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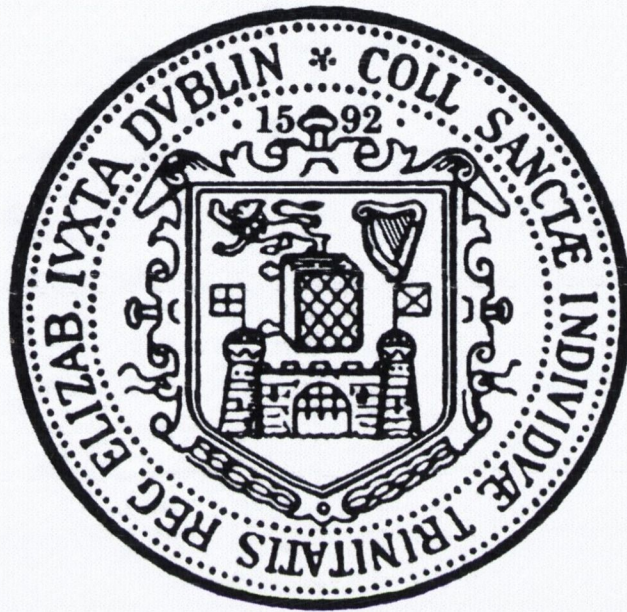
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**INTERFERON-ALPHA (IFN- α) - INDUCED COGNITIVE AND
AFFECTIVE IMPAIRMENTS: CONTRIBUTIONS OF PREFRONTAL
CORTEX AND HIPPOCAMPAL FORMATION**



A thesis submitted to the University of Dublin for the Degree of Doctor of
Philosophy

By

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April 2009

Under the supervision of Prof. Shane O'Mara

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9492

Declaration

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S Barlow

Sally Barlow
April 2009

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Abstract

Interferon-alpha (IFN- α) - induced Cognitive and Affective Impairments: Contributions of Prefrontal Cortex and Hippocampal Formation

Sally Barlow

Interferon-alpha (IFN- α) is a potent antiviral agent useful in the treatment of many malignancies, but most widely used in the treatment of Hepatitis C. However, sinister adverse events appear as a consequence of IFN- α -treatment. Such side effects include fatigue, fever ('flu-like' symptoms) and more serious symptoms such as memory impairments, anxiety, depression and suicidal ideation. Symptoms of major depression present in ~40% of the clinical population taking this drug. The mechanisms underlying these side effects remain elusive; this thesis aims to unravel mechanisms underlying these adverse events.

This body of research characterises prefrontal impairments as a consequence of IFN- α -treatment. From the data presented in this thesis, together with previous findings from our lab, we suggest that there is an underlying dysregulation of the limbic-cortical pathway. Suppression of hippocampal neurogenesis was observed as a result of IFN- α -treatment at a clinically relevant dose; specifically decrease cell proliferation was observed in the subgranular zone. Moreover, the adverse events associated with affective disorders were rescued effectively with concomitant SSRI treatment. In light of these data, further investigations into the basis of hippocampal deficits using magnetic resonance imaging (MRI) were carried out. These MRI findings indicate a remarkable decrease in hippocampal volume in IFN- α -treated animals. It is plausible that a decrease in neurogenesis may contribute to these alterations in hippocampal volume.

A preliminary microarray study was undertaken, demonstrating differential gene expression in IFN- α -treated animals. With further validation, these alterations in gene expression may reveal a potential mechanism of action of SSRI treatment. In addition, potential targets may be used for future research in further characterising the molecular basis underlying the adverse effects of IFN- α -treatment.

While much progress has been made to elucidate the mechanisms underlying the adverse events of IFN- α -treatment, the molecular basis of these events remain unknown. The results revealed here may provide some potential pathways on route to discovering the route of dysfunction associated with IFN- α .

Abbreviations

3OHK	3-hydroxy-kynurenine
5-HT	5-Hydroxytryptamine
5HTT	5-Hydroxytryptamine transporter
5HTTLPR	5-Hydroxytryptamine transporter Promotor
AAF	IFN-Alpha Activated Factor
Acg	Precentral Anterior cingulated gyrus
ADHD	Attention Deficit Hyperactivity Disorder
ASY	Asymmetry
BBB	Blood Brain Barrier
BET	Brain Extraction Tool
BrdU	Bromo-Deoxyuridine
BV	Brain Volume
BW	Body Weight
Cacnali	Calcium Channel voltage Dependent alpha 1 subunit
CBF	Cerebral Blood Flow
Chrm2	Cholinergic Receptor, muscarinic
CML	Chronic Myeloid Leukaemia
CMS	Chronic Mild Stress
COX2	Cyclooxygenase 2
Cxcr3	Chemokine Motif Receptor 3
DBH	Dopamine Beta Hydroxylase
EEG	Electroencephalographic
EPM	Elevated Plus-Maze
FGF	Fibroblast Growth Factor
Flx	Fluoxetine
FST	Forced Swim Test
GCL	Granule Cell Layer
HC-PFC	Hippocampo-PFC
HCV	Chronic Hepatitis C
HPA	Hypothalamic-Pituitary-Adrenal Axis
I.V.	Intravenous
ICAM-1	Intracellular Adhesion Molecule 1
IDO	Indoleamine-2, 3, dioxygenase
IFN	Interferon
IFNAR	Type 1 IFN Receptors
IFN- α	Interferon-alpha
IFN- β	Interferon-beta
IFN- γ	Interferon-gamma
IFN- ϵ	Interferon-epsilon
IFN- τ	Interferon-tau
IFN- ω	Interferon-omega
IL	Infralimbic
IL	Interleukin
IRFs	Interferon Regulatory Factors
ISA	Image Sequence Analysis
ISGF3	IFN-Stimulated Gene Factor 3
ISGs	Interferon-Stimulated Genes
ISRE	IFN-Stimulated Regulatory Element
JAK	Janus Kinases
Kmo	Kynurenine 3-monooxygenase
Kyn	Kynurenine
KynU	Kynureninase

