1997; Miller et al., 2009). Type II IFN, otherwise known as IFN- γ , is another cytokine that may be germane to this discussion (Maes et al., 2011b). To wit, elevated concentrations of IFN- γ have been detected in the blood and CSF of depressed patients (Maes et al., 1994, Simon et al., 2008; Dahl et al., 2014), and several different classes of antidepressants were shown to antagonize IFN- γ activity (Kubera et al., 2000b; Diamond et al., 2006). Moreover, variation in the IFN- γ gene was reported to modify amygdalar reactivity to emotional stimuli (Redlich et al., 2015) as well as depression risk in IFN- α -treated hepatitis C patients (Oxenkrug et al., 2011). Consistently, acute exposure to an IFN- γ adenovector provoked long-lasting hedonic-like deficits (Kwant and Sakic, 2004) whereas genetic ablation of IFN- γ or its receptor attenuated some of the depressive-like neurochemical, immunological and behavioural effects of chronic stress (Litteljohn et al., 2010b, 2014) or *M. bovis* BCG infection (O'Connor et al., 2009b). And more recently, IFN- γ was implicated as an important contributor to the depressive-like behavioural effects of long-term LPS administration in rats (Fischer et al., 2015).

Regarding the molecular and cellular pathways underlying the putative depressogenic effect of IFN- γ , much has been made of the cytokine's potent stimulatory action on the inflammatory enzyme IDO, which shuttles TRP down the KYN pathway and away from the 5-HT synthesizing pathway (Miller et al., 2009). Specifically, it's

been suggested that an increase in IFN- γ levels, as might occur in response to bacterial or viral infection (Schroder et al., 2004) or in the context of chronic stress (Tian et al., 2014), an ongoing medical illness and even normal aging (Bandrés et al., 2000; Pollard et al., 2013), could influence affective states via an IDO-dependent reduction in 5-HT synthesis and/or the elaboration of neuroactive glutamatergic compounds, such as 3-HK and QUIN; this has largely been borne out by the research enterprise (Müller and Schwarz, 2007; Oxenkrug, 2011; Maes et al., 2011b; Myint et al., 2013; Young et al., 2016). However, IDO-related serotonergic dysfunction has not always been observed in the context of IFN- γ -associated depressive sequelae (Dantzer et al., 2008; O'Connor et al., 2009b) and it is evident that other signalling pathways must also contribute to the cytokine's behavioural effects.

In this regard, surprisingly few studies have sought to characterize the prospective influence of IFN- γ on HPA axis and brain regional monoamine activity, perturbations of which are extremely common and pathophysiologically important in depression (Hamon and Blier, 2013; Horowitz and Zunszain, 2015). Moreover, the few studies that have actually investigated this research question have generated somewhat contradictory results. For instance, while de Metz et al. (1999) showed that IFN- γ administration in humans augmented plasma concentrations of ACTH and cortisol, Besedovsky et al. (1986) failed to observe analogous changes in mice. And though our own work with IFN- γ KO mice has indirectly implicated the cytokine in the modulation of central noradrenergic, dopaminergic and serotonergic systems (Litteljohn et al., 2010b, 2014), direct evidence of such, beyond the aforementioned IDO-associated changes, is scarce if not wanting (Clement et al., 1997; Färkkilä et al., 1998; Morikawa et al., 1998). This

paucity of knowledge or at least lack of consensus surrounding IFN- γ stands in stark contrast to the many studies detailing the potent neuroendocrine and monoamine regulatory effects of other depression-linked cytokines (Dunn et al., 2005a; Anisman, 2009). Moreover, it's unclear whether exogenously administered IFN- γ can interact with psychologically relevant stressors to influence depression-related pathophysiological domains and behavioural states, as has been reported for TNF- α , IL-1 β and several other cytokines (Merali et al., 1997; Hayley et al., 2003). Once again, our own studies investigating chronic stressor effects in IFN- γ KO mice have hinted at such a possibility (Litteljohn et al., 2010b, 2014).

In the present investigation we assessed the individual and combined neuroendocrine and central monoamine effects of systemically administered IFN- γ and acute restraint stress among 4-5 month old male and female littermate mice. Our main hypotheses were as follows: 1) acutely administered IFN- γ will stimulate HPA axis and brain regional monoamine activity in a manner reminiscent of other depression-linked cytokines; 2) synergistic effects will be evident for IFN- γ and restraint stress; and 3) sexual dimorphism will be apparent for at least some of these effects.

4.3 Methods

4.3.1 Animals

Four-to-five month old male and female WT littermate mice were obtained from our in-house LRRK2 heterozygous breeding colony, which had been backcrossed onto the C57BL/6J genetic background for > 15 successive generations (in-house and by the strain's donating investigator and commercial supplier, i.e., The Jackson Laboratory, Bar Harbor, ME). These animals were weaned at 21 days and maintained on a 12 h light/dark

cycle (lights on at 0800 h) in standard polycarbonate enclosures (27 × 21 × 14 cm) as same-sex littermate groupings of 2-3. A diet of standard laboratory mouse chow (Harlan Laboratories, WI) and water was provided ad libitum, and room temperature maintained at ~ 21 °C. All experimental procedures were approved by the Carleton University Committee for Animal Care and complied with the Canadian Council on Animal Care's guidelines on the ethical use and care of animals in research.

4.3.2 Procedure

Male and female mice were randomly assigned to one of 8 experimental conditions ($n = 7-9$), as provided for by our 2 (Sex: male, female) × 2 (Injection: vehicle, IFN- γ) × 2 (Stressor: control, restraint) experimental design ($N = 65$). The experimental timeline is shown in Figure 4.1a. All mice were acclimated to the behavioural testing room for ~ 16 h, in their usual cages and with their usual cage-mates. Commencing at 0830 h on the day of experimentation and at an interval of every 10 min, mice were individually removed from the grouped enclosures and singly housed in freshly made cages. As before, food and water were provided ad libitum. Immediately upon being introduced into the new cage, animals were injected intraperitoneally with IFN- γ or vehicle (see below).

Over the ensuing 60 min, spontaneous locomotor activity was assessed using a Micromax infrared beam-break apparatus positioned exterior to the home-cage (AccuScan Instruments, Columbus, OH). A subset of the animals was then transferred to a nearby room and administered a 15 min restraint stressor. At 78 min post-injection (i.e., 3 min following termination of the stressor), mice were rated for sickness behaviours and immediately sacrificed by rapid decapitation; blood and brain tissue were collected for

later corticosterone and neurochemical determinations. Sickness behaviours were rated on a 3-point scale with respect to curled body posture, ptosis, piloerection, lethargy, and overall non-responsiveness (where 0 = no symptom, 1 = one symptom present, 2 = two symptoms presents, 3 = three or more symptoms) (Anisman et al., 2008c). In order to minimize the effects of diurnal variations, experiments were carried out between the hours of 08:30 and 12:30.

4.3.3 Cytokine injections and acute restraint stress

Mouse recombinant IFN- γ (25000 IU, R&D Systems) was reconstituted in a phosphate-buffered saline (PBS) solution containing 0.1% bovine serum albumin (BSA, Sigma Aldrich). The vehicle contained a matching amount of BSA. All injections were administered in a volume of 0.3 ml. The cytokine dose used in the present study falls within the range of IFN- γ doses previously reported to cause neurochemical, neuroendocrine and behavioural alterations in mice (Saito et al., 1991; Crnic and Segall, 1992; Cano et al., 2005; Hozumi et al., 2008). Although 25000 IU IFN- γ represents a physiologic dose that is expected to approximate tissue IFN concentrations during viral infection (Heremans et al., 1980; Crnic and Segall, 1992), the existing in vivo data are somewhat limited and a worthwhile next step will be to characterize the brain and behavioural effects of varying doses of IFN- γ , as well as different routes of delivery, dosing regimens and sampling time points.

The flat-bottom restraint stressor apparatus comprised a 4 × 12 cm semi-circular Plexiglas tube, with a tail restraint fashioned from the same material. Tails were also taped to prevent mice from turning. A 10-15 min application of this neurogenic stressor

has been shown to reliably and sex-dependently stimulate HPA axis and brain regional monoamine activity (Anisman et al., 2001; Jacobson-Pick, 2013).

4.3.4 Brain dissection technique and HPLC determination of central amine and metabolite concentrations

Brains were extracted and dissected and tissue specimens maintained according to the methods described in §2.3.4. In this study we collected the PVN, LC, CeA, PFC, and dorsal hippocampus (from here on out used interchangeably with HC). Regional brain levels of NE, 5-HT and DA, and their respective metabolites MHPG, 5-HIAA, and DOPAC and HVA, were determined by HPLC as previously described (§2.3.7).

4.3.5 Plasma corticosterone assay

Corticosterone determinations were carried out with commercially available radioimmunoassay kits (ICN Biomedicals, CA, USA) as described in Chapter 2 (§2.3.5).

4.3.6 Statistical analyses

The corticosterone and monoamine data were analyzed by 2 (Sex) × 2 (Injection) × 2 (Stressor) factorial ANOVA, followed where appropriate by Tukey-Kramer's multiple comparison procedure ($\alpha = .05$). As the home-cage activity measurements were obtained prior to stressor application, these data and were analyzed by a Sex × Injection between subjects ANOVA. Second-order interactions were interpreted by analyzing the separate familywise error-controlled two-way ANOVAs at each level of the moderator variable; i.e., by running a simple interaction effects analysis for Sex and then following up where appropriate with Tukey-Kramer's post-hoc tests ($\alpha = .05$). All *F*-tests and pairwise comparisons were conducted using the pooled error term from the relevant overall ANOVA. Data points exceeding 2.5 standard deviations from the mean were

considered outlier values and omitted from the analyses. During the course of tissue dissection and monoamine determination a few samples (CeA, PVN and LC) were lost due to error. Data were evaluated and plotted using StatView version 6.0 and GraphPad Prism 6 (La Jolla, CA), respectively.

4.4 Results

4.4.1 Lack of sickness-type behaviours following systemic administration of IFN- γ

Although the ANOVA for home-cage activity produced a significant Sex \times Injection interaction ($F_{1, 60} = 5.57, p < .05, \eta^2 = .085$), the follow-up tests were not statistically significant (Tukey-Kramer HSD: $p > .05$) (Fig. 4.1b). As expected, neither IFN- γ nor restraint stress provoked sickness behaviours in mice of either sex ($F_s < 3$). The females all received scores of 0 (on a 3-point scale) and the overall mean score in the males was 0.09 ± 0.09 .

4.4.2 Individual and interactive effects of IFN- γ and restraint stress on plasma corticosterone levels

The ANOVA for plasma corticosterone levels uncovered significant main effects of Sex, Injection and Stress ($F_{S1, 57} = 28.31, 29.61$ and 109.31 , respectively; $p < .0001, \eta^2 = .12, .13$ and $.46$, respectively), as well as a significant Injection \times Stress interaction ($F_{1, 57} = 5.30, p < .05, \eta^2 = .022$). As shown in Figure 4.2 and confirmed by the follow-up tests, the corticosterone-elevating effect of Stress was independent of Sex and Injection whereas that of IFN- γ appeared to be contingent on subsequent stressor exposure ($p < .05$). However, it should be noted that the main effect of Injection accounted for $\sim 13\%$ of the total variability in plasma corticosterone scores whereas the Injection \times Stress interaction term accounted for roughly 2%. Moreover, visual inspection of the data

suggests that IFN- γ and Stress additively (and not synergistically) increased plasma corticosterone levels in the female mice, among whom corticosterone levels were elevated overall ($p < .05$ relative to male littermates).

4.4.3 IFN- γ and stress influenced monoamine activity within the PVN, LC and CeA in a largely non-interactive and Sex-independent manner

There were no significant differences between groups for NE levels in the PVN (Fig. 4.3a). However, concentrations of the primary NE metabolite MHPG were elevated overall in response to the acute restraint stressor ($F_{1,47} = 22.74, p < .0001, \eta^2 = .30$) (Fig. 4.3b). And while IFN- γ did not alter NE activity within this brain region, the cytokine treatment significantly reduced PVN 5-HT levels irrespectively of both Sex and Stress ($F_{1,47} = 4.60, p < .05, \eta^2 = .078$) (Fig. 4.3c). Yet, as shown in Figure 4.3d, 5-HIAA accumulation was unaffected by IFN- γ treatment, and together these data are consistent with an IFN- γ -induced increase of PVN 5-HT turnover (i.e., 5-HIAA:5-HT) (main effect of IFN- γ : $1.94 \pm .22$ vs. $2.31 \pm .28$; $F_{1,47} = 4.34, p < .05, \eta^2 = .073$). In regards to PVN DA activity, while neither DA nor DOPAC varied significantly across groups ($F_s < 3$, Fig. 4.3e, f), the ANOVA for HVA levels revealed a significant Sex \times Injection interaction ($F_{1,47} = 4.11, p < .05, \eta^2 = .07$). As shown in Figure 4.3g and confirmed by the multiple pairwise comparisons, while IFN- γ did not significantly alter PVN HVA concentrations among mice of either sex (i.e., relative to vehicle-treated mice of the same sex), the cytokine did tend to have an opposing effect on metabolite levels in the male and female mice (IFN- γ treated males vs. females: $p < .05$, consistent with a disordinal interaction).

Within the LC, NE concentrations did not vary in a statistically meaningful way as a function of Sex, Injection or Stress ($F_s < 4.2$, Tukey-Kramer post-hocs: $p > .05$, data not shown). However, as was the case in the PVN, LC MHPG levels were increased overall in the stressor-treated animals (main effect of Stress: $3.78 \pm .41$ vs. $5.89 \pm .95$; $F_{1, 44} = 11.50$, $p < .01$, $\eta^2 = .20$).

A similar pattern of effects was observed with regards to noradrenergic activity in the CeA: unchanged NE levels and enhanced MHPG accumulation overall following restraint ($F_{1, 50} = 6.56$, $p < .05$, $\eta^2 = .01$) (Fig. 4.4a, b). In addition, MHPG levels were lower overall among the female mice ($F_{1, 50} = 5.31$, $p < .05$, $\eta^2 = .082$), and separate ANOVAs revealed a similar effect of Sex for both CeA DA and DOPAC ($F_{S1, 50} = 4.04$ and 5.90 , $p < .05$, $\eta^2 = .066$ and $.08$, respectively) (Fig. 4.4e, f). Although NE activity within the CeA was unchanged by IFN- γ , the cytokine treatment robustly and independently (i.e., of Sex and Stress) augmented CeA levels of not only 5-HT and 5-HIAA ($F_{S1, 50} = 13.02$ and 6.57 , $p < .001$ and $.05$, $\eta^2 = .20$ and $.098$, respectively), but also DA, DOPAC and HVA ($F_{S1, 50} = 5.08$, 7.69 and 14.05 , $p < .05$, $.01$ and $.001$, $\eta^2 = .083$, $.11$ and $.21$, respectively) (Fig. 4.4c-g). In effect, IFN- γ increased the tone of these monoamine systems within the CeA of both the male and female mice.

4.4.4 Individual and interactive effects of Sex, Injection and Stress on monoaminergic activity in the HC and PFC

Within the dorsal HC, the concentrations of NE, 5-HT and 5-HIAA were unaltered between groups ($F_s < 3.8$, Fig. 4.5a, c, d). In contrast, the ANOVA for MHPG yielded a significant main effect of Injection ($F_{1, 57} = 5.58$, $p < .05$, $\eta^2 = .07$) as well as a significant Sex \times Stress interaction ($F_{1, 57} = 4.67$, $p < .05$, $\eta^2 = .059$). As shown in Figure

4.5b and confirmed by the follow-up tests, while IFN- γ increased MHPG levels in both the male and female mice, the restraint stressor did so only in the former ($p < .05$). It will be noted, however, that the male-specific MHPG-boosting effect of stress appeared to be most prominent in the IFN- γ co-treated animals (the second-order interaction was not statistically significant: $F_{1, 57} = 1.82, p = .183, \eta^2 = .023$).

Similarly, within the PFC, concentrations of NE were unchanged and those of MHPG augmented overall in response to IFN- γ treatment ($F_{1, 57} = 5.79, p < .05, \eta^2 = .075$) (Fig. 4.6a, b). The restraint stressor also enhanced prefrontal MHPG accumulation, but unlike in the dorsal HC this effect occurred independently of Sex (main effect of Stress: $F_{1, 57} = 11.30, p < .01, \eta^2 = .15$). There were no between-group differences in the PFC concentrations of either 5-HT or DA (Fig. 4.6c, e). And while the male mice had unaltered levels of 5-HIAA and HVA, in the female PFC accumulation of both these metabolites was significantly increased by IFN- γ ($p < .05$ relative to vehicle-treated females) (Sex \times Injection interaction: $F_{S1, 57} = 5.71$ and $4.21, p < .05, \eta^2 = .07$ and $.056$, respectively) (Fig. 4.6d, g). The pattern of PFC DOPAC changes was somewhat more complex, as the ANOVA produced a significant 3-way interaction between Sex, Injection and Stress ($F_{1, 57} = 10.02, p < .01, \eta^2 = .104$). The follow-up analyses uncovered a significant Injection \times Stress interaction in the male mice ($F_{1, 57} = 17.87, p < .05$) and a lack of significant ANOVA effects in the females. As shown in Figure 4.6f, the stressor markedly enhanced prefrontal DOPAC levels in the male mice ($p < .05$ relative to non-stressed controls), an effect that was abrogated by pre-treatment with IFN- γ and absent altogether in the female mice.

4.5 Discussion

Although IFN- γ has been suggested to play a role in depressive-like pathology, there is limited information concerning the cytokine's effects on neuroendocrine and brain regional monoamine function; that is, beyond those protracted changes attributable to IFN- γ -induced IDO signalling (e.g., reduced 5-HT synthesis, KYN pathway-related excitotoxicity and degeneration of monoamine and other neurons) (Tu et al., 2005; Maes et al., 2011b; Oxenkrug, 2011). Moreover, it's unclear whether IFN- γ can interact synergistically in this regard with psychological stressors, as has been reported for TNF- α , IL-1 β and IFN- α (Hayley et al., 2003). As summarized in Table 4.1, we presently report that systemically administered IFN- γ (25000 IU) robustly influenced monoaminergic neurotransmission in a network of stressor-sensitive limbic areas; i.e., the PVN, LC, CeA, dorsal HC, and PFC (Jankord and Herman, 2008; Brown and Morey et al., 2012; Rive et al., 2013). A majority of these effects were consistent with a stimulation or potentiation of monoamine activity/tone, and several of them appeared to be sex-specific (PVN HVA and PFC 5-HIAA and HVA in the females, PVN 5-HT in the males). Importantly, these effects are largely reminiscent of the neuroendocrine and monoamine changes induced by other depression-linked inflammatory cytokines in comparable acute exposure experimental paradigms (Anisman et al., 2005; Dunn et al., 2005a; see §1.2.3.1).

Consistent with the earlier report of Weinberger et al. (1988), our data do not support an activity-suppressing or sickness-inducing effect of acute systemic IFN- γ exposure (although these authors did observe reduced open field activity with repeated IFN- γ injections). However, in our study there was clearly a trend towards reduced home-

cage activity in the IFN- γ -treated male mice (supported by the significant Sex \times Injection disordinal interaction) and at least one other group has documented altered locomotor activity in response to acute systemic IFN- γ treatment (Crnic and Segall, 1992). However, in this latter study IFN- γ was administered and home-cage activity assessments initiated immediately preceding dark onset, which for the nocturnal mouse may correspond to the most sensitive window for detecting depression-like alterations in activity (consistent with neurovegetative symptoms) (Crnic and Segall, 1992).

As expected, acute restraint markedly increased HPA axis output and stimulated brain regional noradrenergic and, to a far lesser extent, dopaminergic activity, and again several of these effects were sex-specific (Table 4.1) (Jacobson-Pick et al., 2013; Kawakami et al., 2013). Yet, the cytokine and stressor treatments had mostly non-overlapping effects (PFC DOPAC, HC and PFC MHPG, and plasma corticosterone being the exceptions), which itself is not entirely surprising (Song et al., 1999), and there was very little statistically compelling evidence of synergy between the two; indeed, this was restricted to circulating corticosterone levels. Here, our findings indicate that while acute systemic IFN- γ (25000 IU) may not alone be capable of activating the murine neuroendocrine stress axis (although it appeared to do so in the female mice), which is consistent with the negative findings in male mice reported by Besedovsky et al. (1986), the cytokine is yet able to sensitize HPA responses to psychologically relevant stressors. How exactly IFN- γ comes to modulate corticosterone levels in vivo is not entirely clear, although a number of early human studies attest to this being a real phenomenon (Goldstein et al., 1987; Holsboer et al., 1988; Späth-Schwalbe et al., 1989). Potential non-mutually exclusive mechanisms, each of which derives some measure of support from the

available human and/or animal data, include: 1) a direct influence of IFN- γ on PVN neurons, pituitary corticotrophs, and/or corticoadrenal cells (Holsboer et al., 1988; Vankelecom et al., 1990; Katahira et al., 1998; Labeur et al., 2008); 2) the involvement of secondary mediators, such as IL-6 and prostaglandins (Yamaguchi et al., 1991; de Metz et al., 1999; Tu et al., 2005); and 3) indirect actions of IFN- γ or secondary mediators at vagal afferents, CVOs (particularly the median eminence), and/or any number of upstream brainstem integration nuclei (e.g., bed nucleus of the stria terminalis) (Robertson et al., 2000; Dunn, 2000; Ziegler and Herman, 2002).

Yet, there was clearly a trend in our brain monoamine data towards an IFN- γ \times stressor synergism, and again there appeared to be a degree of sex-specificity in this regard. Specifically, while in the PFC the cytokine-plus-stressor treatment appeared to synergistically increase MHPG levels to a similar extent in the male and female mice (Injection \times Stress interaction: $\eta^2 = .028$), in the HC and CeA this trend appeared to be most prominent in the males (Sex \times Injection \times Stress interactions for HC MHPG and 5-HIAA: $\eta^2 = .023$ and $.037$, respectively) and females, respectively (Injection \times Stress interactions for CeA 5-HIAA and DOPAC: $\eta^2 = .034$ and $.043$). Thus, we suggest that the paucity of significant interaction effects observed in the present study may reflect a lack of statistical power, and the totality of the evidence at least hints at a possible sensitizing or permissive influence of IFN- γ on select central monoamine responses to psychological stress. It ought also to be underscored that in the current study we only tested a single cytokine dose, stressor duration, and interval between treatment exposures; varying these and other parameters in future work (e.g., route of delivery, dosing regimen, stressor

modality, order of treatments) will lead to more light being shed on the prospective interactive effects of IFN- γ and stress.