LHV	Left Hippocampal Volume
LTP	Long Term Potentiation
MD	Mediodorsal Thalamus
MIU	Million International Units
mPFC	Medial Prefrontal Cortex
MRI	Magnetic Resonance Imaging
NFκB	Nuclear Factor Kappa B
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OFC	Orbitofrontal Cortex
OVLT	Organum Vasculosum Laminae Terminalis
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral blood mononuclear cells
PCA	Principle Component Analysis
pDC	Plasmacytoid dendritic cells
Pdgfrb	Platelet Derived Growth Factor Receptor
PEG-IFN-α	Pegylated IFN-alpha
PFC	Prefrontal Cortex
Ppp2r2c	Protein Phosphatase 2
PPRs	Pattern Recognition Receptors
PrL	Prelimbic
PTKs	Protein Tyrosine Kinases
RARE	Rapid Acquisition Relaxation Enhancement
RF	Radio Frequency
RHV	Right Hippocampal Volume
RNA	Ribonucleic Acid
ROI	Region of Interest
rtPCR	Real Time Polymerase Chain Reaction
s.c.	Subcutaneously
Sal	Saline
SEM	Standard Error of the Mean
SGZ	Subgranular Zone
SPECT	Single Photon Emission Computed Tomography
SSRI	Selective Serotonin Reuptake Inhibitor
STATs	Signal Transducer and Activators of Transcription
SVR	Sustained Virological Response
SVZ	Subventricular Zone
T1	Longitudinal Plane Relaxation Time
T2	Transversal Plane Relaxation Time
TE	Echo Time
THV	Total Hippocampal Volume
Tjp-1	Tight Junction Protein 1
TLR-3	Toll-Like Receptor 3
TNF	Tumor Necrosis Factor
Tpo	Thyroid Peroxidase
TR	Inversion Time
Trp	Tryptophan
VBM	Voxel-Based Morphometry
VEGF	Vascular Endothelial Growth Factor
WPM	Water Plus-Maze

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Chapter I

Literature Review

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A FIFTY-year-old woman living in Japan is infected with a potentially fatal virus, hepatitis C. Doctors bombard her body with a powerful drug to boost her immune response. The drug beats back the virus, but has horrific side effects. She becomes inexplicably moody, rapidly sinking into a depression so savage that the woman douses herself in oil and sets herself alight.

Fortunately, her suicide attempt fails and she recovers fully. But the woman's terrifying experience is not unique.

Extract from (Fukunishi et al., 1998)

1.1. Interferon- α and the Immune System

Viral Attack

It is taken for granted that our bodies will be able to effectively protect us from viral and pathological challenges. Inevitably, at times vulnerability strikes and we are exposed to pathogens that require medical intervention.

Immune activation results in the induction of signalling cascades of cytokines, chemokines and the stimulation of inflammatory responses. An exaggerated immune response can also elicit an adaptive immune response, moderating future reactions to the pathogen. Interferon (IFN), a specific class of pro-inflammatory cytokine is the principal mediator of the innate immune system. Cytokines are vitally important for immune system regulation which becomes evident when deficits in their induction arise. Patients with a mutation in the UNC93B gene are deficient in the production of Type I IFNs and consequently demonstrate a high susceptibility to infectious agents (Casrouge et al., 2006). The immune system exhibits a delicate balance and it is undeniable that inappropriate or persistent activation of immune responses can lead to behavioural adaptations in both humans and animals (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). These adaptations may present in the form of sickness type behaviour such as anhedonia and fatigue.

1.2. Interferon

What are Interferons?

Isaacs and Lindenmann discovered in 1957 a cytokine aptly termed Interferon, whose name is derived from its '*interference*' in the viral replication process. A series of investigations into viral interference of influenza virus in chick chorioallantoic membranes revealed this novel chemical (Isaacs & Lindenmann, 1957). However, IFN research was remarkably stalled for 20 years until the molecular techniques caught up to support the successful purification of homogenous IFN. Successful purification, cloning, and synthesis of IFN by Nagata et al in 1980 was pivotal in revealing the true properties of IFNs (Pestka, Krause, & Walter, 2004).