In an intriguing turn, the one instance where we uncovered a significant 3-way interaction was consistent not with a sex-specific synergism between IFN- γ and stress (as hypothesized), but rather the IFN- γ -mediated *blunting* of a male-specific stressor-induced increase in prefrontal DOPAC (Table 4.1). It is not immediately clear what could have accounted for this surprising finding. One possibility is that IFN- γ may have induced an early rise in glucocorticoids, which through genomic signalling routes could have attenuated the PFC dopaminergic response to subsequent stressor exposure. Indeed, GR-mediated suppression of neuronal excitability is a well-established genomic feedback mechanism (de Kloet et al., 2008), although at < 1.5 h post-injection we are likely still in the excitatory phase of GR-mediated transcriptional regulation (e.g., down-regulation of MAO) (Morsink et al., 2006). Alternatively, at 18 min post-stressor initiation, it is possible that IFN- γ -and-stressor-induced glucocorticoids were acting via rapid non-genomic mechanisms to suppress prefrontal DA activity; i.e., involving cytosolic mineralocorticoid receptors or as yet unidentified membrane-bound steroid hormone receptors. Even though most of the available evidence points to a permissive effect of such signalling, e.g., enhanced glutamate release and modulation of neuron potassium (K^+) conductances (Karst et al., 2005), in the hypothalamus this feedback is mostly inhibitory (de Kloet et al., 2008) and only recently Teng et al. (2013) reported that glucocorticoids enhance GABAergic (inhibitory) transmission in the PFC (involving a phospholipase C–diacylglycerol-dependent pathway). In effect, rapid glucocorticoid-mediated alterations to ionic conductances and/or the balance between excitatory and

inhibitory synaptic inputs could have attenuated prefrontal DA release in the IFN- γ -plus-stressor-treated mice (which as will be recalled had the highest levels of plasma corticosterone).

4.5.1 Examination of possible mechanisms subserving the neuroendocrine and central monoamine effects of IFN- γ

Such discussion brings us to a critical point of consideration: how is IFN- γ causing these acute monoamine effects, most of which are consistent with *increased* neurotransmitter activity? Indeed, acting mainly through IDO-dependent routes and the increased expression and/or activity of monoamine reuptake transporters (Morikawa et al., 1998; Zhu et al., 2006), IFN- γ and other pro-inflammatory cytokines are considered to *reduce* the synaptic availability of monoamines and thus contribute to affective states (Miller et al., 2009; Felger and Lotrich, 2013). Such mechanisms are obviously at odds with the rapid stimulatory effects of pro-inflammatory cytokines, which in the short-term are expected to contribute to allostatic load and in the long run promote allostatic overload and the development of psychological pathology (McEwen, 2000).

Given the important role of IFN- γ in activating immune cells and orchestrating inflammatory responses (Schroder, 2004), a most plausible scenario has systemically administered IFN- γ signalling through circulating immune cells as well as brain microvasculature endothelial cells and afferent nerves (e.g., the vagus) to increase their production and release of inflammatory and oxidative factors; e.g., prostanoids, ROS and pro-inflammatory cytokines/chemokines, including IFN- γ itself. These, in turn, could recruit and activate perivascular macrophages and brain-resident glia or else act directly on neurons to alter ion conductances, prostaglandin and NO mobilization, and other

intracellular second messenger cascades, with the collective effect being that of neuronal excitation (Masana et al., 1990; Tancredi et al., 1992; Wilkinson et al., 1993; Davis et al., 2006; Desson and Ferguson, 2003; Huang et al., 2011; Galic et al., 2012). Consistent with such a mechanism, we report in Chapter 5 that IFN- γ (25000 IU) increased the circulating levels of both IL-6 and MCP-1/CCL2. Yet, IFN- γ can itself enter the brain via active transport or diffusion across CVOs, such as the median eminence and subfornical organ (Anisman et al., 2008b), which raises the possibility that the cytokine may act directly on CNS neurons to influence monoamine function (i.e., a cell autonomous mechanism). Indeed, IFN- γ receptors are functionally expressed in neurons throughout much of the brain, particularly at axon terminals and postsynaptic sites, and under basal conditions this signal far exceeds that seen in microglial cells (Robertson et al., 2000). This suggests that neurons may be exquisitely sensitive to small changes in local concentrations of IFN- γ .

Presumably, such direct neuronal targeting by IFN- γ , at least in the short term, will result in membrane depolarization and action potential generation/propagation, as discussed above for cytokines in the general case (Born et al., 1989; Galic et al., 2012). For instance, Mizuno et al. (2008) reported that IFN- γ acts through a unique, neuron-specific receptor complex comprising the IFN- γ receptor and the GluR1 AMPA receptor subunit to increase Ca^{2+} influx and NO production (Fig 4.7). Beyond alterations of neuron electrophysiological properties, IFN- γ could directly and rapidly influence monoamine neurotransmitter reuptake via non-canonical (and non-genomic) activation of intracellular kinases, most notably PKA, PI3K, PKC, and ERK1/2 (Fan et al., 1988; Liu et al., 2004; Platanius, 2005; O'Donnell et al., 2015). Indeed, all of these kinases, and in

particular the latter two, have been linked to diminished neuronal DAT, NET and SERT activity and expression (Morón et al., 2003; Ramamoorthy et al., 2011) (Fig. 4.7). Interestingly, since PKA has also been shown to phosphorylate the PD-related multi-domain protein LRRK2 at Ser935, which some groups have taken to indicate increased LRRK2 kinase activity (but which recent evidence suggests may actually foretell the opposite: Muda et al., 2014), it is possible that IFN- γ could be acting through PKA to perturb LRRK2 kinase-dependent functions in the presynaptic compartment (Belluzzi et al., 2016).

Another fascinating possibility is that IFN- γ could be acting in axonal processes to regulate rapid protein synthesis and, hence, synaptic activity (e.g., β -catenin) (Perry and Fainzilber, 2013; Taylor et al., 2013); potentially this could involve IFN- γ signalling through a non-canonical PI3K/Akt/mTOR/p70S6K pathway (Platanias, 2005). Long considered implausible, recent and emerging evidence suggests that local translation of trafficked mRNAs is an important plastic regulatory mechanism in central and peripheral axons, facilitating rapid and spatially appropriate responses to environmental and developmental stimuli (Jung et al., 2012; Perry and Fainzilber, 2013). And while IFN- γ specifically has not yet been shown to activate local translational programs in CNS neurons, other cytokines/growth factors were found to do so (Jung et al., 2012). Moreover, only in February of this year Song et al. (2016) reported that IFN- β , and to a lesser extent IFN- γ , induced local antiviral transcriptional responses in PNS axons.

A final point to consider here is why the female mice in the present study displayed an increased sensitivity to the PVN- and- PFC-targeting effects of IFN- γ (Table 4.1). One possibility is that systemically administered IFN- γ induced a more robust

cytokine/inflammatory response in the female mice, which then reverberated through CNS monoamine circuits. In this regard, Scotland et al. (2011) reported that female rats and mice have an increased number of peritoneal and pleural leukocytes, and that resident macrophages from these animals display an enhanced immunogenicity (increased ROS production, phagocytosis and TLR expression). However, these female-specific immunophenotypic alterations were not associated with heightened cytokine levels/responses (Scotland et al., 2011). Alternatively, our findings could reflect underlying sex differences in the electrophysiological properties of specific neuron types in the PVN and PFC, which presumably become “unmasked” or magnified upon IFN- γ stimulation. In this regard, Dorris et al. (2015) recently showed that striatal medium spiny neurons isolated from prepubertal female mice are more excitable than male neurons, although it would appear that such differences vary drastically according to developmental period and brain region (Willett et al., 2016).

4.6 Conclusion

To summarize, acute systemic IFN- γ (25000 IU) stimulated monoaminergic activity within a number of stressor-sensitive limbic brain regions, including the PVN, CeA and PFC. These effects are largely reminiscent of the monoamine changes induced by other depression-linked inflammatory cytokines in comparable acute exposure experimental paradigms (Dunn et al., 2005a; Anisman, 2009). However, in our hands, IFN- γ did not synergize with an acute restraint stressor to further augment brain monoamine activity. We do, however, report a synergistic effect of IFN- γ and stress on plasma corticosterone levels, suggesting that the cytokine can sensitize HPA axis responses to psychological stress. Overall, the pattern of HPA axis and brain regional

monoamine variations induced by IFN- γ is consistent with a role for the cytokine in the regulation of emotional and cognitive processes and complex behavioural states.

Table 4.1. Summary of significant experimental findings

Experimental endpoint	ANOVA main effects and interactions						
	Main			Interaction			
	Sex	Injection	Stress	Sex × Inj	Sex × Str	Inj × Str	Sex × Inj × Str
Plasma corticosterone	×		×			×	
PVN							
MHPG			×				
5-HT		×					
HVA				×			
LC							
MHPG			×				
CeA							
MHPG	×		×				
5-HT		×					
5-HIAA		×					
DA	×	×					
DOPAC	×	×					
HVA		×					
Dorsal HC							
MHPG		×			×		
PFC							
MHPG		×	×				
5-HIAA				×			
DOPAC							×
HVA				×			

Data were analyzed by ANOVA followed by Tukey-Kramer post-hoc tests. Results were considered significant at the $\alpha = .05$ level. Blank cells represent either non-significant ANOVA main or interaction effects, significant ANOVA interactions absent statistically significant post-hoc results, or significant lower order main or interaction effects subsumed by higher order interactions.

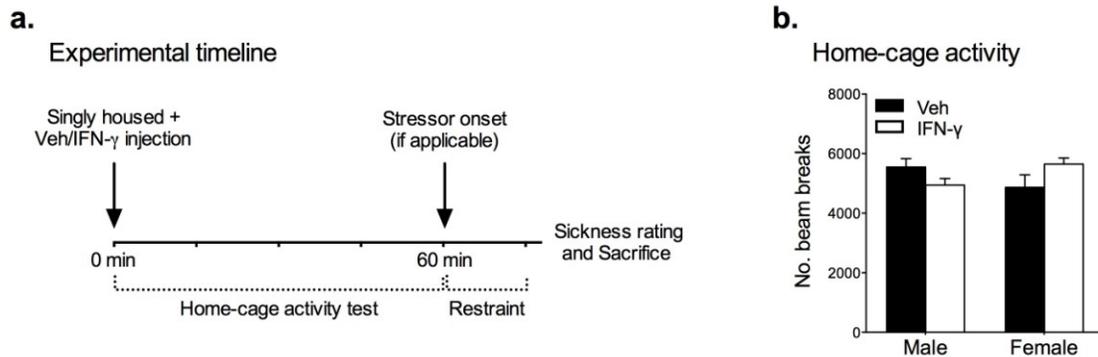


Figure 4.1. Experimental outline and spontaneous home-cage activity. (a) *Experimental timeline*: Commencing at 0830 h and at an interval of every 10 min, mice were individually removed from their grouped enclosures, singly housed in fresh cages and injected intraperitoneally with mouse recombinant IFN- γ (25 000 IU) or vehicle solution. Home-cage activity assessments were made during the subsequent 60 min period, after which a subset of the animals was administered a 15 min restraint stressor. Mice were sacrificed by rapid decapitation 78 min post-injection; i.e., 3 min following termination of the stressor. (b) *Home-cage activity*: Despite a significant Injection \times Stress interaction (two-way ANOVA, $p < .05$, see text), the post-hoc tests did not reveal any statistically significant pairwise differences. Data are shown as mean \pm SEM.

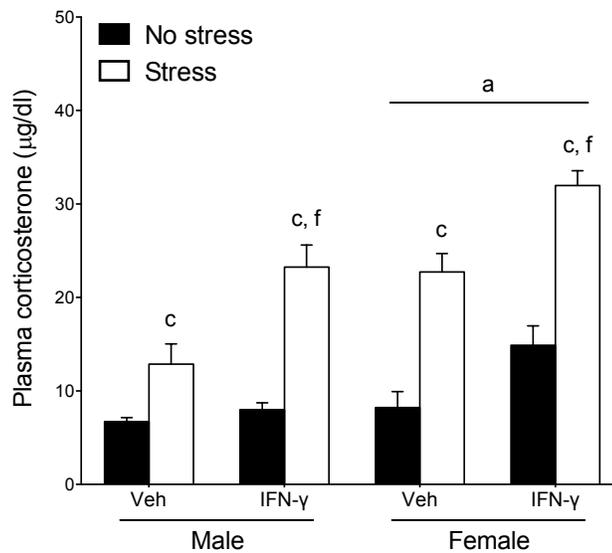


Figure 4.2. Plasma corticosterone concentrations varied as a function of Sex, Injection and Stress. Plasma corticosterone levels were elevated overall in the female mice and in response to the acute restraint stressor. Although IFN- γ did not on its own increase corticosterone concentrations, the cytokine interacted synergistically with the restraint stressor to further augment stress hormone levels. Data are shown as mean \pm SEM. ^a p < .05 relative to the male mice (collapsed across Injection and Stress); ^c p < .05 relative to non-stressed mice (collapsed across Sex and Injection); ^f p < .05 relative to stressed vehicle-treated mice (collapsed across Sex).

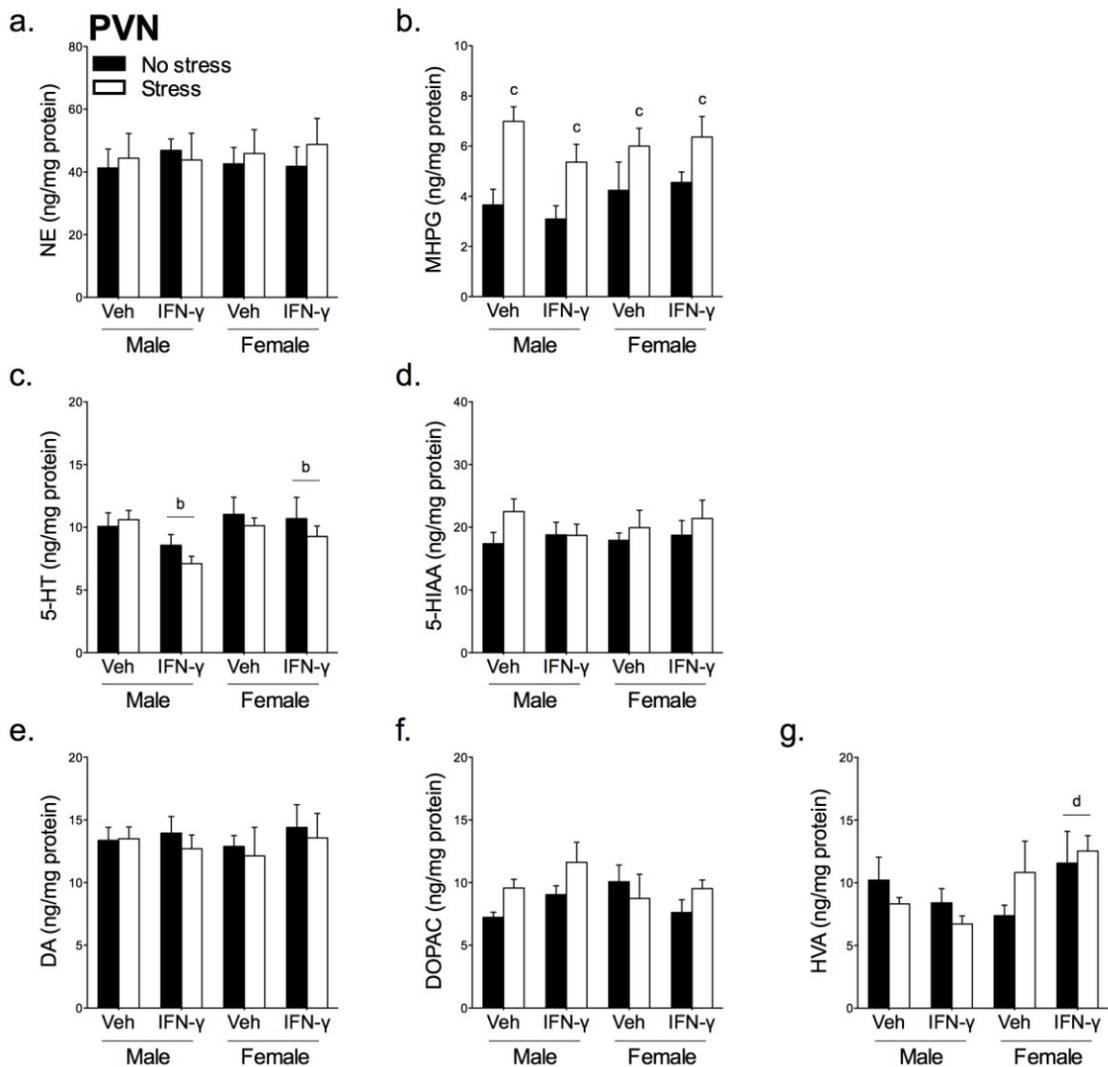


Figure 4.3. IFN- γ and stress influenced PVN monoaminergic activity in a largely sex-independent manner. Within the PVN, MHPG levels were increased overall following exposure to stress (*b*) whereas 5-HT levels were diminished overall in response to IFN- γ treatment (*c*). While the restraint stressor did not influence DA activity in the PVN, IFN- γ had opposing effects on HVA accumulation among the male and female mice (*g*). Data are shown as mean \pm SEM. ^b $p < .05$ relative to vehicle-treated mice (collapsed across Sex and Stress); ^c $p < .05$ relative to non-stressed mice (collapsed across Sex and Injection); ^d $p < .05$ compared to IFN- γ -treated male mice (collapsed across Stress).

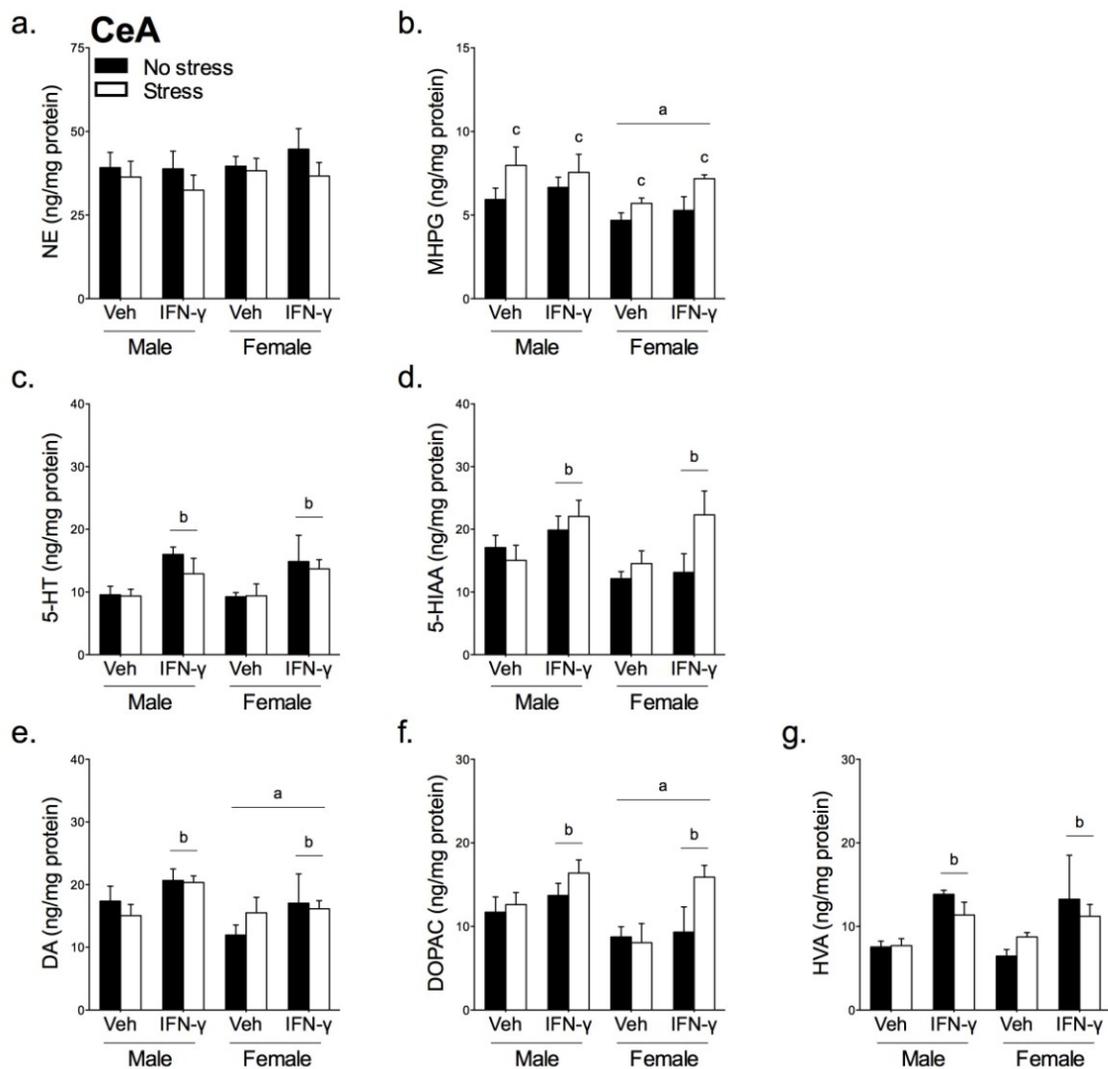


Figure 4.4. Non-interactive effects of Sex, IFN- γ and acute restraint stress on CeA monoamine neurotransmitter activity. The cytokine and stressor treatments independently altered monoaminergic activity in the CeA: whereas IFN- γ increased 5-HT, 5-HIAA, DA, DOPAC, and HVA concentrations (c-g), the restraint stressor enhanced MHPG accumulation. Additionally, CeA levels of MHPG (b), DA (e) and DOPAC (f) were diminished overall in the female mice (compared to the male littermates). Data are shown as mean \pm SEM. ^a $p < .05$ relative to the male mice (collapsed across Injection and Stress); ^b $p < .05$ relative to vehicle-treated mice (collapsed across Sex and Stress); ^c $p < .05$ relative to non-stressed mice (collapsed across Sex and Injection).