IFNs are pleiotropic in nature, exhibiting many beneficial properties such as antiviral activity, immunomodulation and antiproliferative actions (Grandvaux, tenOever, Servant, & Hiscott, 2002). It was also the first cytokine to be discovered and

characterised (Gutterman, 1994). IFNs comprise a glycoprotein family consisting of 7 species in humans, IFN- α (alpha), - β (beta) - ϵ (epsilon), - κ (kappa), - ω (omega), - τ (tau), and - γ (gamma) (Pestka et al., 2004). They may be further subdivided into three main classes based on their ligand-receptor interactions, which also dictate antigenicity. Type I IFNs comprise all of the above species except IFN- γ which is a type II IFN. Additionally, a further category has been determined sequentially named Type III (IFN-like receptors), consisting of interleukin 29 (IL-29), IL-28A and IL-28B (Pestka, 2007).

Type I IFNs demonstrate genetic and structural homology with a molecular weight ranging from 15-24kDa (Chevaliez & Pawlotsky, 2007). IFN- α subtypes are secreted by leukocytes and are the most prolific in nature with 13 subtypes identified (de Weerd, Samarajiwa, & Hertzog, 2007; Pestka et al., 2004). The classification of IFNs is shown in table 1 below.

Table.1. Interferon family members. Adaptation from (Pestka et al., 2004)

Ligand	Alternative Name	Receptor Chain 1	Receptor Chain 2	Signal Transduction Pathways
Type I IFN	IFN- α IFN- β IFN- δ IFN- ϵ IFN- κ IFN- τ IFN- ω Limitin	IFN- α R1	IFN- α R2	Jak1, Tyk2 Stat1, Stat2, Stat3 Stat4, Stat5 P13K Akt NF κ B MAPK p53
Type II IFN	IFN γ	IFN- γ R1	IFN- γ R2	Jak 1, Jak 2 Stat 1, Stat 3, Stat 5 P13K Akt NF κ B MAPK
IL-28A IL-28B IL-29	IFN- λ 2 IFN- λ 3 IFN- λ 1	IL-28R1	IL-10R2	Jak 1, Tyk2 Stat1, Stat2, Stat3 Stat 5

A general discussion of the action of IFNs will be included in this thesis, however, emphasis will be limited to an in depth assessment of the mechanisms of action of interferon-alpha (IFN- α) and provided in the context of the CNS. Exhaustive analysis of the other IFNs is beyond the scope of this thesis however, IFN- β and IFN- γ will be mentioned where appropriate.

1.3. Viral Induction of Interferons

The immune system does not lie dormant in the absence of infection; IFNs circulate at very low levels in the peripheral nervous system, conferring protective potential.

Moreover, Taniguchi and Takaoka (2001) describe another role for this weak signal in priming the immune system, this preparatory role or as they describe, 'revving up' mechanism, charges the system ready to spring into action upon viral invasion (Takaoka & Yanai, 2006; Taniguchi & Takaoka, 2001).

Infectious agents such as viruses are the main inducers of IFNs, however, there are also other candidates such as microorganisms, growth factors, tumours and other cytokines (DeMaeyer & De Maeyer-Guignard, 1988). The principal type I IFN-producing cells in the periphery and most potent producer of IFNs are circulating plasmacytoid dendritic cells (pDC), however, these are absent in the brain parenchyma (Paul, Ricour, Sommereyns, Sorgeloos, & Michiels, 2007). Nevertheless, astrocytes, microglia, macrophages and neurons all have the potential to stimulate IFN production in response to viral infection, although a limited production is evident in neurons (Delhay et al., 2006).

Figure 2 shows the process of viral recognition, this is initiated by pattern recognition receptors (PPRs) on the host cell which recognise pathogen-associated molecular patterns (PAMPs) exhibited by infectious agents (Haller & Weber, 2007). Cell surface PPRs such as Toll-like receptor 3 (TLR3) and specific cytoplasmic PPRs, helicases, retinoic-acid-inducible protein 1 (RIG-1) and Melanoma-differentiation associated gene 5 (Mda5) stimulate the up-regulation of interferon regulatory factors (IRFs). Expression of IRF-3 and IRF-7 aid in the induction of interferon stimulated genes (ISGs), which transcribe IFN- α to mount a response (Taniguchi & Takaoka, 2002).

1.4. IFN- α signalling pathways

Interferon-alpha receptors

Type I IFN receptors (IFNAR) comprise of two ubiquitously expressed subunits IFNAR1 and IFNAR2. IFN- α binds to the receptor in between the two subunits, and forms a trimeric structure. Varying pharmacological profiles of the type I IFNs result from the different affinities of the subtypes with the receptors (Paul et al., 2007).