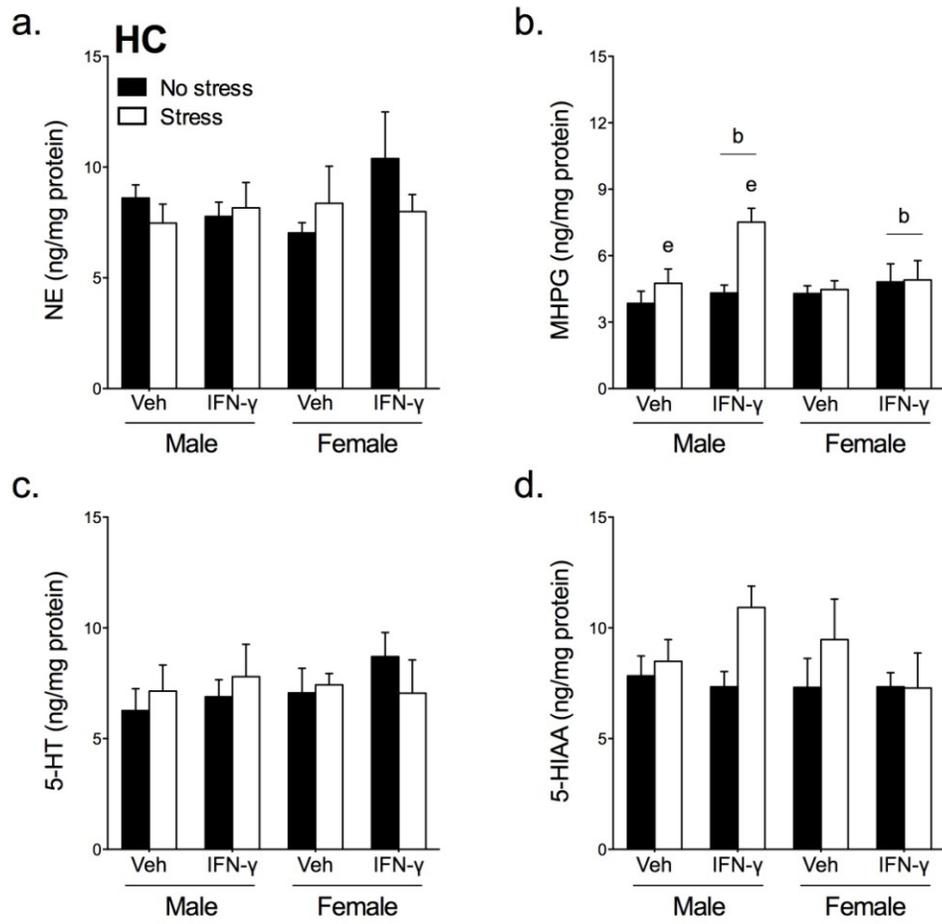


Figure 4.5. Male mice displayed increased sensitivity to the HC noradrenergic effects of acute restraint stress but not IFN- γ . Within the dorsal HC, IFN- γ increased MHPG levels in the male and female mice alike whereas the acute restraint stressor did so only in the former (*b*). Data are shown as mean \pm SEM. ^b*p* < .05 relative to vehicle-treated mice (collapsed across Sex and Stress); ^e*p* < .05 relative to non-stressed male mice (collapsed across Injection).

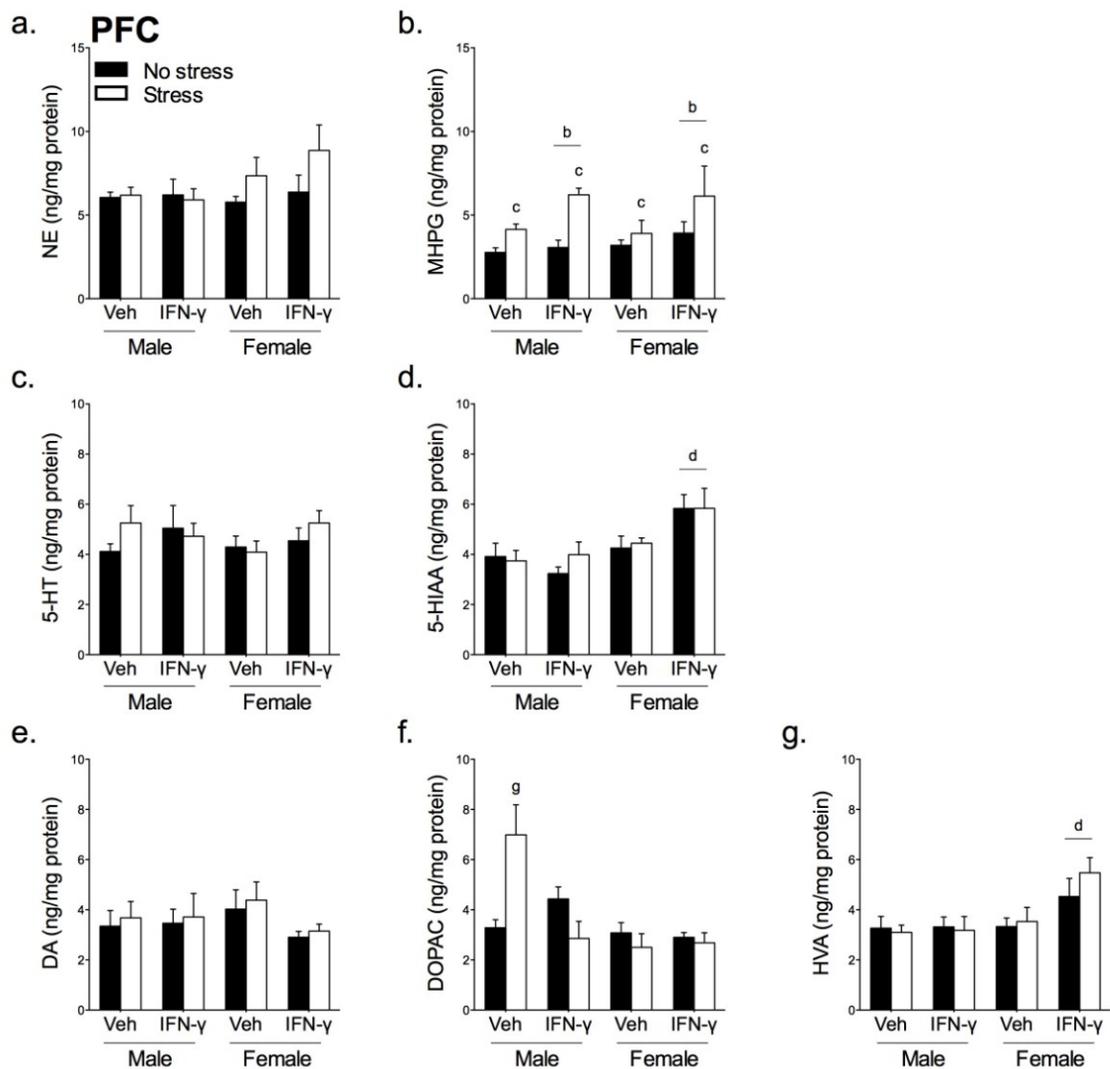
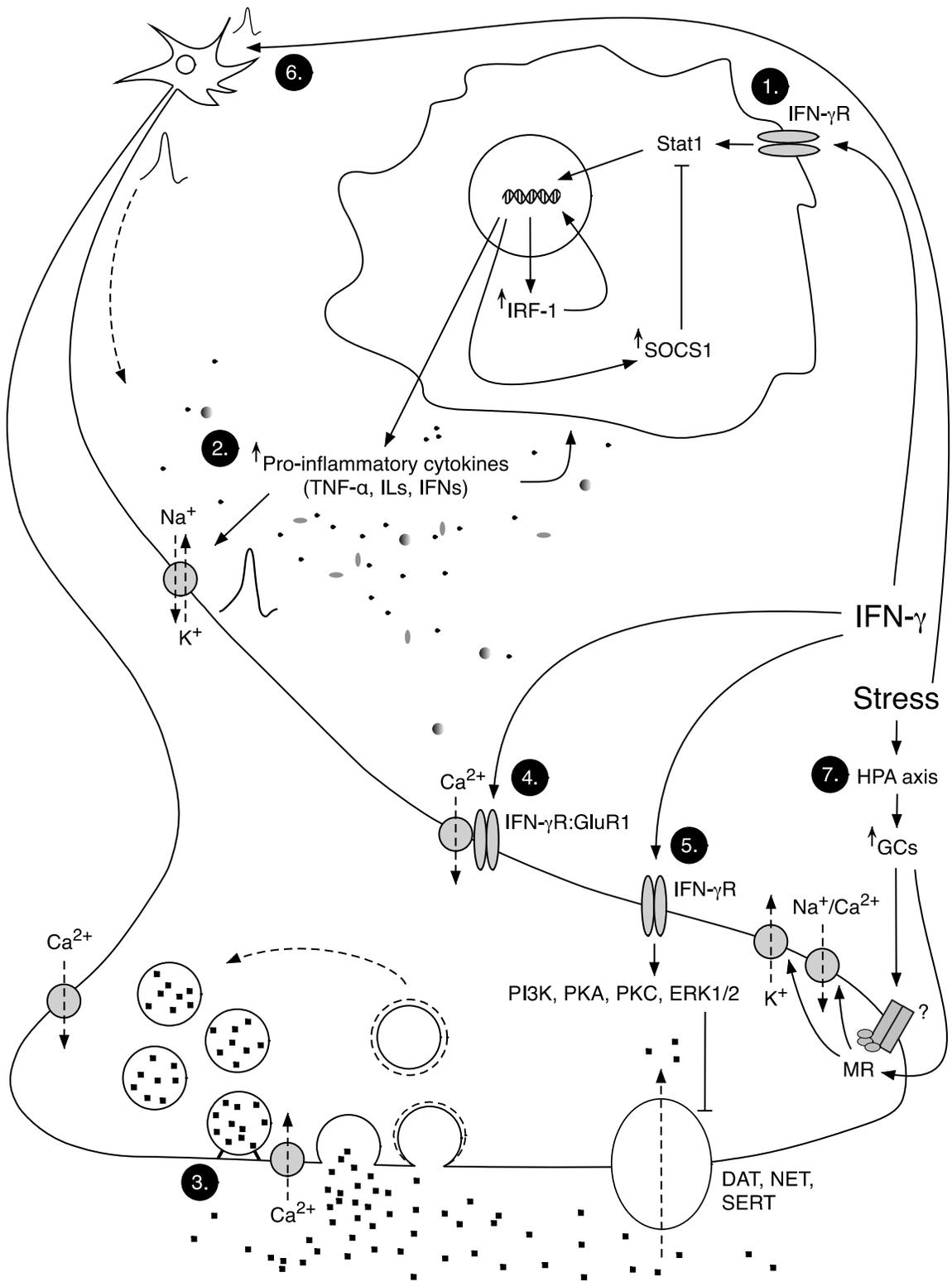


Figure 4.6. Monoaminergic activity within the PFC: individual and combined effects of Sex, IFN- γ and acute restraint stress. Accumulation of the primary NE metabolite MHPG was enhanced overall following exposure to both IFN- γ and acute restraint stress (*b*). The cytokine treatment also elevated PFC levels of 5-HIAA and HVA, but only in the female mice (*d*, *g*). In contrast, the acute restraint stressor augmented PFC DOPAC accumulation in the male mice only, and this effect was curiously abrogated by pre-treatment with IFN- γ (*f*). Data are shown as mean \pm SEM. ^b p < .05 relative to vehicle-treated mice (collapsed across Sex and Stress); ^c p < .05 relative to non-stressed mice (collapsed across Sex and Injection); ^d p < .05 compared to vehicle-treated female mice (collapsed across Stress); ^e p < .05 compared to non-stressed vehicle-treated male mice.

Figure 4.7. Simplified overview of the cellular and molecular signalling mechanisms underlying the rapid brain regional monoaminergic effects induced by systemic IFN- γ . (1) Systemically administered IFN- γ signals through the canonical Jak/Stat pathway in circulating and brain-resident immune cells to increase the synthesis and release of inflammatory/oxidative factors, including pro-inflammatory cytokines, prostaglandins and ROS (the latter two are not shown). A number of IFN- γ regulated genes are transcription factors, e.g., IFN response factor-1 (IRF-1), which induce a further round of inflammatory transcription. IFN- γ is subject to negative feedback regulation by suppressor of cytokine signalling-1 (SOCS1). (2) Secondly released cytokines and other inflammatory/oxidative factors act directly on neurons to alter ionic conductances and the mobilization of prostanoids and other second messenger cascades, with the collective effect being that of neuronal excitation/action potential generation and propagation. (3) This leads to the calcium (Ca^{2+})-triggered exocytotic release of monoamine neurotransmitters into the synaptic cleft and the subsequent binding of transmitter molecules to post-synaptic receptors and pre-synaptic autoreceptors (ligand binding is not shown here). (4) IFN- γ can also act directly on neurons (i.e., without first signalling through immune cells) to cause membrane depolarization and excitation; e.g., IFN- γ -induced Ca^{2+} influx mediated by a neuron-specific IFN- γ R:GluR1 complex. (5) Additionally, IFN- γ can inhibit the monoamine reuptake transporters DAT, SERT and NET via non-genomic pathways involving the Jak-mediated phosphorylation of the kinases PI3K, PKA, PKC, and ERK1/2. (6) As described in the text, a subset of the animals also received a 15 min restraint stressor. As mice were sacrificed 18 min post-initiation of stress, we were not modelling genomic actions of glucocorticoids (GC) or other stress hormones (CRH, vasopressin). Instead, in our acute experimental paradigm, restraint stress is expected to cause neuronal excitation and monoamine release. (7) Additionally, stress-induced glucocorticoids may be inducing rapid non-genomic neuronal effects through cytosolic mineralocorticoid receptors (MRs) and/or as yet unidentified steroid hormone membrane receptors; shown here is a prospective GC-mediated stimulation of G-protein gated sodium (Na^+)/ Ca^{2+} (excitatory) and inwardly rectifying potassium (K^+) (inhibitory) channels. Rapid non-genomic GC signalling can also influence monoamine neurotransmission by increasing glutamatergic (excitatory) or GABAergic (inhibitory) inputs (e.g., in the case of the latter, via GABA/glycine receptors on dendrites and somata); this is not depicted here (see text). Solid arrows (solid arrowhead) indicate positive/stimulatory relationships (activation of receptors, signalling factors/pathways, transcription). Solid lines with blunted ends indicate negative/inhibitory relationships. Broken arrows (solid arrowhead) indicate movement across time and/or space. Small upwards arrows (swept arrowhead) indicate increased protein levels. Grey-filled circles = voltage-gated or G-protein-gated ion channels; grey-filled ellipses = IFN- γ receptor or as labelled; grey-filled rectangles with question mark = possible G-protein-coupled GC receptor.



5 Chapter. Effects of acute systemic LPS or IFN- γ in LRRK2 G2019S transgenic mice: circulating corticosterone and cytokines, brain regional monoamine activity, and behaviour

5.1 Bridging statement

Accumulating evidence suggests that IFN- γ contributes to depression and related stress-associated conditions (O'Connor et al., 2009b; Maes et al., 2011b). Acting mainly through IDO-dependent routes, IFN- γ is considered to reduce the synaptic availability of monoamines and thus contribute to affective states (Miller et al., 2009; §1.3.3). In Chapters 2 and 3 we describe experiments indirectly implicating IFN- γ in some of the depression-like effects of chronic stress and in Chapter 4 we provide evidence directly linking systemically administered IFN- γ to the stimulation of brain regional monoamine activity and the sensitization of HPA responses to acute stress. Presumably such mechanisms will contribute to allostatic load and, eventually, allostatic overload and the development of psychological pathology (McEwen, 2000).

In the next chapter we use this acute IFN- γ exposure model to examine the possibility that the recently described IFN- γ -LRRK2 signal transduction pathway may contribute to monoaminergic and HPA axis dysfunction and, hence, depression and other non-motor symptoms in PD. Indeed, while the precise cell-specific roles of LRRK2 have yet to be conclusively determined, mounting evidence indicates that LRRK2 acts in immune cells to positively regulate inflammation (e.g., phagocyte induction, pro-inflammatory cytokine elaboration) (Russo et al., 2014) and in neurons to accelerate synaptic vesicle exo-endocytosis (Belluzzi et al., 2016).

While IFN- γ is capable of upregulating LRRK2 in immune cells and total soluble LRRK2 levels may induce toxicity via an as yet unknown mechanism (Skibinski et al., 2014), a prospective IFN- γ -LRRK2 signalling contribution to non-motor symptoms in PD may be most relevant in the case of toxic gain-of-function LRRK2 variants. In

particular, recent evidence suggests that the common PD-linked LRRK2 G2019S mutation is associated with a heightened risk of depression (Shanker et al., 2011) as well as impaired hippocampal neurogenesis and perturbations of glutamatergic and dopaminergic neurotransmission (Winner et al., 2011; Beccano-Kelly et al., 2014; Sweet et al., 2015). Thus, in the following chapter we describe a “proof-of-concept” study aimed at assessing whether mice overexpressing LRRK2 G2019S will display altered monoaminergic, plasma corticosterone and circulating cytokine responses to IFN- γ and LPS (which can likewise upregulate and phospho-activate LRRK2: Kim et al., 2012). Specifically, we posit that IFN- γ /LPS signals through mutant LRRK2 in immune cells to induce the exaggerated synthesis and release of pro-inflammatory cytokines, which, in turn, act on neurons to perturb monoamine activity via the routes described previously (Chapter 4). Notably, since LRRK2 also plays an important role at presynaptic terminals, probably acting in a kinase-dependent manner to accelerate synaptic vesicle exo-endocytosis (Belluzzi et al., 2016), we suggest that neuronally expressed kinase-hyperactive LRRK2 G2019S will only serve to amplify the monoaminergic response to IFN- γ /LPS.

5.2 Abstract

Mutations in LRRK2 are implicated in familial and sporadic PD; the most common of these is the gain-of-function LRRK2 G2019S variant. Accumulating evidence suggests that LRRK2 G2019S may facilitate inflammatory mechanisms of neurodegeneration as well as the development of non-motor symptoms such as depression and cognitive impairment. Inasmuch as immune-inflammatory processes are also implicated in depression, it is possible that LRRK2 G2019S could promote depressive-like pathology, at least in part, by potentiating inflammation-driven changes in brain neurochemistry. Here we take the first steps towards testing this hypothesis by: 1) characterizing behavioural and physiological aspects of the phenotype in mice genetically engineered to overexpress LRRK2 G2019S; and 2) ascertaining whether these mutant mice display exaggerated neuroendocrine-immune and central monoaminergic responses to acute systemic IFN- γ or LPS. As expected, LPS and, to a lesser extent, IFN- γ induced circulating cytokine, corticosterone and brain regional monoamine changes (LC, PVN, dorsal and ventral HC, and PFC); the endotoxin also markedly influenced home-cage activity and sickness behaviour. Yet, while the LRRK2 G2019S transgenic mice displayed heightened monoaminergic activity within several of these cognitive and affective brain areas, overexpression of the mutant transgene largely failed to modify the effects of LPS or IFN- γ . There were, however, two notable exceptions in the monoamine data: 1) LRRK2 G2019S overexpression facilitated the IFN- γ -induced increase in ventral HC NE utilization but 2) appeared to blunt the prefrontal dopaminergic response to LPS. The present findings are among the first to implicate LRRK2 G2019S in the modulation of monoaminergic activity in stressor-sensitive extra-nigrostriatal brain regions, both in

the basal state and in response to acute systemic immune challenge. These data could have implications for our understanding of the mechanisms underlying depression and other non-motor symptoms in both LRRK2-related and sporadic forms of PD.

5.3 Introduction

Motor function deficits, including bradykinesia, progressive tremor and rigidity, are among the most recognizable clinical features of PD (Schneider and Obeso, 2015). However, a large proportion of PD patients will also experience neuropsychiatric problems, including anxiety, depression and cognitive disturbances (Chen et al., 2015). These co-morbid or non-motor symptoms, some of which may actually precede the onset of motor decline (Chen et al., 2015; Gustafsson et al., 2015), are major determinants of poor quality of life among PD patients (Duncan et al., 2014). While PD-associated depression can have several different and potentially overlapping causes, mounting evidence suggests that monoaminergic dysfunction in, and even degeneration of, various interconnected cortical, limbic and brainstem regions likely plays an important role (Summerfield et al., 2005; Buddhala et al., 2015; Engeln et al., 2015).

Mutations in the LRRK2 gene, which encodes a large multimeric protein whose enzymatic core comprises Roc-COR tandem GTPase and serine/threonine kinase domains, have been linked to both familial and sporadic forms of PD (Cookson, 2015; Wallings et al., 2015). The most frequent of these mutations is the gain-of-function G2019S substitution, which causes an increased LRRK2 kinase activity (West et al., 2005; Smith et al., 2006). Although LRRK2-associated PD has generally been considered to resemble idiopathic disease (e.g., Healy et al., 2008), there is evidence to suggest that PD patients harbouring the G2019S mutation may actually have more frequent, severe and/or earlier-occurring neuropsychiatric symptoms (Goldwurm et al., 2006; Marongiu et al., 2006; Belarbi et al., 2010; Johansen et al., 2011; Marras et al., 2011; Mirelman et al., 2015; Thaler et al., 2016). Moreover, non-manifesting carriers of the LRRK2 G2019S

mutation displayed altered brain regional activity and inter-regional functional connectivity (van Nuenen et al., 2012; Thaler et al., 2013; Helmich et al., 2015), coupled with lowered cognitive performance (Thaler et al., 2012) and a markedly increased risk of premorbid affective disorders (Shanker et al., 2011).