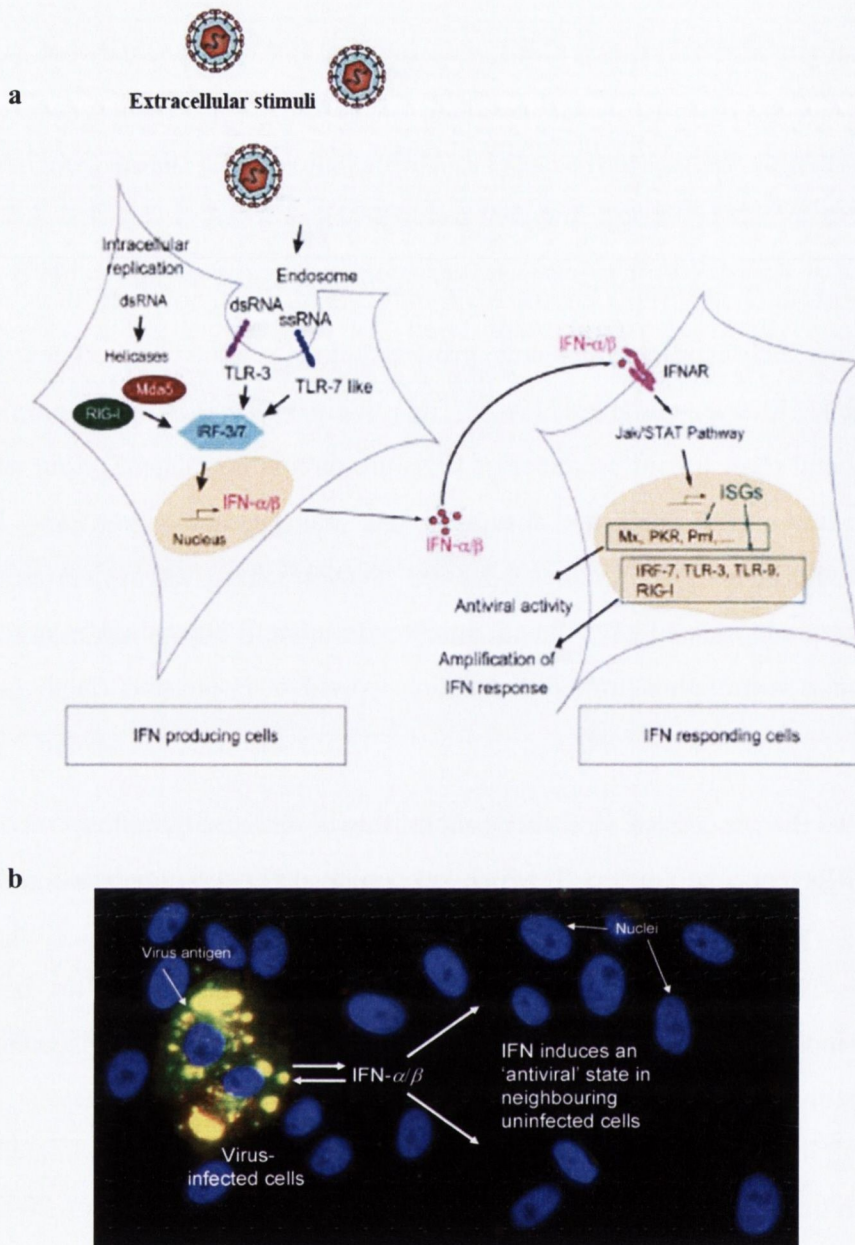


Fig.2. a) Type I IFN production and response. IFN production (left panel) is stimulated when the surface TLRs detect the pathogenic stimuli. Intracytoplasmic helicases sense the viral replication and activate signalling cascades. These converge on the activation of IRF-3 and/or IRF-7; this triggers IFN expression and secretion. IFN response (Right panel) occurs when IFN binds to IFNAR activating the JAK/STAT pathway. This leads to transcriptional up-regulation of hundreds of interferon stimulated genes (ISGs). Upper panel from (Paul et al., 2007) **b)** Annotation of the cellular process of viral response and IFN-induction. lower panel from (Randall & Goodbourn, 2008).

The JAK-STAT Pathway

IFN- α signals via the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, IFNRAs lack intrinsic kinase activity and therefore rely on the

Janus Kinases (JAKs) to phosphorylate receptors (de Weerd et al., 2007). The intracellular domains of the IFNRAs dictate the activation of the JAK/STAT pathway. IFNRAs are associated with Janus protein tyrosine kinases (JAK PTKs), IFNAR1 with Tyk2 and IFNAR2 with Jak1. The JAK PTKs phosphorylate a single tyrosine residue; there are two members of the signal transducer and activators of transcription (Stats), Stat 1 and Stat 2. Phosphorylation results in the production of a homotrimer and heterotrimer of Stat elements. The homotrimeric arrangement, namely IFN- α -activated factor (Monk et al. 2006) is composed of two Stat 1 elements, whereas the heterotrimeric arrangement, IFN-stimulated gene factor 3 (ISGF3) is composed of one Stat 1 and one Stat 2 and IRF-9 (Takaoka & Yanai, 2006). Figure 3 below illustrates the translocation of the complex into the nucleus. This subsequently binds to the IFN-stimulated regulatory element (ISRE) stimulating the production of Interferon stimulated genes (ISGs).