Consistent with a possible role for LRRK2 in emotional and cognitive functions, the PD-linked kinase is expressed in neurons over a wide range of brain regions, including not only the SNc and dorsal striatum but also the HC, PFC, hypothalamus, and LC (Melrose et al., 2006; Simón-Sánchez et al., 2006; Taymans et al., 2006; Higashi et al., 2007). Moreover, recent reports indicate that LRRK2 G2019S overexpressing mice display impaired hippocampal neurogenesis and perturbations of regional glutamatergic neurotransmission (Winner et al., 2011; Beccano-Kelly et al., 2014; Sweet et al., 2015), together with age-dependent cognitive and affective behavioural deficits (Melrose et al., 2010; Beccano-Kelly et al., 2015; Volta et al., 2015). In contrast, LRRK2 inhibition enhances hippocampal neurogenesis and neurite outgrowth (Paus et al., 2013) and causes relatively modest alterations to synaptic vesicle exo-endocytosis (Beccano-Kelly et al., 2014); this latter effect is consistent with a loss of kinase-dependent facilitation of synaptic vesicle recycling (Belluzzi et al., 2016).

LRRK2 is also highly expressed in circulating and tissue immune cells, including monocytes, B lymphocytes, macrophages, microglia, and astrocytes (Miklossy et al., 2006; Hakimi et al., 2011; Kubo et al., 2016), and its expression is markedly inducible by IFN- γ and LPS (Thévenet et al., 2011; Gillardon et al., 2012; Moehle et al., 2012). Perhaps not surprisingly, then, mounting evidence indicates that LRRK2, in both its native and mutated forms, contributes to the positive regulation of inflammation (Russo

et al., 2014, 2015; Puccini et al., 2015). For instance, Dzamko et al. (2016) recently showed that asymptomatic LRRK2 G2019S mutation carriers exhibit signs of increased peripheral inflammation (including elevated levels of IL-1 β) while Moehle et al. (2015) reported that rats overexpressing the mutant transgene display exaggerated neuroinflammatory responses and neurodegeneration following intra-nigral administration of LPS.

As immuno-inflammatory processes are also implicated in depression (Miller et al., 2009), it is possible that LRRK2 G2019S could promote non-motor PD symptoms, at least in part, via a facilitation or potentiation of inflammation-driven changes in brain monoamine neurotransmission and neuroendocrine function. Here we take the first steps towards testing this hypothesis by: 1) characterizing select behavioural (home-cage activity, sickness behaviour) and physiological aspects (circulating corticosterone and cytokines, brain regional monoamine activity) of the phenotype in LRRK2 G2019S transgenic mice (GS-Tg); and 2) ascertaining whether these GS-Tg animals will, as hypothesized, display exaggerated neuroendocrine-immune and central monoaminergic responses to acute systemic IFN- γ or LPS.

5.4 Methods

5.4.1 Animals

Development of the bacterial artificial chromosome (BAC) GS-Tg mouse, which overexpresses the mouse LRRK2 G2019S mutant protein ~ 6-8 fold greater than normal, has been described elsewhere (Li et al., 2010b). These mice show an age-dependent dysfunction of the nigrostriatal DA system, including reduced striatal DA levels, in the absence of a frank loss of SNc dopaminergic neurons or striatal DA terminals (Li et al.,

2010b). A total of 42 male GS-Tg ($n = 21$) and non-transgenic (non-Tg, $n = 21$) littermate mice, aged 5-8 weeks, were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were shipped to our facility in same-sex and same-litter groupings of at least 2 per container. Upon arrival, the mice were housed in microisolator cages in same-litter groups of 2 or 3 and maintained on a 12-h light/dark cycle with lights on at 0800 h. A diet of standard laboratory mouse chow (Harlan Laboratories, WI) and water was provided ad libitum and room temperature maintained at ~ 21 °C. All experimental procedures were approved by the Carleton University Committee for Animal Care and complied with the guidelines set out by the Canadian Council for the Use and Care of Animals in Research. Animals were 12-15 weeks old at the time of experimentation.

5.4.2 Procedure

Mice were randomly assigned to one of six experimental conditions ($n = 7$), as provided for by our 2 (Genotype: non-Tg, GS-Tg) \times 3 (Treatment: vehicle, IFN- γ , LPS) factorial design. All mice were acclimated to the home-cage activity testing room for ~ 16 h. Commencing at 0830 h on the day of experimentation and at 10 min intervals, mice were individually removed from their grouped enclosures and singly housed in fresh cages; as before, food and water was provided ad libitum. Immediately upon cage transfer, mice were administered a single intraperitoneal injection, in a volume of 0.3 ml, of a) a PBS solution containing 0.1% BSA (Sigma Aldrich) (i.e., the vehicle); b) recombinant murine IFN- γ (25000 IU, R&D Systems; reconstituted in 0.1% BSA); or c) LPS (10 μ g, L8274: E. coli serotype O26:B6, Sigma Aldrich; in 0.1% BSA). Drug doses were chosen based on earlier reports indicating that systemic administration of IFN- γ or LPS at these or similar doses influenced stress hormone levels, central monoamine

activity and behaviour (Crnic and Segall, 1992; Lacosta et al., 1999; Gibb et al., 2008). Over the ensuing 90-min period, spontaneous locomotor activity was assessed with a Micromax infrared beam-break apparatus positioned exterior to the home-cage (AccuScan Instruments, Columbus, OH). Sickness ratings were recorded for each mouse at 75 and 90 min post-injection. Per the method of Anisman et al. (2008c), sickness behaviours were scored on a 3-point scale (where 0 = no symptom, 1 = one symptom present, 2 = two symptoms presents, 3 = three or more symptoms) with respect to curled body posture, ptosis (drooping eyelids), piloerection, lethargy, and overall non-responsiveness. At 90-min post-injection, mice were sacrificed by rapid decapitation and blood and brain tissue collected for later biochemical analysis. In order to minimize the effects of diurnal variations, experiments were carried out between the hours of 08:30 and 12:30.

5.4.3 Brain dissection technique and HPLC determination of central amine and metabolite concentrations

Brains were excised and sectioned and tissue samples maintained according to the methods provided in §2.3.4. For this study we collected and analyzed the PVN, LC, ventral HC (containing the ventral domain of the CA1 field), dorsal HC, and PFC. Regional brain levels of NE, 5-HT and DA, and their respective metabolites MHPG, 5-HIAA, and DOPAC and HVA, were determined by HPLC as described elsewhere (§2.3.7).

5.4.4 Plasma corticosterone and cytokine determinations

Corticosterone determinations were carried out as previously described (§2.3.5). The plasma levels of 5 different cytokines, namely MCP-1/CCL2, TNF- α , IL-1 β , IL-6,

and IL-10, were determined by multiplex analysis using the Luminex 100 suspension-based bead array system (Luminex Corp., Austin, TX) and a custom multiple cytokine detection kit (MILLIPLEX MAP Mouse Cytokine/Chemokine Kit, Millipore, Cat. #MPXMCYTO-70K). The assay was performed according to the kit manufacturer's instructions (www.millipore.com/userguides) and, unless otherwise indicated, all reagents were provided in the multiplex kit. Samples were diluted by a factor of 2. Measurements falling below or above the assay's working range for a given analyte were assigned a value of one-half the lower limit of quantitation or twice the upper limit of quantitation, respectively. Six samples from each of the 6 treatment groups were run in duplicate and the data averaged prior to statistical analysis; the remaining samples (1 per group) were analyzed in singlicate.

5.4.5 Statistical analyses

The monoamine, cytokine and corticosterone data were analysed by 2×3 ANOVAs followed where appropriate by Tukey-Kramer post-hoc tests; i.e., for Genotype × Treatment interactions and Treatment main effects absent a significant disordinal interaction ($\alpha = 0.05$). Plasma cytokine concentrations were transformed by natural logarithm (ln) to improve normality and homoscedasticity. Two-factor repeated measures ANOVAs were used to analyze the sickness behaviour and home-cage activity data, the latter having first been divided into 3 consecutive 30 min epochs. A few of the PVN and LC tissue samples were lost during the course of tissue dissection or monoamine determination. Data points exceeding 2.5 standard deviations from the mean were considered outlier values and omitted from the analyses. Data were evaluated using

a StatView (version 6.0) statistical software package and visualized with GraphPad Prism 6 (La Jolla, CA).

5.5 Results

5.5.1 LRRK2 G2019S overexpression did not modify LPS-induced changes in behaviour and plasma corticosterone levels

The repeated measures ANOVA for home-cage locomotor activity yielded a significant main effect of Treatment ($F_{2, 36} = 21.93, p < .0001, \eta^2 = .54$), as well as a significant Treatment \times Time interaction ($F_{4, 72} = 13.77, p < .0001, \eta^2 = .12$). The follow-up tests revealed that LPS progressively suppressed home-cage activity in non-Tg and GS-Tg mice alike ($p < .05$ relative to mice treated with vehicle or IFN- γ). Although IFN- γ also appeared to suppress home-cage activity in the GS-Tg animals, this effect was not significant ($p > .05$); Figure 5.1a presents the home-cage activity data collapsed across Time (i.e., the 3 consecutive 30 min epochs). As for sickness behaviour, the repeated measures ANOVA detected a significant main effect of Treatment ($F_{2, 36} = 379.05, p < 0.0001, \eta^2 = .95$). As shown in Figure 5.1b, which also presents time-averaged data (i.e., across the 75 and 90 min post-injection measurements), this was attributable to the robust genotype-independent sickness-inducing effect of LPS ($p < .05$ relative to mice treated with vehicle or IFN- γ).

Paralleling the behavioural findings, plasma corticosterone concentrations were significantly increased among the LPS-treated animals irrespective of LRRK2 G2019S overexpression (main effect of Treatment: $F_{2, 36} = 72.68, p < .0001, \eta^2 = .79$; Tukey-Kramer HSD: $p < .05$ relative to vehicle or IFN- γ) (Fig. 5.1c). IFN- γ did not significantly impact corticosterone levels ($p > .05$); however, as can be seen in Figure 5.1c, levels of

the stress hormone were increased more than 2-fold among the GS-Tg mice compared to the vehicle-treated GS-Tg controls.

5.5.2 LRRK2 G2019S overexpression did not modify the plasma cytokine effects of LPS and IFN- γ

The multiple separate ANOVAs revealed a significant main effect of Treatment on the natural log-transformed levels of all 5 of the cytokines assayed: TNF- α ($F_{2, 36} = 223.94, p < .0001, \eta^2 = .92$), MCP-1 ($F_{2, 36} = 188.06, p < .0001, \eta^2 = .91$), IL-6 ($F_{2, 36} = 70.34, p < .0001, \eta^2 = .78$), IL-1 β ($F_{2, 36} = 3.40, p < .05, \eta^2 = .15$), and IL-10 ($F_{2, 36} = 99.53, p < .0001, \eta^2 = .84$). As shown in Figure 5.2a-d and confirmed by the follow-up tests, whereas MCP-1 and IL-6 were increased following treatment with either IFN- γ or LPS ($p < .05$ relative to all other groups), only the endotoxin significantly augmented TNF- α and IL-10 ($p < .05$ relative to all other groups). Although IL-1 β also varied according to a main effect of Treatment, the follow-up tests were not significant (Tukey-Kramer HSD: $p > .05$; Fig. 5.2e).

Notably, even as IFN- γ provoked a significant increase in the levels of MCP-1 and IL-6, in both cases the effect of LPS was larger by several orders of magnitude. In fact, in the case of IL-6, all but one of the LPS-treated specimens exceeded the assay's dynamic range; these samples were assigned values twice the assay's upper limit of quantitation for IL-6 (8425.99 pg/ml). Thus, while the analyses showed that LPS (and IFN- γ) increased IL-6 levels among both non-Tg and GS-Tg mice, we cannot rule out the possibility that the mutant transgene may have influenced the magnitude of this change.

5.5.3 LPS-induced PVN and LC monoaminergic changes were independent of LRRK2 G2019S overexpression

Table 5.1 summarizes the brain regional monoamine and metabolite concentration data as a function of LRRK2 G2019S overexpression and LPS or IFN- γ treatment. Within the PVN and LC, endotoxin but not IFN- γ provoked a number of changes in monoamine neurotransmitter concentration and utilization, all of which were independent of LRRK2 G2019S overexpression. Specifically, the multiple separate ANOVAs revealed a significant main effect of Treatment for PVN DA ($F_{2, 29} = 4.93, p < .05, \eta^2 = .23$); PVN 5-HIAA ($F_{2, 29} = 4.23, p < .05, \eta^2 = .22$); LC NE ($F_{2, 35} = 5.15, p < .05, \eta^2 = .19$); and LC MHPG ($F_{2, 35} = 4.60, p < .05, \eta^2 = .19$). The follow-up tests indicated that LPS diminished DA and NE concentrations within the PVN and LC, respectively ($p < .05$ relative to vehicle-treated mice), whilst significantly enhancing 5-HIAA and MHPG accumulation within these respective brain regions ($p < .05$ relative to vehicle-treated mice and, in the former case, IFN- γ -treated animals also). As noted previously, one LC and several PVN specimens were lost during the course of tissue dissection or monoamine determination (with an n of 5, the IFN- γ /GS-Tg condition was the group most affected); this had the effect of reducing statistical power. These data should therefore be interpreted with a measure of caution and replication studies are certainly warranted. In particular, there were several instances where monoamine or metabolite levels appeared to be altered specifically or preferentially in the IFN- γ -treated GS-Tg mice (i.e., consistent with a Genotype \times Treatment interaction): PVN DA ($F_{2, 29} = 2.00, p = .15, \eta^2 = .09$), PVN MHPG ($F_{2, 29} = .44, p = .65, \eta^2 = .07$) and LC MHPG ($F_{2, 35} = 1.20, p = .31, \eta^2 = .05$) (Table 5.1).

5.5.4 Limited evidence of altered HC and PFC monoaminergic responses to LPS and IFN- γ among mice overexpressing LRRK2 G2019S

As depicted in Figure 5.3, LRRK2 G2019S overexpression markedly influenced NE and 5-HT activity in the dorsal HC. Indeed, the multiple separate ANOVAs revealed that the GS-Tg mice, irrespective of LPS or IFN- γ treatment, had significantly higher levels of NE, 5-HT and 5-HIAA relative to their non-Tg littermates (main effect of Genotype: $F_{s1, 36} = 16.16, 8.57$ and 15.72 ; $p < .001, .01$ and $.001$; $\eta^2 = .23, .17$ and $.26$, respectively). Additionally, while none of 5-HT, 5-HIAA and MHPG was significantly affected by treatment with LPS or IFN- γ ($F_s < 3, p > .05$), the cytokine did augment dorsal HC NE concentrations among mice of either genotype (main effect of Treatment: $F_{2, 36} = 7.78, p < .05, \eta^2 = .22$; Tukey Kramer HSD: $p < .05$ relative to vehicle) (Fig. 5.3a).

Within the ventral HC, which specializes in emotional information processing vs. the mainly cognitive functions performed by the dorsal HC (Fanselow and Dong, 2010), NE concentrations were, once again, increased overall in the GS-Tg mice ($F_{1, 36} = 6.54, p < .05, \eta^2 = .14$) (Fig. 5.4a). As for MHPG levels, the ANOVA yielded significant main effects of Genotype and Treatment ($F_{s1, 36} = 11.80$ and $7.36, p < .01, \eta^2 = .15$ and $.21$, respectively), as well as a significant Genotype \times Treatment interaction ($F_{2, 36} = 8.19, p < .01, \eta^2 = .21$). Visual inspection of the data suggested that LPS caused an increase of ventral HC MHPG among mice of either genotype, whereas IFN- γ only raised metabolite levels in the GS-Tg animals (Fig. 5.4b); this was confirmed by the main effect contrasts ($p < .05$ relative to vehicle-treated mice) and multiple pairwise comparisons ($p < .05$ relative to vehicle-treated mutant mice), respectively. In regards to 5-HT activity in the ventral HC, while levels of the parent amine were unchanged between groups ($F_s < 1, p > .05$) (Fig. 5.4c), 5-HIAA concentrations varied as a function of the significant

interaction between Genotype and Treatment ($F_{2, 36} = 4.52, p < .05, \eta^2 = .19$). While the Tukey-Kramer multiple comparisons procedure failed to reveal any statistically significant differences, a less conservative simple main effects approach (i.e., testing 9 simple main effects whilst maintaining the familywise α level at .05) did uncover a significant difference between vehicle-treated GS-Tg and non-Tg controls ($F_{2, 36} = 4.15, p < .05$) (Fig. 5.4d).

With respect to monoaminergic activity in the PFC, the data clearly point to an activating influence of LPS (but not IFN- γ). Indeed, while there were no significant between-group differences in prefrontal NE levels ($F_s < 2, p > .05$), MHPG concentrations were markedly augmented by LPS (main effect of Treatment: $F_{2, 36} = 5.19, p < .05, \eta^2 = .20$; Tukey-Kramer HSD: $p < .05$ relative to vehicle and IFN- γ) (Fig. 5.5a, b). Similarly, both 5-HT and 5-HIAA were increased overall in response to the endotoxin treatment (main effect of Treatment: $F_{s2, 36} = 3.73$ and $5.55, p < .05$ and $.01, \eta^2 = .19$ and $.22$, respectively; Tukey-Kramer HSD: $p < .05$ relative to vehicle) (Fig. 5.5c, d). The pattern of dopaminergic changes within the PFC was a little more complex: while parent amine levels did not differ across groups ($F_s < 2, p > .05$) (Fig. 5.5e), concentrations of DOPAC varied according to the significant interaction between Genotype and Treatment ($F_{2, 36} = 5.85, p < .01, \eta^2 = .22$). The post hoc tests revealed that LPS increased PFC DOPAC levels in the non-Tg animals ($p < .05$ relative to non-Tg controls) and that LRRK2 G2019S overexpression abrogated this effect (Fig. 5.5f). Prefrontal HVA concentrations were also elevated among the LPS-treated animals, but in this case the ANOVA did not reveal any further modulation by LRRK2 G2019S overexpression (main effect of Treatment: $F_{2, 36} = 4.77, p < .05, \eta^2 = .17$) (Fig. 5.5g). Levels of HVA

were, however, significantly higher overall among the GS-Tg mutants (main effect of Genotype: $F_{2, 36} = 8.32, p < .01, \eta^2 = .15$).

Thus, aside from blunting the LPS-induced rise in DOPAC levels, LRRK2 G2019S overexpression appears to have had little modulatory influence on prefrontal monoamine changes induced by the endotoxin treatment. However, closer inspection of the data reveals a trend towards exaggerated LPS-induced PFC effects in the GS-Tg mice more generally. In particular, the LPS-induced enhancement of MHPG and HVA accumulation appears to have been far more pronounced in the GS-Tg mice than in the non-Tg animals (Fig. 5.5b, g), and indeed the effects were 3-5× larger in the former group: ~ 75% vs. ~ 15% increase in MHPG and ~ 100% vs. ~ 42% increase in HVA, relative to same-genotype controls. And from a statistical standpoint, while the ANOVA for neither MHPG nor HVA yielded a significant Genotype × Treatment interaction ($F_{s_{2, 36}} = 2.82$ and $1.57; p < .15$ and $< .23; \eta^2 = .08$ and $.055$, respectively), in both cases the interaction term accounted for a considerable proportion of the total variance in the model (~ 5-8%, consistent with a small-to-medium sized effect). A similar logic can be seen to apply to prefrontal 5-HT levels; however, this pattern was absent if not slightly reversed with respect to 5-HIAA levels. It will be interesting to see if these effects hold up under larger-scale replication, as they appear to hint at a brain-region specific neurotransmitter-modulatory role for LRRK2 G2019S (which is consistent with published evidence: Sweet et al., 2015).

5.6 Discussion

5.6.1 Influence of LRRK2 G2019S overexpression on the behavioural and physiological effects of LPS and IFN- γ : an examination of potential mechanisms

As summarized in Table 5.2, LPS (10 μ g) elicited profound sickness behaviour and home-cage hypolocomotion, markedly increased circulating corticosterone and cytokine levels, and stimulated monoamine activity in several stressor-sensitive brain regions. In contrast, IFN- γ (25000 IU) failed to significantly influence sickness-type behavioural responses or plasma corticosterone levels (though there were trends in the data), and had relatively few cytokine and brain monoaminergic effects. Although the latter results appear to contradict somewhat our findings from Chapter 4, it will be noted that the most pronounced effects of IFN- γ in that study were in the CeA, which in the current study we did not assess. Moreover, among the brain regions common to both studies (PVN, LC, dorsal HC and PFC), the IFN- γ effects reported in Chapter 4 were either specific to females (PVN HVA and PFC 5-HIAA and HVA) or clearly most robust in the IFN- γ -plus-stressor-treated mice (PFC and dorsal HC MHPG); a similar logic applies to the corticosterone data. Thus, the datasets presented in Chapters 4 and 5 are consistent with each other and further highlight the apparent sex-and-brain region-specific nature of IFN- γ 's repertoire of action. Nevertheless, the robust behavioural and physiological response to LPS seen in the current study was largely as expected (Lavicky and Dunn, 1995; Hayley et al., 2008; van Heesch et al., 2014), but what was not was the general *lack* of a modifying influence of LRRK2 G2019S overexpression. Indeed, this was perhaps the most striking finding of the present investigation.

A couple of notable exceptions in the monoamine data do, however, stand out. Specifically, whereas the GS-Tg mice displayed enhanced ventral HC MHPG accumulation following IFN- γ administration, these animals actually showed a blunted LPS-induced rise in PFC DOPAC levels. There were also several non-significant trends

towards exaggerated IFN- γ /LPS-induced monoaminergic responses within the GS-Tg PFC, LC and PVN (Genotype \times Treatment $\eta^2 \geq .05$, Table 5.1). Overall, then, our findings appear to confirm LRRK2 as an in vivo target of IFN- γ and LPS and extend the repertoire of these signalling pathways to include the modulation of monoaminergic activity in select stressor-sensitive brain areas.

Recent and emerging evidence suggests that the common PD-linked LRRK2 mutations G2019S and R1441G promote exaggerated inflammatory responses to immune challenge, including increased cytokine production (Gillardon et al., 2012), potentiated chemotaxis (Moehle et al., 2015), and the enhanced activity of major intracellular inflammatory signalling pathways (e.g., NF- κ B, p38 MAPK, JNK) (Gloeckner et al., 2009; Kim et al., 2012). It is therefore reasonable to suggest that an amplified cytokine response to LPS and IFN- γ may have contributed to the monoamine-modulatory effects of LRRK2 G2019S in the present study (i.e., an “immune cell-centric” mechanism). However, as summarized in Table 5.2 and discussed later in more detail, the GS-Tg and non-Tg mice did not differ with respect to either basal or LPS/IFN- γ -induced circulating cytokine levels. Yet, these data do not necessarily speak to cytokine concentrations in the brain proper (Litteljohn and Hayley, 2012) and we cannot rule out the possibility that overexpression of LRRK2 G2019S may have enhanced or otherwise altered LPS/IFN- γ -induced changes in local brain cytokine networks (Gibb et al., 2011).

Importantly, since LRRK2 is expressed both in immune cells and neurons, it is possible that cytokines are not simply acting on neurons to alter monoaminergic activity but may actually be signalling *through* neuronal LRRK2 to accomplish such ends. In this regard, a growing body of evidence points to a role for LRRK2 in the presynaptic

compartment, where it appears to interact with, and in many cases phosphorylate, various exocytic and endocytic proteins [e.g., synaptic vesicle protein 2A (SV2A), snapin, NSF, endophilin A, clathrin, dynamin-1, Rab5b] (Shin et al., 2008; Matta et al., 2012; Yun et al., 2013; Cirnaru et al., 2014; Parisiadou et al., 2014; Piccoli et al., 2011, 2014; Belluzzi et al., 2016). Although we are nowhere near consensus on the normal synaptic role(s) of LRRK2 and a lot of the evidence appears contradictory, one leading theory is that LRRK2 generally acts to accelerate synaptic vesicle exo-endocytosis (Migheli et al., 2013; Arranz et al., 2015; Belluzzi et al., 2012). For instance, LRRK2 interacts with and likely phosphorylates SV2A (Piccoli et al., 2011, 2014), which should increase the latter's binding to synaptotagmin (the Ca^{2+} sensor for Ca^{2+} -triggered exocytosis) and lead to enhanced neurotransmitter release (Russo et al., 2012). Moreover, Belluzzi et al. (2016) only very recently demonstrated that LRRK2 normally phosphorylates NSF, which increases the rate of soluble NSF attachment protein receptor (SNARE) complex disassembling and ultimately frees SNARE proteins to begin the exocytotic process anew (and/or partake in clathrin-mediated endocytosis: Zhang et al., 2013). Extrapolating these findings to the case of kinase-hyperactive LRRK2 G2019S, one can expect to find abnormally fast synaptic vesicle exo-endocytosis (Belluzzi et al., 2016). Consistently, LRRK2 G2019S has been associated with increased basal and/or evoked glutamatergic and, to a lesser extent, dopaminergic transmission (Zhou et al., 2011; Migheli et al., 2013; Beccano-Kelly et al., 2014; Plowey et al., 2014) whereas LRRK2 silencing tends towards the opposite (Piccoli et al., 2011; Cirnaru et al., 2014).

Thus, even in the absence of an exaggerated cytokine response to LPS/IFN- γ , the GS-Tg mice, by virtue of an enhanced LRRK2 kinase activity, may yet display

exaggerated monoaminergic responses to pro-inflammatory cytokine signals. Viewed in this way, such non-mutually exclusive cell autonomous (“neuron-centric”) and non-cell autonomous (“immune cell-centric”) mechanisms can be seen to share a common physiological outcome: perturbed neurotransmission (Fig. 5.6). Moreover, their concerted action might even be considered to amplify the initial immune signal: firstly by augmenting the release of cytokines from LPS/IFN- γ -stimulated LRRK2 G2019S immune cells and secondly by perturbing LRRK2 kinase-dependent synaptic effects.

5.6.2 Exploring the potential functional significance of altered central monoaminergic responses to IFN- γ and LPS in GS-Tg mice

Whatever the precise cellular and molecular mechanisms, what is important to highlight here is that the ventral HC results, and specifically those data indicating a selective enhancement of MHPG accumulation in the IFN- γ -treated GS-Tg mice, appear to support our position that LRRK2 G2019S can enhance monoaminergic changes induced by an immune stressor. As will be recalled, the ventral HC (or ventral subiculum) specializes in emotional information processing (Fanselow and Dong, 2010) and is recognized to play an important role in anxiety, depression and the antidepressant response (O’Leary and Cryan, 2014). In particular, glutamatergic ventral HC afferents to the reward-related nucleus accumbens can uniquely regulate susceptibility to chronic social defeat stress (which models depressive-like illness in mice), such that enhancing transmission along this pathway is pro-susceptible/pro-depressive whereas reducing it is pro-resilient (Bagot et al., 2015). Although LC noradrenergic signalling to ventral HC neurons can have both inhibitory and excitatory effects, in vivo electrical stimulation of the rat LC activated more than twice as many ventral HC neurons as it inhibited (Lipski

and Grace, 2013). This suggests that the overall effect of LC-ventral HC noradrenergic transmission may be heavily slanted towards activation. As ventral HC MHPG accumulation presumably reflects increased NE release from LC afferents, the present data raise the possibility that IFN- γ could be driving depressive-like behavioural responses via a LRRK2 G2019S-associated potentiation of LC-ventral HC noradrenergic signalling (and the modulation of downstream reward-related neural circuits). However, it ought to be highlighted that this is the only instance where we observed an exacerbation of IFN- γ action in the present study (although though there were clearly trends in the LC and PVN) (Tables 5.1 and 5.2).

If the ventral HC data can be seen to support our general argument that LRRK2 G2019S potentiates the central effects of immune stressors, then the prefrontal DA results, in particular the LRRK2 G2019S-mediated blunting of LPS-induced DOPAC accumulation, seem to do anything but. However, an alternate reading of the data that takes into account changes in both HVA and DOPAC appears to tell a very different story. As will be recalled, not only did prefrontal HVA levels trend towards being enhanced in the LPS-treated GS-Tg mice, but the ratio of HVA-to-DOPAC, which may indicate the extracellular lifetime of DA (as DA is generally metabolized to HVA by COMT in the extracellular space and to DOPAC by MAO upon reuptake into the presynapse) (Bast et al., 2002), was also far greater ($\sim 3 \times$) in these animals. These results raise the possibility that the LPS-treated GS-Tg mice had a higher proportion of extracellular compared to intracellular DA, which is consistent not with a blunted dopaminergic response to LPS but rather an *enhancement* thereof. Interestingly, temporary overexpression of human LRRK2 G2019S in rats was noted to impair striatal

DAT activity (Zhou et al., 2011) and several reports have implicated reduced DAT function in LRRK2-related PD (Adams et al., 2005; Sossi et al., 2010). While DAT expression in the PFC is low (Sesack et al., 1998) and DA clearance in this region appears mainly to be subsumed by the norepinephrine transporter (NET) (Yamamoto and Novotney, 1998; Morón et al., 2002), it is tempting to speculate that our data may reflect a G2019S-associated impairment in PFC DA clearance. Such an effect might actually be consistent with a prospective G2019S-related exacerbation of LPS-induced central inflammatory responses given that cytokines, while capable of increasing the expression and function of SERT and possibly DAT (Felger and Lotrich, 2013), have actually been shown to suppress NET expression in cultured sympathetic neurons (Pellegrino et al., 2011).

5.6.3 LRRK2 G2019S overexpression in 3-to-4 month old male mice: towards tentative monoaminergic and immunological phenotypes

As can be seen in Table 5.2, another main finding of the present study was that LRRK2 G2019S overexpression caused a number of phenotypic variations in brain monoamine neurotransmitter systems (but not plasma corticosterone or cytokines, see below). Strikingly, all such changes were consistent with enhanced central monoaminergic activity in the GS-Tg mice: increased noradrenergic and serotonergic neurotransmission in dorsal and ventral HC, coupled with a heightened dopaminergic drive in the PFC [HVA levels but also (DOPAC+HVA)/DA]. These results are remarkable insofar as they: 1) provisionally implicate LRRK2 G2019S in the regulation of dopaminergic and non-dopaminergic monoamine systems in cognitive and emotional brain areas outside of the nigrostriatal DA pathway (whether during development and

maturation and/or in adulthood); and 2) attest to the manifestation of these changes by early adulthood, i.e., before the end of the 4th month of life. In regards to the first point, there are actually very few *in vivo* studies that have assessed the role of LRRK2 and its mutations in neurons outside of the nigrostriatal DA system (e.g., Sweet et al., 2015), and most of what we know in this regard comes from *in vitro* studies of primary neuronal cultures and/or invertebrate model systems (in particular cortical glutamatergic neurons). A major strength of our study, then, is that by investigating the monoaminergic effects of LRRK2 G2019S in a network of stressor-sensitive extra-nigrostriatal brain areas we can be considered to have potentially shed new light on the neural substrates contributing to depression and other non-motor PD symptoms (Sweet et al., 2015). And with respect to the second point, of the handful of *in vivo* studies that have linked LRRK2 G2019S (over) expression to altered nigrostriatal dopaminergic and, more recently, hippocampal glutamatergic transmission, this has generally been seen in older mice (usually ≥ 12 months) (Li et al., 2010b; Melrose et al., 2010; Chou et al., 2014; Lee et al., 2015; Liu et al., 2015b; Sweet et al., 2015; Volta et al., 2015).

As already mentioned, despite the need for more empirical testing, several lines of evidence have suggested that LRRK2 G2019S may cause perturbations to the pre-synaptic exo-endocytic machinery (Belluzzi et al., 2012, 2016). We speculate here that the increased monoaminergic tone observed in the young adult GS-Tg mouse, which can perhaps best be conceptualized as an early neuronal contribution to allostatic load (Beccano-Kelly et al., 2014), could reflect a biological perturbation to the synaptic release machinery, perhaps in conjunction with an altered intracellular distribution of synaptic vesicles (more vesicles located in close proximity to the presynaptic active zone:

Migheli et al., 2013) (Fig. 5.6). Alternatively or additionally, since LRRK2 G2019S (over) expression in mice has been linked to a multitude of mostly age-related CNS pathological changes (e.g., autophagic-lysosomal dysfunction, abnormal intracellular trafficking, impaired neurogenesis, increased tau phosphorylation and microglial activation) (Lin et al., 2010; Melrose et al., 2010; Ramonet et al., 2011; Winner et al., 2011; Sepulveda et al., 2013), we cannot discount the possibility that the presently described hypermonoaminergic phenotype could have arisen as compensation for incipient disease processes (e.g., Ballanger et al., 2016).

Finally, as shown in Table 5.2, both LPS and IFN- γ altered circulating cytokine levels and LRRK2 G2019S failed to modulate these effects. These data suggest that overexpression of the mutant transgene in 3-4 month old male mice is not associated with a systemic pro-inflammatory phenotype, whether under basal conditions or in response to acute immune challenge. Although these null results were somewhat unexpected, especially in view of the recent report of Dzamko et al. (2016) documenting increased IL-1 β levels in asymptomatic LRRK2 G2019S mutation carriers, it will be noted that Lopez de Maturana et al. (2014) failed to detect a robust cytokine- or- COX-2-modifying influence of LRRK2 G2019S in LPS-stimulated dermal fibroblasts (from PD patients and controls) while Gardet et al. (2010) did not find a difference in NF- κ B activation in HEK293 cells expressing LRRK2 G2019S or WT protein. Moreover, Moehle et al. (2015) recently reported that adult GS-Tg mice (the same strain used here) displayed unaltered complete blood chemistry and that LPS-stimulated thioglycollate-elicited primary GS-Tg macrophages did not produce elevated levels of pro-inflammatory cytokines and chemokines. Thus, while LRRK2 G2019S is clearly implicated in

exaggerated (neuro) inflammatory responses (Kim et al., 2012; Daher et al., 2014, 2015; Moehle et al., 2012, 2015; Dzamko et al., 2016), uncertainty remains as to the primary underlying cellular and molecular mechanisms; e.g., elaboration of cytokine cascades, as suggested by the recent work of Dzamko et al. (2016) and the earlier work of Kim et al. (2012), or potentiation of immune cell mobility and chemotactic responses, as indicated by the study of Moehle et al. (2015).

5.7 Conclusion

The present findings are among the first to implicate LRRK2 G2019S in the modulation of monoaminergic neurotransmission in a network of stressor-sensitive limbic regions, both in the basal state and in response to acute systemic immune challenge. Our data suggest that IFN- γ and LPS may partially act through toxic gain-of-function LRRK2 variants to influence regional brain monoamine signalling and, hence, affective states. Whether this primarily involves a cell-autonomous “neuron-centric” or non-cell autonomous “immune cell-centric” mechanism is currently unclear, although we did not find evidence of an altered peripheral cytokine response to IFN- γ /LPS in the GS-Tg mice.

Table 5.1. Brain regional concentrations of NE, 5-HT and DA, and their principal metabolites MHPG, 5-HIAA, and DOPAC and HVA, respectively, as a function of G2019S LRRK2 overexpression and experimental treatment

	Concentration (ng/mg protein)					
	Non-Tg			G2019S LRRK2 Tg		
	Vehicle	LPS	IFN- γ	Vehicle	LPS	IFN- γ
PVN						
NE	25.05 \pm 4.04	26.45 \pm 3.56	39.24 \pm 2.27	28.76 \pm 10.9	39.68 \pm 8.37	47.57 \pm 9.38
MHPG ^c	5.13 \pm 0.78	10.55 \pm 2.85	10.51 \pm 3.33	5.32 \pm 1.54	9.58 \pm 3.55	15.89 \pm 6.88
5-HT	12.53 \pm 1.74	17.66 \pm 2.51	13.10 \pm 1.23	15.56 \pm 2.12	18.99 \pm 2.15	15.54 \pm 2.80
5-HIAA ^a	17.02 \pm 1.78	22.92 \pm 1.65	18.01 \pm 1.83	17.65 \pm 2.95	21.43 \pm 1.87	15.81 \pm 1.75
DA ^{a, e}	16.35 \pm 1.77	9.67 \pm 1.49	14.31 \pm 1.06	17.89 \pm 3.01	13.50 \pm 1.35	10.33 \pm 2.33
DOPAC	9.74 \pm 1.21	17.05 \pm 4.91	12.05 \pm 1.90	10.61 \pm 0.44	15.14 \pm 1.78	11.84 \pm 1.59
HVA	7.34 \pm 1.08	8.66 \pm 1.65	8.51 \pm 0.51	9.58 \pm 1.62	11.19 \pm 0.85	8.19 \pm 1.90
LC						
NE ^a	21.83 \pm 1.50	14.16 \pm 1.28	18.66 \pm 1.28	17.37 \pm 1.40	15.82 \pm 1.56	15.17 \pm 1.59
MHPG ^{a, c}	4.10 \pm 0.50	5.78 \pm 0.67	3.90 \pm 0.31	3.93 \pm 0.49	6.27 \pm 0.96	5.72 \pm 0.89
Dorsal HC						
NE ^{b, c}	5.82 \pm 0.30	6.26 \pm 0.39	6.94 \pm 0.40	6.47 \pm 0.33	7.98 \pm 0.35	7.99 \pm 0.30
MHPG	4.46 \pm 0.92	5.39 \pm 0.91	3.78 \pm 0.29	3.01 \pm 0.20	3.32 \pm 0.55	4.46 \pm 0.78
5-HT ^c	5.89 \pm 0.47	7.00 \pm 0.91	5.91 \pm 0.67	7.10 \pm 0.34	7.49 \pm 0.42	8.31 \pm 0.42
5-HIAA ^c	3.18 \pm 0.29	4.11 \pm 0.53	3.73 \pm 0.44	4.49 \pm 0.26	4.51 \pm 0.23	5.72 \pm 0.43
Ventral HC						
NE ^c	5.95 \pm 0.79	6.54 \pm 0.62	6.89 \pm 0.82	7.17 \pm 0.45	8.65 \pm 0.45	7.54 \pm 0.60
MHPG ^{a, d}	4.48 \pm 0.44	6.25 \pm 0.81	4.59 \pm 0.20	4.99 \pm 0.20	6.19 \pm 0.42	7.97 \pm 0.37
5-HT	5.39 \pm 0.74	6.52 \pm 0.73	6.77 \pm 0.93	5.67 \pm 0.73	6.52 \pm 1.13	5.95 \pm 0.77
5-HIAA ^d	5.17 \pm 0.61	6.04 \pm 0.30	6.70 \pm 0.37	7.10 \pm 0.47	6.19 \pm 0.48	5.86 \pm 0.50
PFC						
NE	7.26 \pm 0.30	8.01 \pm 0.59	7.19 \pm 0.37	6.47 \pm 0.61	7.60 \pm 0.48	7.92 \pm 0.68
MHPG ^{a, e}	3.10 \pm 0.27	3.53 \pm 0.25	3.02 \pm 0.22	2.74 \pm 0.19	4.75 \pm 0.89	2.72 \pm 0.43
5-HT ^a	4.37 \pm 0.53	4.87 \pm 0.29	5.24 \pm 0.33	4.10 \pm 0.35	5.79 \pm 0.64	5.24 \pm 0.43
5-HIAA ^a	2.95 \pm 0.25	4.28 \pm 0.28	3.81 \pm 0.30	3.59 \pm 0.36	4.29 \pm 0.33	3.46 \pm 0.32
DA	2.93 \pm 0.36	4.06 \pm 0.64	3.45 \pm 0.39	2.93 \pm 0.57	3.23 \pm 0.57	3.35 \pm 0.18
DOPAC ^d	2.39 \pm 0.44	5.51 \pm 0.87	3.60 \pm 0.52	4.06 \pm 0.68	3.08 \pm 0.59	2.58 \pm 0.45
HVA ^{a, c, e}	2.99 \pm 0.27	4.24 \pm 0.37	3.55 \pm 0.40	4.16 \pm 0.50	8.18 \pm 1.71	4.76 \pm 1.13

Data are presented as mean \pm SEM and were analyzed by ANOVA followed by the Tukey-Kramer HSD test. Results were considered significant at the $\alpha = .05$ level.

a = Treatment main effect; LPS-induced change

b = Treatment main effect; IFN- γ -induced change

c = Genotype main effect

d = Genotype \times Treatment interaction

e = Trend towards exaggerated IFN- γ - or LPS-induced response in the GS-Tg mice (Genotype \times Treatment $\eta^2 \geq .05$)

Table 5.2. Summary of significant experimental findings

Experimental endpoint	ANOVA main effects and interactions			Genotype × Treatment interaction
	Main effect of Genotype	Main effect of Treatment		
		LPS-induced Δ	IFN-induced Δ	
Behaviour				
Home-cage activity ^a		×		
Sickness		×		
Blood plasma				
Corticosterone		×		
TNF-α		×		
IL-6 ^b		×	×	
IL-1β		×		
MCP-1		×	×	
IL-10		×		
PVN				
5-HIAA		×		
DA		×		
LC				
NE		×		
MHPG		×		
Dorsal HC				
NE	×		×	
5-HT	×			
5-HIAA	×			
Ventral HC				
NE	×			
MHPG		×		×
5-HIAA				×
PFC				
MHPG		×		
5-HT		×		
5-HIAA		×		
DO				×
HVA	×	×		

Data were analyzed by ANOVA followed by the Tukey-Kramer HSD test ($\alpha = .05$). ^aHome-cage activity also varied according to a significant Treatment × Time interaction; as described in the text (p. 183), LPS progressively suppressed home-cage activity among GS-Tg and non-Tg mice alike. ^bA large majority of the LPS-treated IL-6 samples exceeded the assay's upper limit of quantitation; caution should be exercised when interpreting these data.

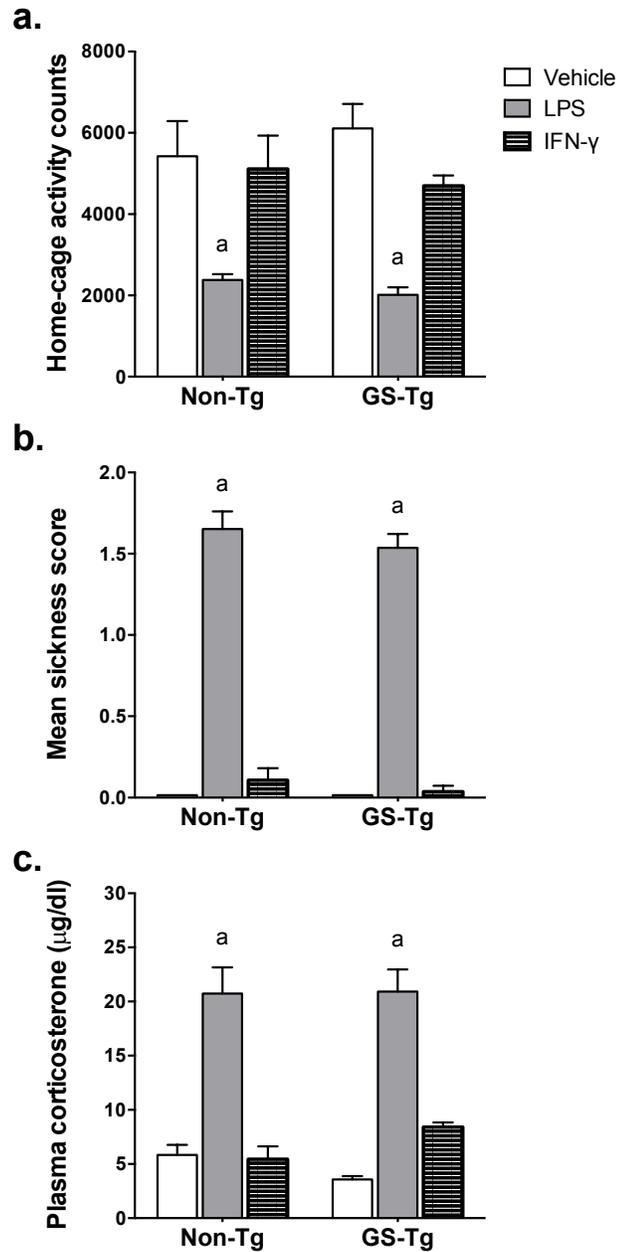


Figure 5.1. LRRK2 G2019S overexpression failed to modify LPS-induced changes in behaviour and plasma corticosterone. The LPS treatment markedly suppressed home-cage activity (*a*), induced sickness behaviour (*b*), and raised plasma corticosterone concentrations (*c*) in GS-Tg and non-Tg mice alike. Data are shown as mean \pm SEM. The behavioural data were averaged across time. ^a $p < .05$ relative to all other mice.