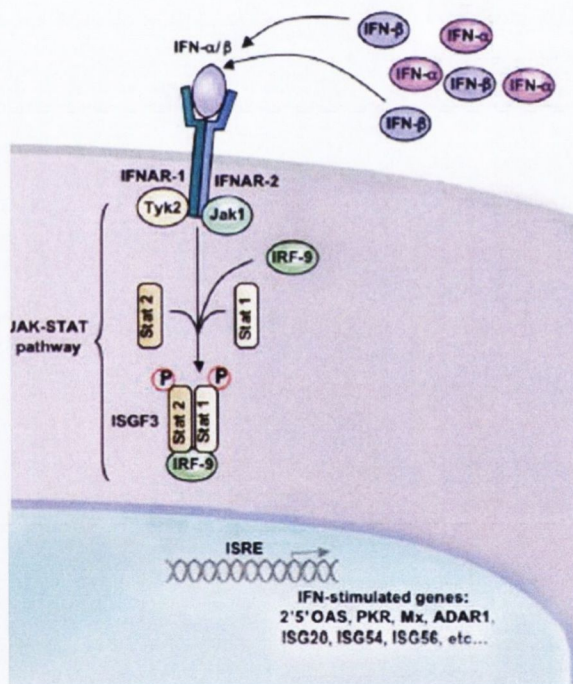


Fig.3. Type I IFN binds to the receptor which subsequently signals the activation of the protein kinases Tyk2 and Jak1. Phosphorylation of the protein kinases results in the formation of a Stat1-Stat2 complex and subsequent binding to the IRF-9 to form the IFN-stimulated gene factor 3 (ISGF3). This complex translocates to the nucleus and binds to the IFN-stimulated response element (ISRE) on specific target genes to direct IFN Stimulated Gene (ISG) expression. Adapted from (Chevaliez & Pawlatsky, 2007).

Studies investigating deficits in IFN reveal the importance of these glycoproteins to the immune system. Type I deficient mice are particularly susceptible to infection (Paul et al., 2007).

1.5. IFN and the Blood-Brain Barrier (BBB)

Access to the brain by foreign substances is restricted by the tight security of the blood-brain barrier (BBB). This acts as an interface between the brain and the periphery impeding the free flow of chemicals from the blood. A barrier breach could elicit an enhanced immune challenge to the brain and result in neurotoxic events. This immunological privilege is advantageous to survival (Paul et al., 2007). The BBB is localised to the endothelial cells of the brain capillaries and is rich in tight junctions. Many diseases can compromise the integrity of the BBB, exposing the brain to vulnerability (meningitis, epilepsy, multiple sclerosis, Alzheimer's disease, and HIV Encephalitis (Hawkins & Davis, 2005). In addition, extreme stress such as acute immobilisation stress in rats may increase the permeability of the BBB (Esposito et al., 2002; Hanin, 1996).

IFN- α is administered systemically, it remains unknown how it produces its consequences on the brain (Wang, Campbell, & Zhang, 2008). Given the properties of the BBB and the size of IFN- α (15-24kDa) it is not known if IFN- α can freely access the brain. However, there are several potential indirect routes in which it may gain entry: 1) passing through the 'leaky' regions; 2) via active transport; 3) via afferent nerve fibres such as the vagus nerve and finally 4) via a compromised BBB. The 'leaky' areas include the posterior pituitary and the circumventricular organs (area postrema, subfornical organ, medial eminence, subcommissural organ, and neurohypophysis). IFN α reaches high levels in the pons and hypothalamus where the BBB is more permeable (Dafny, 1998). BBB breakdown has been implicated in the pathoetiology of cognitive impairment and depression (Bonaccorso et al., 2001).

Interestingly, one study found that seizures in a patient given IFN- α also co-occurred with BBB breakdown (Pavlovsky et al., 2005). IFN- α can also induce an increase in expression of soluble intracellular adhesion molecule 1 (sICAM-1). sICAM-1 levels are an indicator of increased permeability of the BBB, and may be correlated with acute and chronic stress exposure and the subsequent presentation of anxiogenic or depressive symptomology (Schaefer et al., 2004). Conflicting evidence, indicates that

IFN- α and IFN- β (and also glucocorticoids) may tighten the BBB, increasing the electrical resistance across the endothelium (de Boer & Gaillard, 2006). Nevertheless, this study was conducted *in-vitro* and may not reflect the complex dynamics of an intact nervous system. In transgenic mice over-expressing IFN- α there is an up-regulation of integrins in the BBB aiding in strengthening the integrity of the barrier (R. Milner & Campbell, 2006).

These contradictory results indicate that the true relationship of IFN- α and the BBB still remains unknown. However, it remains possible that the breakdown of the BBB is a precipitating factor in the development of severe adverse events associated with IFN- α -treatment.