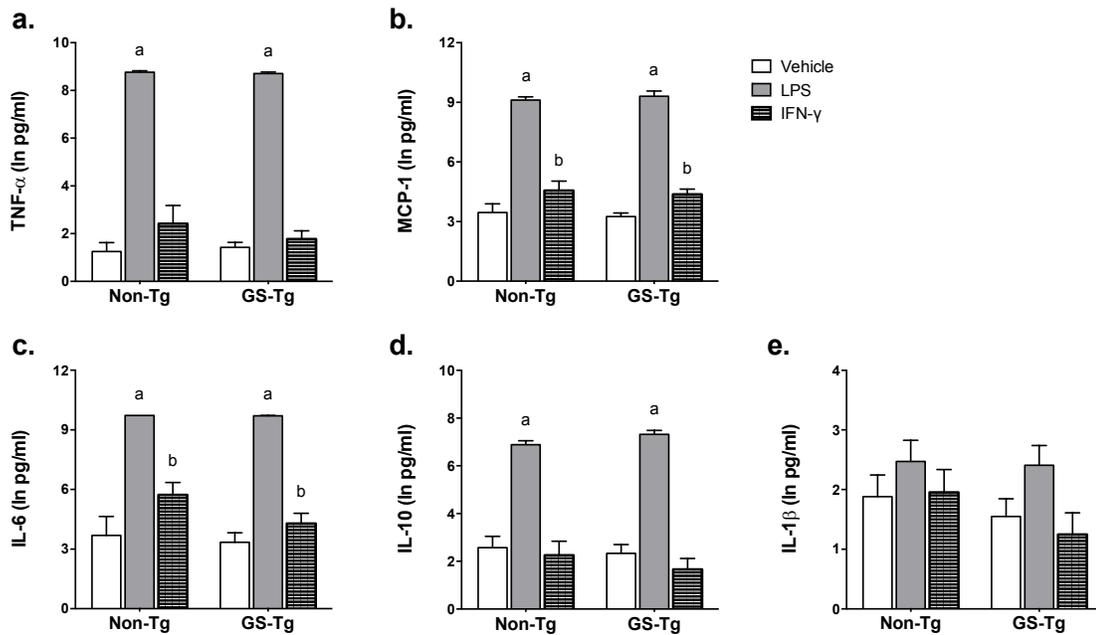


Figure 5.2. LPS and IFN- γ increased circulating cytokine levels irrespective of LRRK2 G2019S overexpression. In both the GS-Tg and non-Tg mice, LPS increased the natural log-transformed (ln) plasma levels of TNF- α , MCP-1, IL-6, and IL-10 (a-d); there were no significant between group differences in IL-1 β (e). In addition, both MCP-1 (b) and IL-6 (c) were augmented by IFN- γ among mice of either genotype. Data are shown as mean \pm SEM. ^a p < .05 relative to vehicle-treated and IFN- γ -treated mice; ^b p < .05 relative to vehicle-treated and LPS-treated mice.

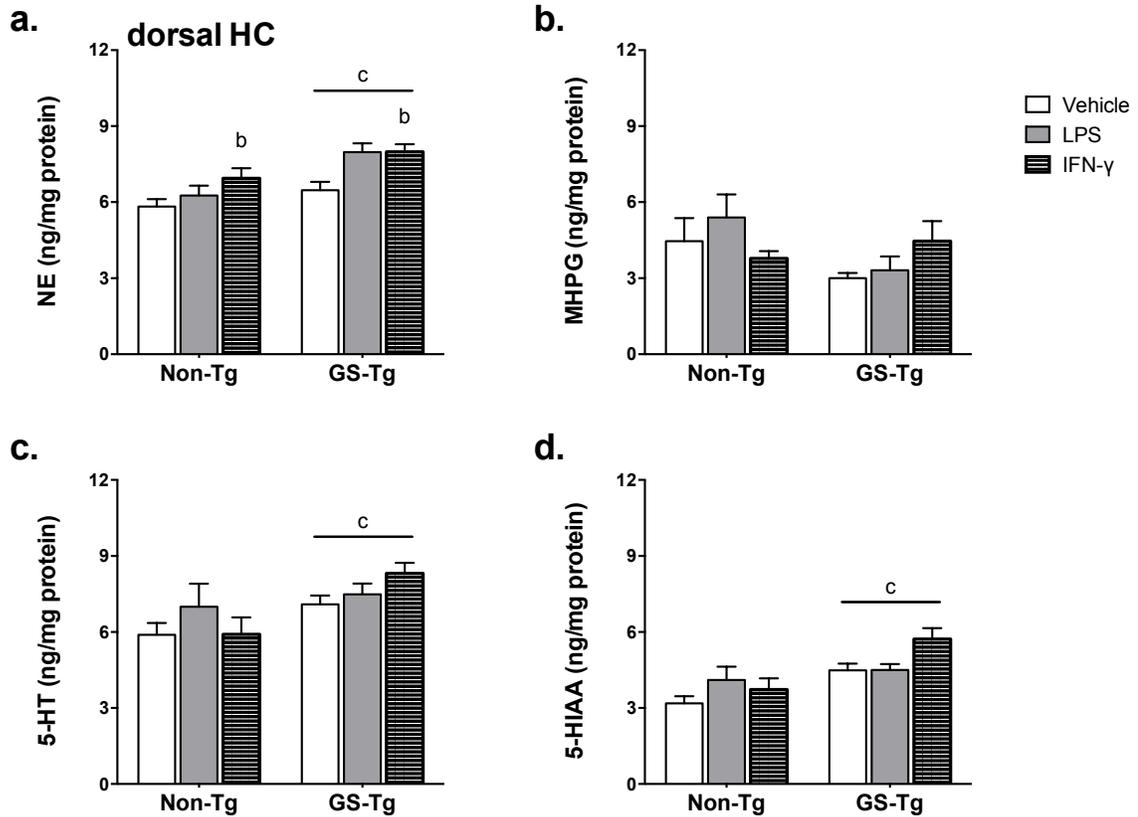


Figure 5.3. Noradrenergic and serotonergic activity within the dorsal hippocampus: influence of LPS, IFN- γ and LRRK2 G2019S overexpression. Within the dorsal HC, NE concentrations were significantly increased following IFN- γ administration in the GS-Tg and non-Tg mice alike (*a*). As well, compared to their non-Tg littermates, the GS-Tg mice had elevated overall levels of dorsal hippocampal NE (*a*), 5-HT (*c*) and 5-HIAA (*d*). There were no between-group differences in the levels of MHPG (*b*). Data are shown as mean \pm SEM. ^b $p < .05$ relative to vehicle-treated mice; ^c $p < .05$ relative to non-Tg mice.

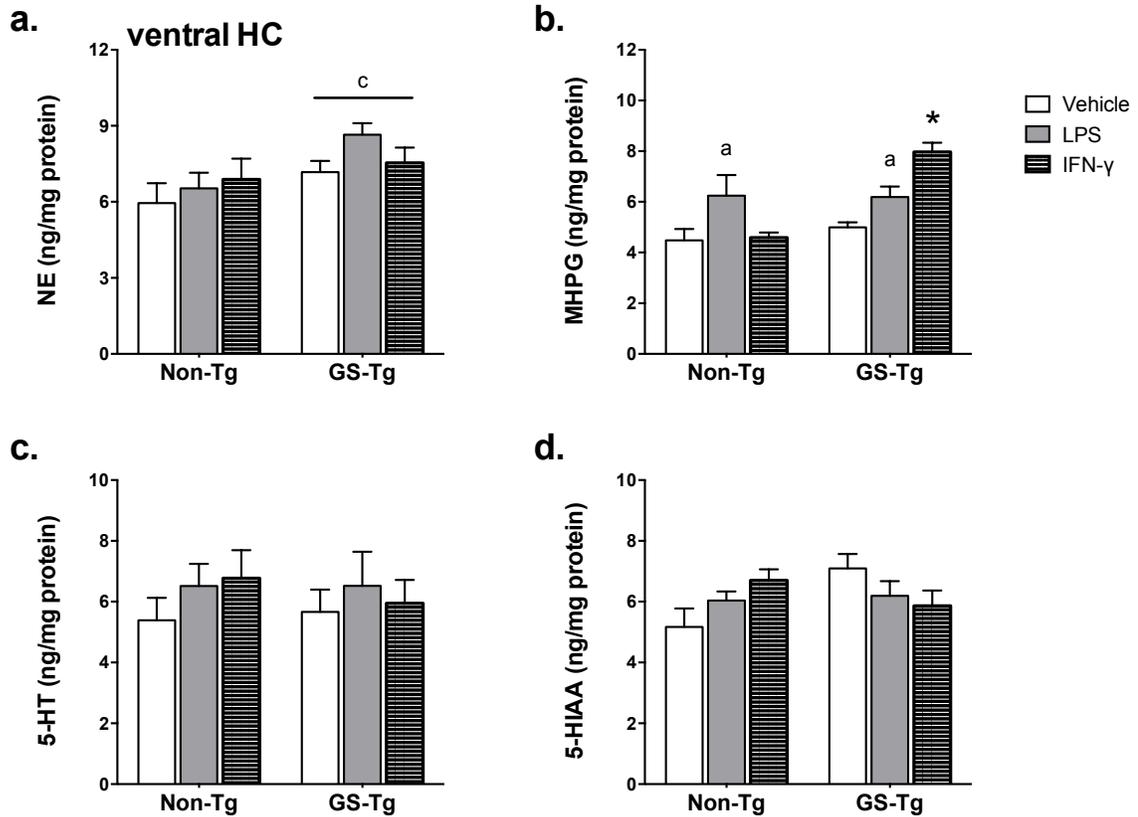


Figure 5.4. Noradrenergic and serotonergic activity within the ventral hippocampus: influence of LPS, IFN- γ and LRRK2 G2019S overexpression. Within the ventral HC, NE content was increased overall among the GS-Tg mice (a). While LPS augmented MHPG concentrations in both the GS-Tg and non-Tg mice, IFN- γ increased metabolite levels in the mutant mice only (b). There were no significant between-group differences in the levels of 5-HT or 5-HIAA (c, d). Data are shown as mean \pm SEM. ^a $p < .05$ relative to vehicle-treated mice; ^c $p < .05$ relative to non-Tg mice; ^{*} $p < .05$ relative to GS-Tg controls.

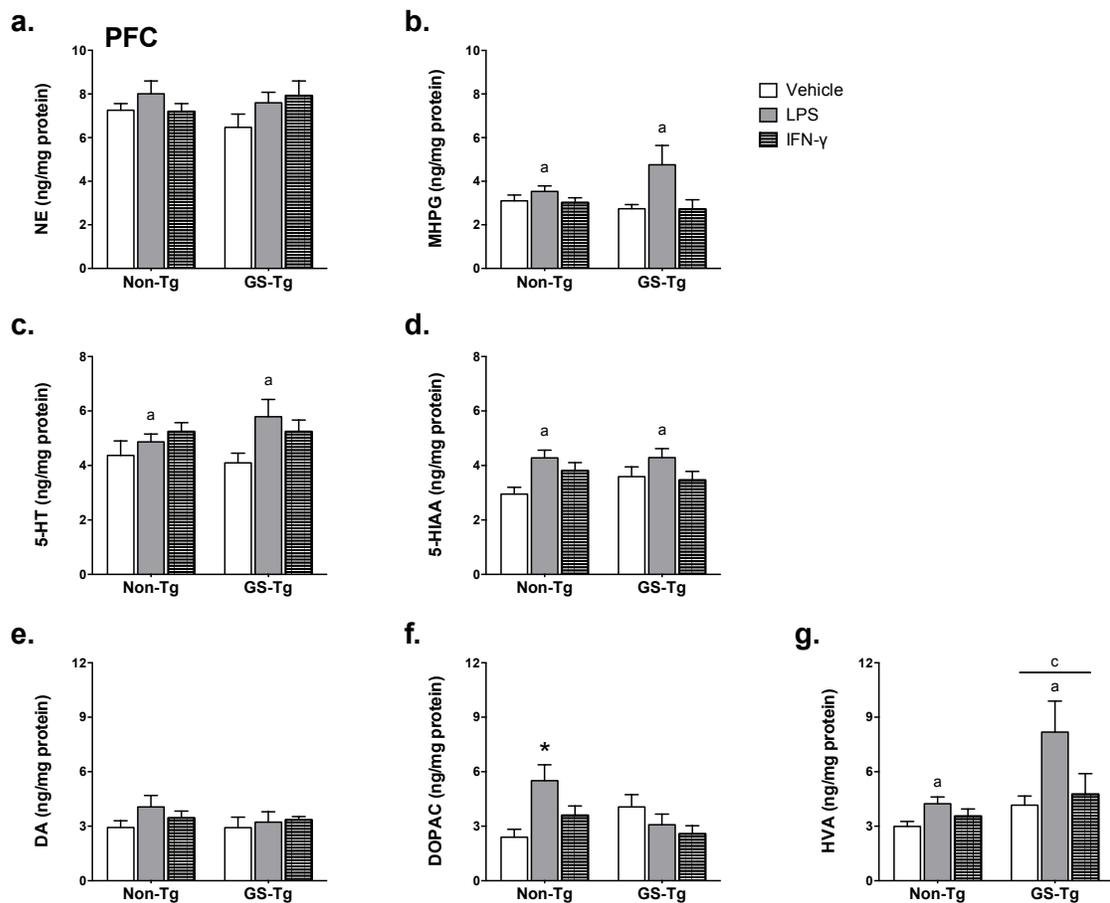
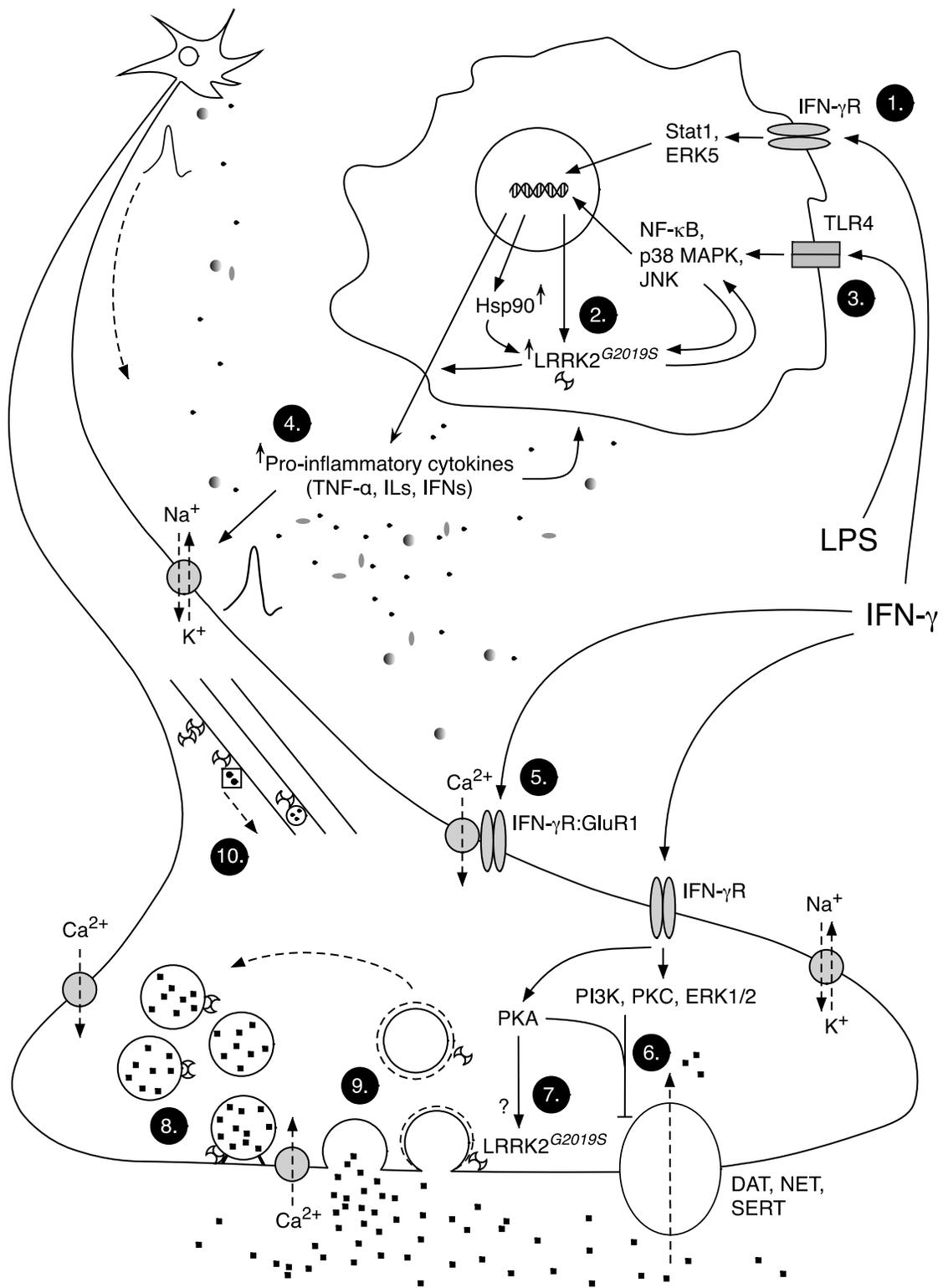


Figure 5.5. Noradrenergic, serotonergic and dopaminergic activity within the medial prefrontal cortex: influence of LPS, IFN- γ and LRRK2 G2019S overexpression. Within the PFC, LPS significantly increased the levels of MHPG, 5-HT, 5-HIAA, DOPAC, and HVA (*b, c, d, f, g*). LRRK2 G2019S overexpression attenuated the LPS-induced increase in DOPAC levels (*f*) and was itself seen to augment HVA concentrations overall (*g*). In contrast to the LPS treatment, IFN- γ did not influence PFC monoaminergic neurotransmission among mice of either genotype. Data are shown as mean \pm SEM. ^a $p < .05$ relative to vehicle-treated controls; ^c $p < .05$ relative to non-Tg mice; ^{*} $p < .05$ relative to non-Tg controls.

Figure 5.6. Potential LRRK2-associated cellular and molecular mechanisms underlying the rapid central monoaminergic effects of systemic IFN- γ . (1) IFN- γ signals through Jak/Stat and/or ERK5 transduction pathways in circulating and brain-resident immune cells to induce the transcription of LRRK2 and other inflammatory genes (e.g., cytokines and the molecular chaperone Hsp90). (2) LRRK2, whether induced de novo or post-translationally stabilized by Hsp90, can stimulate or potentiate intracellular signalling cascades leading to another wave of inflammatory transcription (precise mechanisms have yet to be identified). LRRK2 may also accelerate the release of inflammatory cytokines in secretory vesicles (by regulating aspects of the exo-endocytic release machinery) and facilitate the trafficking of membrane receptors and inflammatory enzymes (via interactions with the cytoskeleton and various Rabs), but again empirical confirmation is needed (Russo et al., 2014). (3) Acting through TLRs on circulating immune and brain endothelial cells, LPS can likewise activate inflammatory transcriptional programs (involving NF- κ B, p38 and JNK), leading to LRRK2 induction and the release of inflammatory/oxidative factors. LPS/TLR4 signalling can also influence LRRK2 post-translationally via phospho-activation by I κ B kinases, which are key positive regulators of NF- κ B, and probably other stress kinases (depicted here as a generic activation of LRRK2 by LPS/TLR signal transduction). This relationship appears to be reciprocal as LRRK2 G2019S can stimulate/potentiate NF- κ B, p38 and JNK, probably by phospho-activating upstream kinases such as Akt, I κ B α and the MAPK kinases -3, -4, and -7 (e.g., Gloeckner et al., 2009). (4) Secondarily released cytokines can then act on neurons to trigger action potentials and monoamine release (e.g., via altering ionic conductances and second messenger cascades). Since most evidence suggests that LRRK2 G2019S exacerbates inflammatory responses, the expectation is that cytokine-induced monoaminergic responses will likewise come to be exaggerated. (5, 6) IFN- γ can also act directly on neurons, i.e., without first signalling to immune cells, to promote monoamine release and the rapid inhibition of monoamine transporters (via non-genomic mechanisms). (7) Although it is possible that IFN- γ could be acting via PKA to phosphorylate LRRK2 at presynaptic terminals (and in immune cells, which is not shown), it is unclear whether this will have a stimulatory or inhibitory effect on LRRK2 kinase activity. Indeed, LRRK2 is an important but probably functionally redundant player in the neuronal presynaptic compartment, where it is believed to contribute to (8) synaptic vesicle mobilization/clustering (e.g., in the reserve and readily releasable pools) and exocytosis (docking, priming and/or fusion), as well as (9) endocytosis and recycling (direct recycling or through an early endosomal intermediate; only the former is shown here). Several (but not all) of these LRRK2-associated synaptic events are dependent on LRRK2 kinase activity, which suggests that kinase-hyperactive mutants, such as LRRK2 G2019S, may act to perturb synaptic neurotransmission. (10) LRRK2 has also been implicated in the trafficking of newly synthesized synaptic proteins to axon terminals (e.g., axonal transport of synaptic vesicle precursors and monoamine synthetic enzymes). Solid arrows (solid arrowhead) indicate positive/stimulatory relationships. Solid lines with blunted ends indicate negative/inhibitory relationships. Broken arrows (solid arrowhead) indicate movement across time and/or space. Small upwards arrows (swept arrowhead) indicate increased protein levels; dashed circle in step 9 = clathrin coat; grey-filled circles = voltage-gated ion channels; grey-filled ellipses = IFN- γ receptor or as labelled; grey-filled rectangles = TLR4.



6 Chapter. General Discussion

The presently described thesis work adds to the literature on cytokine influences in depression by furthering our growing understanding of the important but sometimes overlooked contributions of the Th1-type cytokine IFN- γ . Not only do we provide compelling evidence that IFN- γ mediates some of the depressive-like effects of chronic stress (Chapters 2 and 3), but we also demonstrate the cytokine's ability to proactively influence depression-relevant pathophysiological domains (monoamine and HPA axis systems, peripheral cytokine networks) (Chapters 4 and 5) and suggest a possible role for the PD-linked kinase LRRK2 in this regard (Chapter 5). Indeed, when we started down this path, IFN- γ had yet to garner significant consideration as a major player in the psychoneuroimmunology of depression, although there were certainly early indications that the cytokine could be involved (Krishnan et al., 1987; Maes et al., 1994; Seidel et al., 1995; Iwagaki et al., 1997; Färkkilä et al., 1988). Our own journey really began with the surprising observation that mice genetically lacking IFN- γ were protected from some of the anxiety- and- depression-like behavioural and limbic monoaminergic effects of the PD-linked pesticide paraquat (Litteljohn et al., 2009).

6.1 IFN- γ contributes to chronic stressor effects

Given the primacy of psychologically relevant stress in depression (Hill et al., 2012) and the important mediating role of cytokines in this regard (Hayley et al., 2005), it made sense to begin by probing for possible contributions of IFN- γ to stressor effects. In Chapter 2 we show that IFN- γ KO blunted some of the corticosterone, cytokine and brain regional dopaminergic effects of chronic stress, but surprisingly not stressor-induced depressive-like behaviours. In Chapter 3 we focus specifically on the contributions of IFN- γ to stress-induced memory impairment, revealing that a lack of the cytokine not

only protected against stressor-induced memory dysfunction but also appeared to facilitate memory performance following stress. It is not entirely clear why this should have occurred, but we do entertain the possibility that increased LC-HC noradrenergic signalling may have played a role. Importantly, however, IFN- γ deficiency was not universally beneficial and we report in Chapters 2 and 3 that under basal conditions the KO mice actually had increased plasma corticosterone levels, heightened brain regional noradrenergic (CeA, LC and HC) and serotonergic activity (CeA), and impaired hippocampus-dependent memory function. These findings are consistent with reports indicating that genetic ablation of IFN- γ in mice is associated with increased emotional reactivity and impaired hippocampal neurogenesis (Kustova et al., 1998; Campos et al., 2014), and provide a compelling parallel to recent human neuroimaging work implicating the cytokine in the regulation of amygdala reactivity to emotional stimuli (Redlich et al., 2015).

Overall, then, the findings of our studies described in Chapters 2 and 3 are consistent with a nuanced role for IFN- γ in stressor-related psychological pathology. On the one hand the cytokine appears to mediate some of the depression-like neurobiological and, to a lesser extent, behavioural effects of chronic stress, and perhaps even restrict adaptive responses to stressor challenge. On the other hand, it would appear that a certain basal level of IFN- γ is required for the homeostatic regulation of specific neuroendocrine, neurotransmitter and behavioural systems. This latter point is consistent with the growing movement to recognize the many often conflicting roles of IFN- γ and pro-inflammatory cytokines/immune activation more generally in cognitive and affective functions (Mühl

and Pfeilschifter, 2003; Yirmiya and Goshen, 2011; Hindinger et al., 2012; Puzzo et al., 2012; O'Donnell et al., 2015).

Although on the whole these data strongly suggest that chronic stress is acting in part *through* IFN- γ to induce depression-like changes, it would be informative to have cytokine/brain mRNA measurements to corroborate this position. Moreover, though in Chapter 2 we assess circulating cytokine levels and actually point to an IFN- γ -dependent increase in serum TNF- α as a potential mechanism contributing to the central effects of chronic stress, it is not certain to what extent cytokine levels in the periphery accurately reflect the situation in the brain (Erickson and Banks, 2011; Litteljohn and Hayley, 2012; Kunugi et al., 2015). Thus, a limitation of our work presented in Chapters 2 and 3 is a lack of evidence that a) directly implicates increased IFN- γ signalling in the chronic stress response (although at least two recent reports appear to substantiate this claim: Liu et al., 2013; Wrona et al., 2014) and b) confirms the presence of inflammation within the brain itself. Brain regional transcriptional profiling of cytokine and other inflammatory genes, together with assessments of microglial activation, inflammasome induction and monocyte trafficking are examples of possible worthwhile “next steps” (Gustin et al., 2013; Thomson et al., 2014; Walsh et al., 2014; Reader et al., 2015). In a similar vein, though in Chapter 3 we do consider alterations of LC-HC noradrenergic signalling and hippocampal BDNF as possible neural substrates for the chronic stress \times IFN- γ KO-induced memory changes, we stop short of providing an actual mechanism.

6.2 Systemic IFN- γ induces central monoaminergic changes

Whereas our findings from Chapters 2 and 3 indirectly implicate IFN- γ in some of the neurobiological and, to a lesser extent, behavioural effects of chronic stress, in

Chapters 4 and 5 we provide direct evidence linking IFN- γ to several depression-relevant pathophysiological domains. Specifically, we report that systemically administered IFN- γ mobilizes peripheral cytokine networks, stimulates monoaminergic activity in stressor-sensitive limbic regions, and proactively sensitizes the plasma corticosterone response to psychological stress. Outside of the ever-growing literature concerning IFN- γ /IDO-dependent reductions in 5-HT biosynthesis (Maes et al., 2011b; Xu et al., 2015; Mahmoud et al., 2016), our data are among the first to directly link IFN- γ to perturbations of these neuroendocrine and neurotransmitter systems (Clement et al., 1997; Färkkilä et al., 1998; de Metz et al., 1999). Indeed, the presently described acute IFN- γ effects are largely reminiscent of those induced by other depression-linked cytokines in similar acute exposure paradigms (Dunn et al., 2005a; Anisman, 2009; Miller et al., 2009). Even the lack of overlap in the profile of brain regional monoamine changes induced by IFN- γ and an acute neurogenic stressor (Chapter 4) is consistent with earlier reports in the cytokine \times stress literature (e.g., IL-1 β and IL-6) (Song et al., 1999).

Consistent with some of the null behavioural findings from Chapters 2 and 3, in our hands, acute systemic IFN- γ did not significantly alter home-cage locomotor activity or induce sickness-type behaviours in mice of either sex. However, as discussed in Chapter 4, the lack of an activity-suppressing effect of IFN- γ may have had to do with the time at which behavioural testing occurred; i.e., at the beginning of the light (sleep) phase vs. during the dark (awake) phase (Crnic and Segall, 1992). Moreover, it should be noted that activity measurements in our acute cytokine exposure studies were collected over a relatively short duration (the 60 min immediately following injection) and only in response to a single cytokine exposure. We certainly cannot rule out changes in

locomotor activity outside of this window of time and/or with repeated cytokine exposures (Weinberger et al., 1988). In fact, in a small pilot study investigating the behavioural effects of sub-chronic exposure to a lower dose of systemic IFN- γ (15000 IU per day \times 6 days + 25000 IU on Day 7, male 5-6 m.o male mice, $n = 8$) we observed a significant cytokine-induced reduction in home-cage activity (Day 6). However, it should be noted that in this study IFN- γ did not induce changes in emotional and cognitive behaviours (nest building, social approach-avoidance and forced swim behaviours, as well as spatial working memory) (Appendix). We therefore advocate here for future work investigating the behavioural and neurobiological effects of repeated administration of different doses of IFN- γ (with and without concurrent psychological stress), which will more closely mimic the course of viral or bacterial illness as well as chronic stressor-induced inflammatory processes (Hayley et al., 2003; Hayley et al., 2013).

6.3 A role for LRRK2 in the central monoaminergic effects of IFN- γ : mechanisms, limitations and future research directions

Beyond characterizing some of the acute depression-relevant monoamine, corticoid and cytokine effects of IFN- γ , in Chapter 5 we provide proof-of-concept for an IFN- γ /LPS-LRRK2 signalling pathway that may be relevant for depression and other non-motor symptoms in PD. Specifically, we show that overexpression of kinase-hyperactive LRRK2 G2019S, which in mice causes impaired hippocampal neurogenesis and perturbations of glutamatergic signalling (Winner et al., 2011; Beccano-Kelly et al., 2014; Sweet et al., 2015) and in humans is associated with increased immunogenicity (Dzamko et al., 2016) and a heightened risk and/or severity of depression (Belarbi et al., 2010), exacerbates some of the brain regional monoaminergic effects of IFN- γ and LPS.

Additionally, in this first-of-its-kind study we provide preliminary evidence suggestive of a mild central hyper-monoaminergic phenotype in the young adult GS-Tg mouse.

However, as detailed in Chapter 5, we cannot be certain whether these effects primarily reflect a) an initial LRRK2 G2019S-associated exaggeration of the IFN- γ /LPS-induced cytokine/inflammatory response, which given the relatively short interval between injection and sacrifice (90 min) is likely to be mediated mainly by phosphorylation events or other forms of post-transcriptional LRRK2 regulation (Gloeckner et al., 2009; Ohta et al., 2011; Gillardon et al., 2012; Kim et al., 2012; Moehle et al., 2012; Hongge et al., 2015), or b) an “unmasking” or potentiation of the perturbative presynaptic action of kinase-hyperactive LRRK2 (i.e., LRRK2 kinase-dependent acceleration of synaptic vesicle cycling upon cytokine-induced neuronal excitation: Belluzzi et al., 2016). Importantly, these non-cell autonomous and cell-autonomous mechanisms do not appear to be mutually exclusive and together they may even be considered to amplify the initial immune signal/stressor (Fig. 6.1).

In contrast, longer intervals between injection and sacrifice or more chronic immune exposures could be expected to engage genomic mechanisms that will likely only serve to reinforce or exacerbate the constitutive toxic gain-of-function effects associated with LRRK2 G2019S kinase hyperactivity (West et al., 2005; Smith et al., 2006). As shown in Figure 6.1, both IFN- γ and LPS can markedly upregulate LRRK2 expression in circulating and brain-resident immune cells (Gardet et al., 2010; Thévenet et al., 2011; Moehle et al., 2012; Kuss et al., 2014), causing them to assume an activated, migratory/phagocytic and pro-inflammatory phenotype (Russo et al., 2014, 2015; Moehle et al., 2015). This increase in LRRK2 protein levels can be achieved either through de

novo LRRK2 transcription and translation or, in the case of IFN- γ , an Hsp90-dependent post-translational stabilization of LRRK2 against proteasomal degradation (the molecular chaperone Hsp90 is an IFN- γ target gene: Stephanou et al., 1999) (Wang et al., 2008; Ko et al., 2009; Thévenet et al., 2011). Notably, while IFN- γ preferentially induces LRRK2, it appears that other inflammatory cytokines may share this capacity to a lesser degree (e.g., TNF- α , IL-1 β and IL-6) (Thévenet et al., 2011; Hongge et al., 2015).

An interesting point of consideration is whether an analogous IFN- γ /cytokine-to-LRRK2 genomic pathway is operational in CNS neurons. As shown in Figure 6.1, if one assumes both that IFN- γ induces mutant LRRK2 in neurons and that some of this newly synthesized or stabilized protein is being trafficked to presynaptic terminals, then such a mechanism could be anticipated to a) further perturb synaptic transmission in the basal state and b) lower the threshold for cytokine/inflammatory or other stimulus-evoked monoaminergic effects (i.e., by further accelerating LRRK2 kinase-dependent synaptic vesicle exo-endocytosis). It warrants mentioning, however, that in monocytes IFN- γ was shown to upregulate LRRK2 via a mechanism involving ERK5 (Kuss et al., 2014), which in adult neurons is generally only expressed at low levels (Di Benedetto et al., 2007). The LRRK2 promoter region has nevertheless been found to contain several IFN response sequences (Gardet et al., 2010; Thévenet et al., 2011), which suggests that IFN- γ may yet be capable of inducing LRRK2 through canonical Jak/Stat signalling in certain cell types. These mechanisms are admittedly speculative and it is early days still in the study of LRRK2 signalling pathways (both upstream and downstream) (Lobbestael et al., 2012; Manzoni et al., 2015).

Particularly fruitful next steps, all of which are envisioned to utilize relevant LRRK2 gain-and-loss-of-function approaches (e.g., genetically modified mice and pharmacological agents targeting specific aspects of LRRK2 biology, e.g., kinase-specific inhibitors: Deng et al., 2011) could include: 1) assessments of monoamine release from brain regional synaptosomes incubated with IFN- γ and other cytokines (Zhu et al., 2006); 2) electrophysiological characterizations of primary neuronal cultures treated with IFN- γ /cytokines (Morini et al., 2015); 3) cell-specific transcriptional profiling of IFN- γ /LPS-induced responses (Rock et al., 2005; Wang and Campbell, 2005); and 4) immunofluorescence co-localization analyses of LRRK2, IFN- γ receptors and/or cell-specific activity markers (e.g., c-fos and cd11b for neurons and microglia, respectively) (Robertson et al., 2000; Davies et al., 2013).

Another worthwhile avenue of future research involves examining whether LRRK2 contributes to the depressive-like immunological, hormonal and neurobiological effects of chronic stress (e.g., cytokine mobilization and microglial activation, circulating corticosterone levels, monoamine neurotransmission, neurogenesis), which is predicted in part by Park and colleagues' work (2013) showing that glucocorticoids markedly upregulated LRRK2 in cultured DA neurons. Moreover, given the apparent convergence of IFN- γ /inflammatory- and- stressor/glucocorticoid-associated signalling pathways at the level of LRRK2 transcription, and the multiple possibilities for cross-talk between signalling factors [e.g., IFN- γ -induced Hsp90 can increase GR affinity for glucocorticoids (Kirschke et al., 2014), which could enhance GR-associated LRRK2 transcription], we submit that studies investigating the interactional effects of processive stressors and chronic IFN- γ /immune activation in different LRRK2 genotypes may be

particularly constructive (loss-of-function KOs vs. WT vs. gain-of-function KIs or Tgs) (e.g., Rudyk et al., 2015) (Fig. 6.1).

While novel, the studies described in Chapters 4 and 5 have several shortcomings. Some of these relate specifically to aspects of our experimental design and methodology.

- 1) A lack of time-series data, which would have allowed us to address potential genotype- or- sex-dependent differences in the temporal dynamics and magnitude of brain monoaminergic, plasma corticosterone and cytokine responses to IFN- γ , LPS and/or restraint stress (Cano et al., 2005; Skelly et al., 2013).
- 2) The absence of dose-response data, although in a small pilot study we did find that a lower dose of IFN- γ (15000 IU) suppressed home-cage activity (Litteljohn and Hayley, unpublished observations; see also Appendix). And 3) Building our case on HPLC determinations of monoamine and metabolite concentrations in post-mortem brain tissues as opposed to in vivo amperometric/voltammetric or microdialysis monitoring of brain regional monoamine release (extracellular amine levels) (van Heesch et al., 2013; Felger et al., 2015).

We also highlight here three limitations that pertain specifically to the GS-Tg study described in Chapter 5, and offer a reasoned rebuttal for each. Firstly, we didn't include an advanced age cohort, and previous research has shown that LRRK2 G2019S interacts with age to modify molecular and neurobehavioural phenotypes (Li et al., 2010b; Ramonet et al., 2011; Zhou et al., 2011; Chen et al., 2012; Sweet et al., 2015). Thus, it is perhaps not all that surprising that we failed to detect a distinct behavioural, neuroendocrine or cytokine phenotype in the 3-4 month old GS-Tg mouse, and we certainly cannot rule out alterations of these systems at a more advanced age. Most of

these studies, however, were designed to investigate the consequences of LRRK2 G2019S alone across time. This makes good sense in the context of modeling PD, where advancing age and LRRK2 G2019S are two of the most important risk factors (Reeve et al., 2014; Cookson, 2015). In contrast, our aim in Chapter 5 was not to model PD or elucidate pathways to neurodegeneration, but to shed new light on potential mechanisms underlying depression and other non-motor PD symptoms. As will be recalled, these symptoms often predate motor dysfunction and disease diagnosis (Chen et al., 2015) and may occur even earlier among LRRK2 G2019S mutation carriers (Shanker et al., 2011; Thaler et al., 2012).

Secondly, it will be noted that the recently described LRRK2 G2019S knock-in mouse offers a closer approximation of the human condition than does the GS-Tg mouse (i.e., a more physiologically relevant system), although neither completely recapitulates the cardinal features of PD (e.g., Longo et al., 2014; Yue et al., 2015). Yet, in **Chapter 5** we were primarily interested in ascertaining whether LRRK2 G2019S, regardless of dose (our particular BAC transgenic mouse exhibits LRRK2 G2019S overexpression ~ 6-8 fold greater than normal: Li et al., 2010b), would modify some of the short-term effects of systemic IFN- γ or LPS (and thus provide a proof-of-concept); phenotyping the 3-4 month old GS-Tg mouse was an important but secondary concern.

And thirdly, we did not include a LRRK2 WT transgenic comparison group (this strain is in cryopreservation at The Jackson Laboratory). This prevents us from concluding with absolute certainty that the observed monoaminergic phenotype is specific for the LRRK2 G2019S mutation. However, it is important to underscore that the originators of the BAC GS-Tg mouse strain that we use in Chapter 5 did not detect a

pathological phenotype in BAC LRRK2 WT overexpressing mice (vs. what was seen in the LRRK2 G2019S overexpressors) (Li et al., 2010b). And while overexpression of WT LRRK2 has certainly been linked to a range of pathological changes (e.g., ROS production, neurite shortening and autophagic deregulation, basal and LPS-induced phosphorylation of p38 and JNK), a common thread in these studies (primarily in vitro and ex vivo) is that LRRK2 G2019S aggravates such pathology (MacLeod et al., 2006; Plowey et al., 2008; Heo et al., 2010; Kim et al., 2012; Orenstein et al., 2013; Lavalley et al., 2016). For example, Hongge et al. (2015) recently reported that WT LRRK2 in cultured human endothelial cells enhanced IL-1 β -mediated induction of vascular cell adhesion molecule 1 (and NF- κ B transcriptional activity) and increased monocyte attachment to these cells; however, these effects were exacerbated by LRRK2 G2019S. And finally, the important study of Skibinski et al. (2014) showing that total soluble LRRK2 levels may be key to predicting toxicity – and perhaps more so than mutant kinase activity per se – was conducted using an in vitro model of neuronal degeneration (with neurons recapitulating age-related pathological features of PD). It is not expected that our acute systemic IFN- γ /LPS model of early non-motor PD symptoms will tap into the same cellular and molecular mechanisms as this neuronal cell culture model of PD.

6.4 Possible therapeutic implications of an IFN- γ -LRRK2 signalling pathway in co-morbid depression

It is very early days yet in the study of depression-related mechanisms in LRRK2-related PD and we must be careful not to overreach based on a few interesting studies (Dächsel et al., 2010; Winner et al., 2011; Sweet et al., 2015; Volta et al., 2015). However, it will be noted that the data presented in Chapter 5 tend to support our position

that inflammatory-driven perturbations of brain regional monoamine neurotransmission may at least partially underlie the heightened risk for depression and cognitive deficits reported among LRRK2 G2019S mutation carriers (perhaps in concert with altered hippocampal neurogenesis and LTP: Winner et al., 2011; Sweet et al., 2015) (Shanker et al., 2011; Mirelman et al., 2015). It follows that anti-inflammatory agents could be particularly useful in treating depression among these individuals. This recommendation is made all the more relevant by the recent study of Dzamko et al. (2016) reporting increased levels of IL-1 β in asymptomatic LRRK2 G2019S mutation carriers. Interestingly, IL-1 β levels in this group also correlated positively with the levels of 17 other cytokines, including TNF- α , IL-6, MCP-1/CCL2, and the IFN- γ -inducing cytokine IL-12 (Dzamko et al., 2016).

Although we make a point here of highlighting evidence suggesting a different clinical presentation of LRRK2-related PD, i.e., more frequent, severe and earlier-occurring cognitive and affective symptoms (Thaler et al., 2012; Mirelman et al., 2015), in almost all other respects (both clinically and pathologically) LRRK2-associated PD is remarkably similar to idiopathic disease (San Luciano et al., 2010; Marras et al., 2011). Moreover, several large-scale genetic studies have identified LRRK2 as an independent genetic risk factor for sporadic PD (Healy et al., 2008; Satake et al., 2009; Simón-Sánchez et al., 2009; Tan et al., 2010; Ross et al., 2011; Liu et al., 2011a). Thus, it can be argued that much of what we learn from studying pathogenic LRRK2 variants is applicable not only to LRRK2-related PD but also the far more common sporadic form of the disease. Accordingly, our argument that immune-driven monoamine disturbances probably contribute to non-motor symptoms in LRRK2 G2019S PD (based on our proof-

of-concept data) suggests that a similar mechanism may be relevant to idiopathic cases, if only to a lesser degree. This reinforces what researchers have been suggesting for some time now regarding monoamine dysfunction as a likely neural substrate of non-motor PD symptoms (Schapira et al., 2006; Buddhala et al., 2015; Engeln et al., 2015) and adds to the growing belief that inflammatory events may be key drivers of such pathology (Pålhagen et al., 2009; Hassin-Baer et al., 2011; Lindqvist et al., 2012; Rudyk et al., 2015).

This line of reasoning may have important therapeutic implications, particularly for depressed PD patients with any number of common co-morbid inflammatory conditions (e.g., rheumatoid arthritis, Crohn's disease, hypertension) (Savoia and Schiffrin, 2006; Pollard et al., 2013; Nalls et al., 2014). Specifically, it could be the case that in these individuals chronic high levels of IFN- γ and other immune factors (e.g., Mogi et al., 2007; Mount et al., 2007) are increasing the soluble levels of normal LRRK2 to such an extent (and over long durations) that we begin to approximate or phenocopy the exaggerated immune-driven monoaminergic dysfunction and presumed depressive-like behaviours associated with toxic gain-of-function LRRK2 G2019S (Fig. 6.1). A similar logic may also apply to the underlying primary degenerative process (Gillardon et al., 2012). And, importantly, as we saw in the preceding section, recent data indicate that total LRRK2 levels may actually be more crucial in determining toxic outcomes than mutant kinase activity itself (Skibinski et al., 2014).