To address the conundrum further, Wang and colleagues (2007), took an alternative approach and assessed the production of Interferon stimulated genes (ISGs) in the brain as an indicator of IFN- α activation. Using *in situ* hybridisation they found that murine IFN- α but not human IFN- α was detected in the brain. Evidence of ISGs was however abolished in STAT1 knockout mice. Therefore this indicated an IFN- α related dependence on STAT1 (Wang et al., 2008). This is one of the first indicators to suggest IFN- α passes the BBB, however, it remains unknown if preclinical use of human IFN- α travels to the CNS in a similar manner.

1.6. Cytokines and Sickness Behaviour

Sickness behaviour symptoms are sometimes alternatively named non-specific behavioural signs of immune activation. They present in behavioural responses such as; malaise, fatigue, sleepiness, anorexia, apathy and irritability. Sickness behaviour can be induced in laboratory animals by administration of proinflammatory cytokines (TNF- α , IL-1, IL6 and IL2) or by administering agents, such as endotoxins and Lipopolysacharride (Menzies et al., 1996) that activate these cytokines (Cunningham & Sanderson, 2008). The expression of non-specific responses to illness such as neurovegetative and cognitive/depressive side effects has been examined intensively by Dantzer et al (2008). Dantzer refers to the development of sickness behaviour characteristics deriving from immune system activation of proinflammatory cytokines in the circulation producing a corresponding 'molecular image' of proinflammatory cytokines activated in the brain (Dantzer et al., 2008). This may aid in providing an immune surveillance for the CNS.

"Sickness behaviour is like fear--it is a state that makes the animal reorganise its priorities."

(Dantzer, 2001)

The evolutionary role of sickness behaviour has been proposed to originate from a necessity of the organism to reserve energy in order to fight infection, in so doing sickness behaviour develops.

Understanding the role of the cytokine IFN- α and the impact of an activated immune system in animal models of sickness behaviour will hold invaluable translative potential.

2. Therapeutic Potential of IFN- α

There are many beneficial properties of IFN- α , namely antiviral activity, immunomodulation and antiproliferative actions. The very nature of these properties ensures the therapeutic relevance of IFN- α in treating a broad range of clinical disease states. IFN- α is a treatment option in several malignancies, skin (melanomas), kidneys, blood (chronic myeloid leukaemia, CML) and lymph nodes (lymphoma) (Loftis & Hauser, 2004; Schaefer et al., 2002). However, by far the largest clinical population needing IFN- α treatment is sufferers of Hepatitis C.

It has recently been suggested that IFN- α 's many beneficial properties make it a candidate for use prophylactically to prevent cancers (Pestka, 2007). Historically, one study investigated the potential use of intranasal IFN- α to prophylactically treat rhinovirus colds (Douglas, 1986), however persistent side effects precluded its future use. The table above indicates the clinical use of IFN- α is widespread. IFN- α therapy is the only FDA approved treatment for Hepatitis C which further emphasises the importance of unravelling the mechanisms of action underlying this treatment.

Table 2. Therapeutic uses of IFN- α . (Adapted from (Schaefer et al., 2002))

<p>Cancers Hairy Cell Leukaemia Multiple myeloma Kaposi's Sarcoma Cervical neoplasia Basal cell carcinoma Squamous cell carcinoma Melanoma Renal cell carcinoma Carcinoid t-cell lymphoma Non-Hodgkin's lymphoma</p> <p>Viral Disorders Condylomata accuminata Veruca Vulgaris HIV Hepatitis B and C</p>	<p>Myeloproliferative disorders Thrombocytosis Chronic myelogenous leukaemia Polycythaemia vera idiopathic thrombocythaemia Myeloid metaplasia</p> <p>Rheumatoid and immune-related disorders Rheumatoid arthritis Systemic sclerosis Lupus erythematosus Behcet's syndrome Atopic dermatitis</p>
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2.1. Chronic Hepatitis C

2.1.1. Incidence and Prevalence

Sufferers of Chronic Hepatitis C (HCV) remain the largest clinical population worldwide (WHO). The disease affects in excess of 170 million people worldwide and promotes a challenge to morbidity and mortality. Figure 4 shows the worldwide alteration of incidence in a 5 year period. The significance of the clinical population results in the ranking of HCV as the leading cause of liver cirrhosis and transplantation in the developed world (Liang & Hoofnagle, 2000). Current guidelines emphasise focus on prophylactic treatments and the development of a vaccines in order to eradicate the disease (Manns et al., 2007).

2.1.2. Modes of Transmission

Most cases of HCV in the developed world are acquired via intravenous (I.V.) drug use (Manns et al., 2007). However there are other routes of infections such as blood transfusions and sexual transmission, although many cases of HCV are idiopathic in nature.

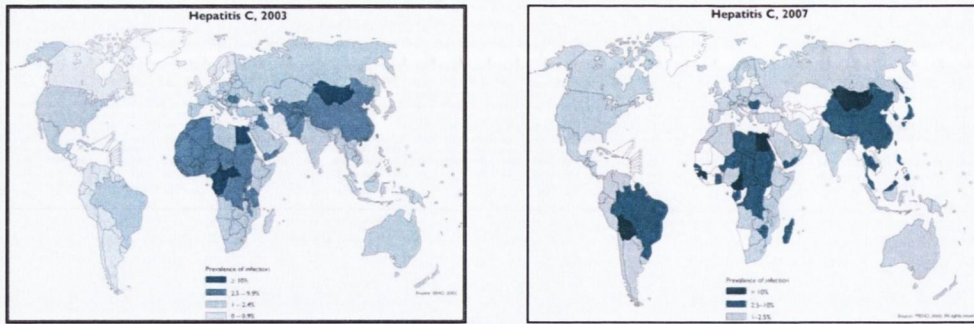


Fig.4. World Health Organisation (WHO) incidence maps demonstrating the worldwide incidence of Hepatitis C. The two panels illustrate the changing incidence of Hepatitis C in 5 year duration.

2.1.3. Management of the Disease

Recovery of the disease is assessed by outcome measurements of sustained virological response (SVR) which indicates clearance of the virus. With a long term 24-48 weeks treatment regimen the disease may be eradicated.

There are many factors that dictate the response of individuals to treatment (Table 3). Factors are either patient-centred or a result of the viral genotype the patient exhibits. Patient-centred factors include, age, alcoholism and co-infection with HIV (Gale & Foy, 2005).

Table.3. Factors that are correlated with a sustained response to PEG IFN in Hepatitis C (Feld & Hoofnagle, 2005)

<p style="text-align: center;">Viral Factors</p> <p style="text-align: center;">Genotypes 2 and 3 (versus genotype 1)</p> <p style="text-align: center;">Lower viral levels</p> <p style="text-align: center;">Greater quasispecies diversity</p> <p style="text-align: center;">Acute versus chronic infection</p> <p style="text-align: center;">Host Factors</p> <p style="text-align: center;">Female Sex</p> <p style="text-align: center;">Younger age</p> <p style="text-align: center;">Less Fibrosis</p> <p style="text-align: center;">Lower body weight and body mass index</p> <p style="text-align: center;">Non-African-American race</p> <p style="text-align: center;">Absence of significant co-morbidities (alcohol abuse , renal disease, HIV infection)</p>

There are many shortcomings in treatment, both resulting from the poor tolerability of IFN- α and proportion of patients who remain refractory to treatment. Interestingly, the global density of receptor expression may confer susceptibility to IFN α response; during chronic hepatitis C infection the IFNAR2 transcript and serum levels were lower in responders than non responders. This indicates that IFNAR receptors suppress the effectiveness of IFN- α -treatment in Hepatitis C patients (Mizukoshi et al., 1999).

In patients who do not respond to treatment there are currently no other pharmacological options available other than trying a different IFN- α formulation or extending treatment duration. Pegylated IFN- α (PEG-IFN- α) and ribavirin may increase SVR rates with retreatment. Successful achievement of SVR is 25-40% higher in patients taking PEG-IFN- α and a 10% increase is demonstrated with retreatment (Manns, 2002).

2.1.4. Treatment

Treatment for Hepatitis C has evolved gradually since the first use of IFN- α monotherapy. IFN- α monotherapy is administered subcutaneously (s.c), thrice weekly in the range of 3-6 million international units (MIU). Approved in 2001, the new gold-standard treatment uses Pegylated IFN- α (PEG-IFN α) plus ribavirin. This new combination is more beneficial in its long-lasting (due to its long half life) nature and is also a more efficacious treatment, increasing the SVR of patients. A requirement for increased treatment response ensures novel treatment strategies are under close scrutiny, an excellent review for these can be found in (De Francesco & Migliaccio, 2005; Houghton & Abrignani, 2005).

2.2. IFN- α -induced Adverse Events

There are many adverse events associated with IFN- α treatment (see table 4); these are widespread and often compromise tolerability to treatment resulting in decreases in treatment compliance.

Table.4. Adverse Events Associated with Interferon-alpha therapy Adaptation of Capuron et al. (2002)

Neurovegetative		Mood/Neurocognitive	
	%		%
Fatigue/Loss of energy	80	<i>Depressive symptoms</i>	
Abnormal Sleep	45	Depressed Mood	60
Psychomotor Retardation	40	Anhedonia	30
Abnormal Appetite	35	Suicidal Thoughts	10
		Feelings of Guilt	5
		<i>Anxious Symptoms</i>	
		Tension/Irritability	50
		Anxious Mood	45
		Fear	15
		<i>Cognitive Symptoms</i>	
<i>Somatic Symptoms</i>		Loss of Concentration	30
Pain	55	Memory Disturbances	15
Gastrointestinal symptoms	50	Word Finding Problems	15
		Episodes of Confusion	10
		Indecisiveness	10

Capuron (2002) systematically divided the adverse events into two syndromes, neurovegetative and mood/neurocognitive. She ascertained that somatic ailments presented early in the course of treatment, (within the first 2 weeks), however, the cognitive effects elicited a delayed onset appearing in the third and fourth week of treatment (Capuron, Gumnick et al., 2002). Identification of adverse events associated with IFN- α -treatment can lead to increased efficacy of treatment and decrease the discontinuation of treatment.