Accordingly, it is tempting to speculate about a possible role for LRRK2 inhibitors, of which there are many in the research and development pipeline (Martin et al., 2016; Taymans and Greggio et al., 2016), as novel antidepressant agents. An

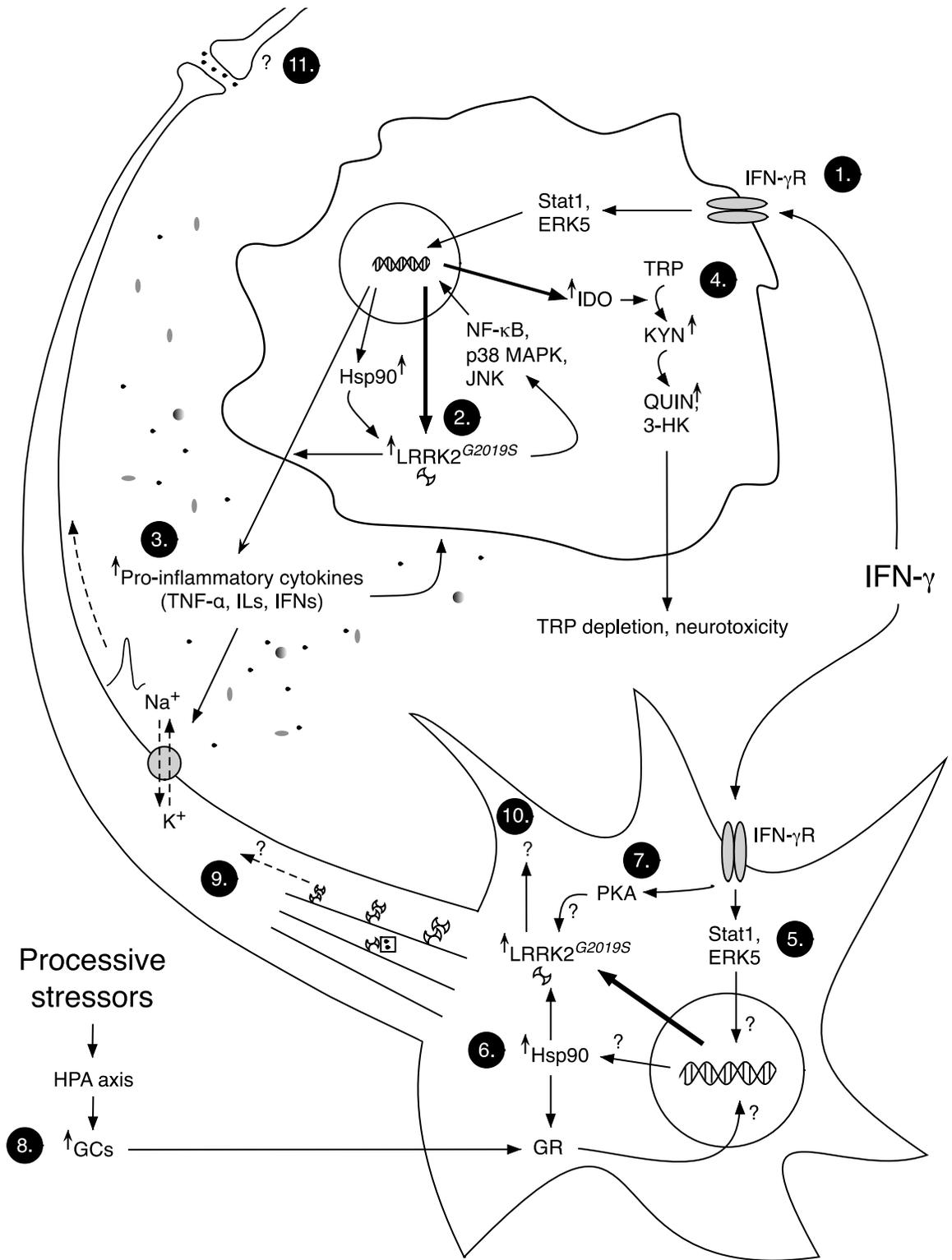
intriguing parallel can be found in the HIV/AIDS literature: while the HIV-1 transactivator of transcription (Tat) protein increased phosphorylated WT LRRK2 levels in microglial cells (Marker et al., 2012), LRRK2 KO protected against some of the Tat-induced neurologic changes in a mouse model of HIV-1-associated neurocognitive disorders (Puccini et al., 2015). However, it should be cautioned that LRRK2 silencing, while not associated with an altered neurobehavioural phenotype in rodents (Andres-Mateos et al., 2009; Volta et al., 2015; Beccano-Kelly et al., 2015), causes biological changes to the presynaptic exo-endocytotic machinery (Cirnaru et al., 2014; Belluzzi et al., 2016) as well as progressive pathological phenotypes in peripheral organs (in particular lung, kidney and liver) (Baptista et al., 2013; Fuji et al., 2015). Additionally, there is growing concern about the immunological implications of LRRK2 inhibition (Liu et al., 2011b; Kubo et al., 2016). Taking the long view then, rather than advocate for LRRK2 silencing as a viable routine treatment of co-morbid depression in PD, we echo the sentiments of Belluzzi and colleagues (2016) and others (e.g., Martin et al., 2016) in calling for studies aimed at identifying LRRK2 interactors, substrates and pathways that could yield novel downstream targets for the development of antidegenerative, and as we submit here, antidepressant therapies.

6.5 Conclusion

Overall the findings presented in this thesis support a role for IFN- γ in depression. Firstly, we show that IFN- γ contributes to the immunological, hormonal, central monoaminergic and, to a lesser extent, behavioural effects of chronic stress. Secondly, we provide evidence directly linking systemically administered IFN- γ to all of these depression-relevant biological readouts. And thirdly, we elucidate a novel role for the

PD-linked gain-of-function mutation LRRK2 G2019S in the central monoaminergic effects of acute systemic IFN- γ and LPS. Extrapolating these findings to the human condition, we suggest that exploring ways to modulate IFN- γ -LRRK2 signalling may hold significant clinical promise in co-morbid depression.

Figure 6.1. Hypothesized working model for IFN- γ /stress-LRRK2 signalling in the modulation of central monoaminergic activity. (1, 2) IFN- γ signals through Jak/Stat and/or ERK5 transduction pathways in circulating and brain-resident immune cells to induce the transcription of LRRK2 and other inflammatory genes (e.g., cytokines and the molecular chaperone Hsp90). (3) In the short-term, secondarily released cytokines cause neuronal excitation and monoamine release. (4) In the long run, however, IFN- γ and other pro-inflammatory cytokines act through genomic mechanisms to diminish the synaptic availability of monoamines; e.g., increased expression of monoamine transporters and inflammatory enzymes (IDO-mediated TRP depletion is shown here). As hypothesized, IFN- γ could induce or stimulate LRRK2 in neurons via any number of potential mechanisms: (5) de novo LRRK2 mRNA and protein synthesis, (6) Hsp90-dependent post-translational stabilization of LRRK2, or (7) phosphorylation by PKA. (8) We also raise the possibility that stress-associated glucocorticoids (GCs) could likewise induce neuronal LRRK2 expression [predicated on the in vitro work of Park et al. (2013)] and that IFN- γ /Hsp90 could potentiate such signalling by increasing GR affinity to GCs (depicted in step 6). (9) If we are to make the argument that LRRK2 is disrupting synaptic vesicle cycling in presynaptic terminals then one must also assume that some of the newly synthesized (mutant) LRRK2 is being trafficked there; this has yet to be empirically tested. (10, 11) However, it should be underscored that LRRK2 plays a role in many other cellular processes (and in compartments other than the presynaptic boutons), several of which are relevant to depression and are therefore worthy of examination in preclinical co-morbid depression models; e.g., neurogenesis (Winner et al., 2011; Paus et al., 2013), LTP (Sweet et al., 2015), autophagy (Plowey et al., 2008), and possibly neurotrophin signalling (Caesar et al., 2014; Dzamko et al., 2016). We suggest that these are important future research directions in LRRK2-related depression and co-morbid illness. Solid arrows (solid arrowhead) indicate positive/stimulatory relationships. Solid lines with blunted ends indicate negative/inhibitory relationships. Broken arrows (solid arrowhead) indicate movement across time or space. Small upwards arrows (swept arrowhead) indicate increased protein levels; grey-filled circles = voltage-gated ion channels; grey-filled ellipses = IFN- γ receptor.



7 Appendix. Supplemental Figures

Presented here are the results of a preliminary study of the behavioural effects induced by subchronic systemic exposure to IFN- γ . At 5-6 months of age, group-housed male WT mice from our in-house LRRK2 heterozygous breeding colony (raised on a C57BL/6J genetic background) were singly housed and randomly assigned to one of two experimental conditions ($n = 8$): recombinant murine IFN- γ (15000 and then 25000 IU, R&D systems; in 0.1% BSA-containing PBS solution) or vehicle (PBS + 0.1% BSA). Injections (volume of 0.3 ml) were administered daily for 7 consecutive days, between the hours of 08:30 and 10:30. On each of experimental days 1 through 6 mice were administered the 15000 IU dose of IFN- γ (or an equivalent volume of vehicle solution); on Day 7 mice were administered the higher 25000 IU cytokine dose.

All mice were assessed in a behavioural test battery comprising the following tests: a) home-cage activity assessments on Days 3 and 6 (23 h assessments commencing ~15-20 min post-injection); b) a nestlet test on Day 4 (1, 3, 5 and 24 h post-injection); c) a social interaction/avoidance test on Day 5 (~ 8 h post-injection); d) spontaneous alternation version of the Y-maze on Day 7 (1 h post-injection); and e) a forced swim test on Day 7 (~ 2 min following completion of the 8-min Y-maze test). Animals were euthanized immediately following the FST. The animals were maintained on a 12-h light/dark cycle with lights on at 0800 h. A diet of standard laboratory mouse chow (Harlan Laboratories, WI) and water was provided ad libitum and room temperature maintained at ~ 21 °C. All experimental procedures were approved by the Carleton University Committee for Animal Care and complied with the Canadian Council on Animal Care's guidelines on the ethical use and care of animals in research.

A.1 Home-cage locomotor activity

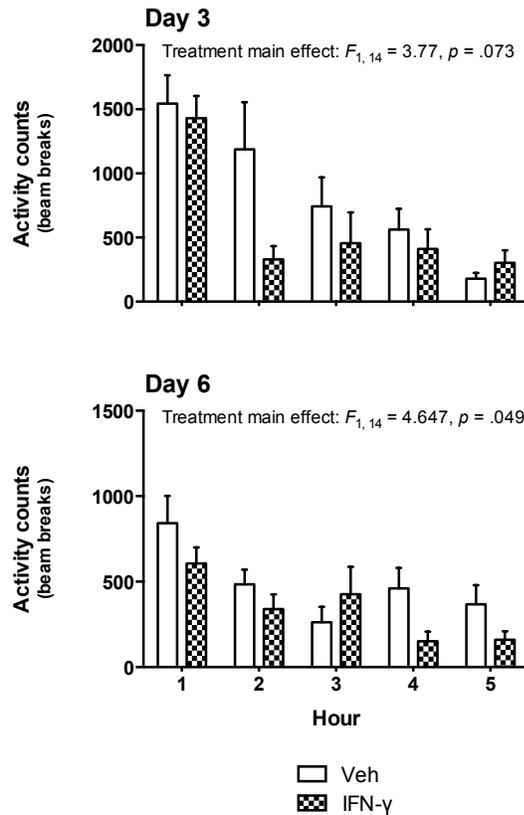


Figure A.1. IFN- γ suppressed home-cage activity in the 5 h immediately following the 6th injection. Measurements of horizontal motor activity were obtained during uninterrupted 23 h periods (commencing at ~ 0900 h) using a Micromax infrared beam-break apparatus (AccuScan Instruments, Columbus, OH) exterior to the home-cage. The ANOVA did not reveal a significant effect of IFN- γ on total (23 h) locomotor activity on either Day 3 or Day 6 (main effect of Treatment: $F_{S1,14} = .244$ and $.072$, respectively, $p > .60$, data not shown). However, when the analyses are restricted to the first 5 h post-injection, IFN- γ can be seen to have caused a significant reduction in Day 6 home-cage activity. Data are shown as mean \pm SEM.

A.2 Nest building behaviour

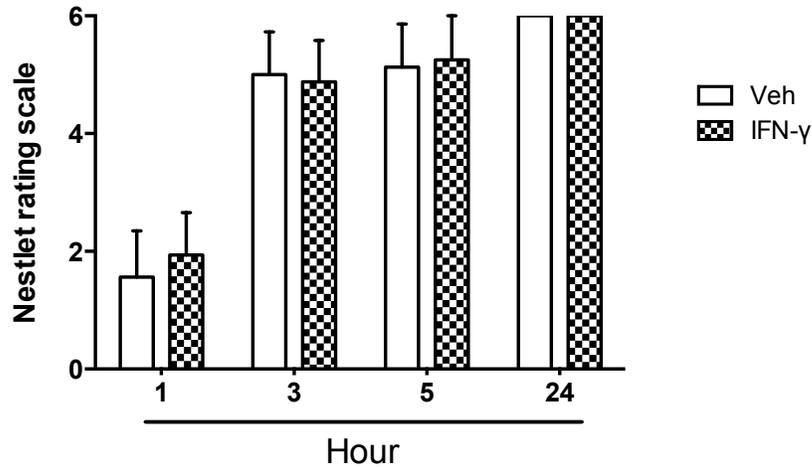


Figure A.2. IFN- γ did not influence nest building behaviour. Nest building is a complex goal-directed behavior driven both by sensorimotor (in particular co-ordinated orofacial/forelimb movement) and motivational systems (Paumier et al., 2013). We were most interested in the contributions of the latter, as motivation disturbance is common in depression and other stressor-associated psychiatric conditions. At approximately 0900 h on Day 4 (i.e., immediately upon completion of the first of the two home-cage activity assessments), mice were transported to their usual housing environment and administered the 4th cytokine or vehicle injection. Animals were then transferred individually into freshly made corncob bedding-lined cages, into which a single cotton nestlet (Ancare, Bellmore, NY) had been placed. Assessments of the quality of the nests were made at 1, 3, 5, and 24 h, using the nestlet rating scale described by Paumier et al. (2013). This scale ranges from 0-6, where 0 indicates the complete absence of a nest and 6 corresponds to a “perfect” nest; i.e., nestlet is 100% shredded and built into a compact and raised 3-dimensional nest that is located in one corner of the cage. Data are shown as mean \pm SEM.

A.3 Social interaction test

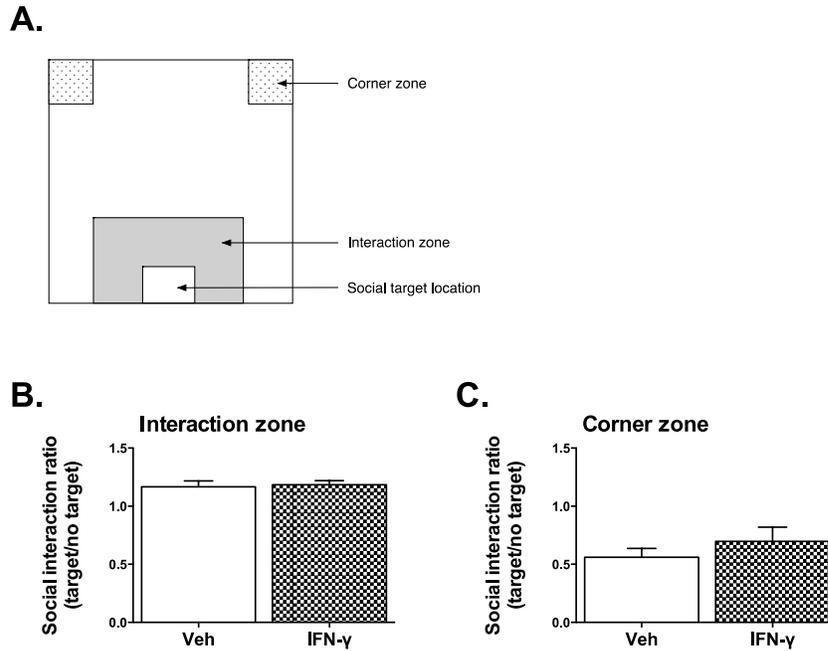


Figure A.3. Social approach-avoidance behaviour was unaltered in mice receiving repeated systemic injections of IFN- γ . Social approach-avoidance behaviour was assessed using a 2-step procedure that has been described elsewhere (Berton et al., 2006). (A) The testing apparatus comprised a 40 cm³ Plexiglas arena, into which a rectangular wire cage had been affixed at one end. During the first test session mice were placed individually into a corner of the arena opposite the location of the empty wire cage and permitted free exploration of the arena for 2.5 min. After spending the next 60 sec in the home-cage, mice were reintroduced into the open field arena (in the same corner as before) and allowed to freely explore their surroundings for 2.5 min. During this second test session the wire cage contained an unfamiliar non-experimental CD1 male mouse (14-18 w.o., Charles River Laboratories, Saint-Constant, QC). The trajectory of the experimental mice during both test sessions was tracked with an overhead video camera connected to an automated video tracking system (SMART-CS, Harvard Apparatus). From these recordings, the duration in the interaction zone (defined as the 8 cm-wide corridor directly surrounding the accessible sides of the wire cage) and the corners (7 \times 7 cm) opposite where the cage was located was determined for each mouse, both in the absence and presence of the social target. Data are presented as social interaction ratios, i.e., time in a given zone “with social target”/time in that zone “without target”. (B, C) IFN- γ did not significantly alter either of the social interaction ratios. Testing was conducted under red light conditions during the final 2 hours of the light cycle (1800-2000 h). Data are shown as mean \pm SEM.

A.4 Spontaneous alternation Y-maze

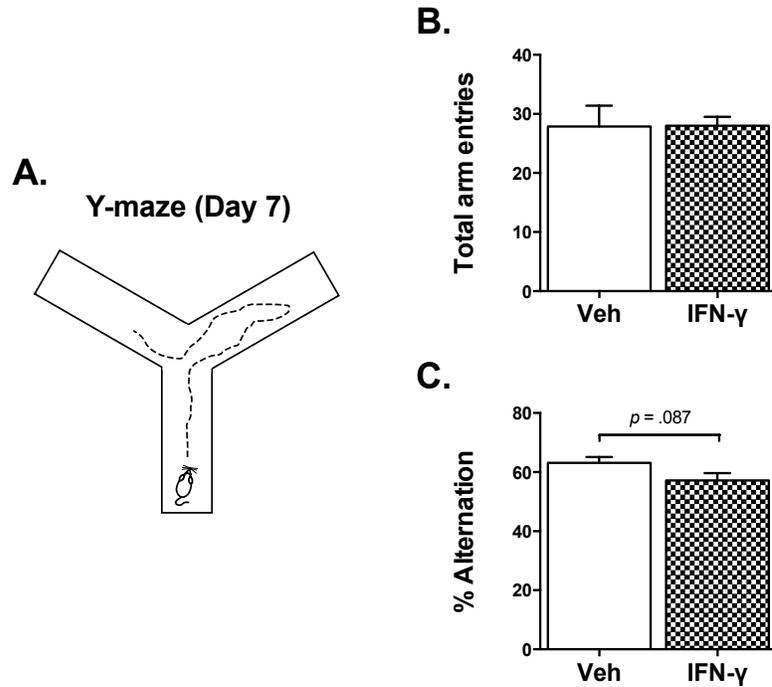


Figure A.4. No effect of IFN- γ on immediate spatial working memory in the spontaneous alternation (free-running) Y-maze. (A) Mice were permitted free exploration of all three arms of the Y-maze for a single 8 min trial. Mice were placed individually in one of three arms ('A', 'B' or 'C'), with head directed away from the center of the maze; the start arm was chosen at random. The testing room was dimly lit and contained global landmarks for orientation (cardboard cut-out shapes affixed to the walls). The animals' path through the maze was tracked by way of an overhead video camera connected to an automated video tracking system (SMART-CS, Harvard Apparatus). Total arm entries (defined as all four legs having entered a given arm) and the order of arm entries were recorded manually for each mouse. We then calculated spontaneous alternation, defined as consecutive entries into each of three arms without repetition (e.g., ABCDC is scored as 2 alternations: ABC and BCD); these data are expressed as % of total arm entries (Sarter et al., 1988). The mice were not acclimated to the testing environment. (B) The IFN- γ treatment did not affect total Y-maze arm entries ($F < 1$). (C) Likewise, % alternation was not significantly different between groups ($F_{1,14} = 3.38, p = .087$). Data are shown as mean \pm SEM.

A.5 Forced swim test

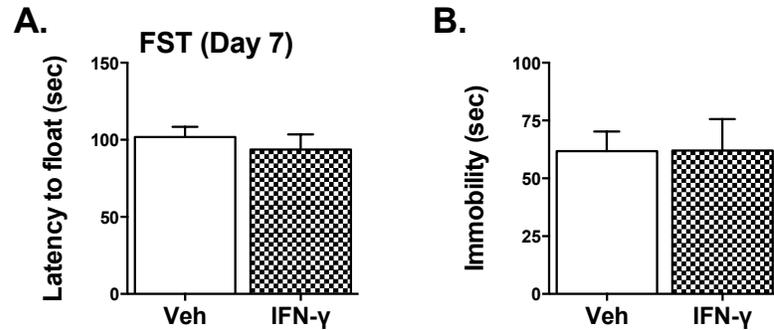


Figure A.5. Forced swim behaviour was unchanged in response to subchronic systemic IFN- γ . Forced swim behaviour was assessed using a modified version of the Porsolt et al. (1977) method. Mice were individually placed in a glass cylinder (20 cm diameter \times 25 cm high) containing tepid water (22 ± 1 °C; 15 cm deep) for 6 min, during which time their behavior was video recorded. Latency to 1st immobility and immobility time during the final 4 min of the test (i.e., floating, with only minimal paw movements necessary to keep the head above water) was determined for each mouse. (A, B) Neither latency to float nor immobility time was altered in response to the subchronic systemic IFN- γ treatment ($F_s < 1$). Data are shown as mean \pm SEM.

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