2.2.1. Neurovegetative Syndrome

This series of symptoms appear to present earlier in treatment and are more persistent in nature. These are often compared to ‘flu-like’ symptoms.

Fatigue and Muscle Aches

Fatigue is the adverse event that presents with the highest percentage (80%) in patients treated with IFN- α therapy. IFN- α -treated patients are somnogenic, indicating a significant overlap of symptoms with major depressive disorder (MDD). This

symptom is a characteristic of sickness behaviour and may be speculated to occur as an energy saving bid in order to fight off infection (Dantzer et al., 2008). Interferon-mediated fatigue may originate from the induction of hypothyroidism or a hyper-responsive hypothalamic-pituitary-adrenal axis (HPA), events to which IFN- α -treated patients are particularly susceptible (Schaefer et al., 2002). The high incidence of fatigue in patients treated with IFN- α poses a challenge for treatment continuation. Dysregulated sleep and sleep deprivation is associated with increased interleukin 6 (IL-6) and nuclear factor kappa B (NF- κ B) in the cortex, both of which are stimulated by the immune system (Capuron et al., 2009). Malik et al (2001) provide an excellent review outlining definition criteria and management strategies for IFN- α -mediated fatigue (Malik, Makower, & Wadler, 2001). The criteria for management of IFN- α -mediated fatigue are dictated by established objective grading systems for fatigue. A baseline for each patient should be recorded in order to determine specific endpoints to treatment interventions on an individual patient basis.

Interestingly, the TNF- α antagonist Etanercept can decrease the incidence of fatigue and asthenia in cancer patients while still maintaining effective treatment (Monk et al., 2006). Additionally the implementation of an exercise regime has been assessed in IFN- α patients, this may be a promising approach in the alleviation of symptoms of fatigue (A. L. Schwartz, 2007). Exercise may alleviate fatigue through increased functional performance but also may increase the feeling of control, independence and self esteem (Dimeo, Schwartz, Wesel, Voigt, & Thiel, 2008).

Fever

The main endogenous pyrogenic mediators are IL-1, IL-6, TNF- α and IFN- α (Cocconi, Lees, Mancilla, Belizario, & Dinarello, 1993). The basis of fever resides in the activation of thermosensitive neurons located in the preoptic/anterior hypothalamus and prostaglandin neurons (Dinarello et al., 1984). Nikoshima and colleagues also found another mode of fever induction, via the opioid receptors which are known to bind IFN α . The opioid receptors are the first line response to fever whereas prostaglandins take over for long term febrile responses (Blatteis & Li, 2000; Nakashima et al., 1995). Systemically administered IFN- α may activate febrile responses by altering firing rates of neurons in the Organum Vasculosum Laminae Terminalis (OVLT) (Shibata & Blatteis, 1991).

Anorexia and appetite regulation

Anorexia is another known adverse event specific to IFN- α which may result in a loss of 15% of body weight (Dafny, 1998). Other pro-inflammatory cytokines (IL1, TNF- α) are also well known mediators of anorexia. The anorexigenic role of IFN α is mediated by inhibition of the lateral hypothalamic area and stimulation of the hypothalamic ventromedial nucleus (Hori, Katafuchi, Take, & Shimizu, 1998; Plata-Salaman, 1996). However, it is important to note pyrexia and increased sleeping also contribute to a potential anorexic effect.

Motor Slowing

Parkinsonian-like symptoms have been linked with IFN- α -treatment (Horikawa, Yamazaki, Sagawa, & Nagata, 1999; Mizoi, Kaneko, Oharazawa, & Kuroiwa, 1997). The effect of IFN α on the Basal Ganglia has recently been unravelled, largely due to an elegant PET (Positron Emission Tomography) imaging study conducted by Capuron et al (2007). This study revealed an IFN- α -induced increase in glucose metabolism in subcortical brain regions, specifically the basal ganglia and cerebellum. This increase was interpreted as indicative of an altered dopamine activity, via the degeneration of inhibitory dopaminergic neurons in the substantia nigra. This disinhibition results in increased activity in the basal ganglia (Capuron et al., 2007). Further support for the implication of decreased dopamine in pathophysiology stems from the rescue of motor slowing with the common Parkinsons' disease treatment Levodopa (Sunami, Nishikawa, Yorogi, & Shimoda, 2000). Additionally, IFN- α has been shown to produce motor slowing in mice and result in a decrease in dopamine (Dunn & Crnic, 1993; Shuto, Kataoka, Horikawa, Fujihara, & Oishi, 1997). Recently, decreased motor speed and correlations with neuropsychiatric symptoms have been found, suggesting a relationship between motor slowing and the development of depression (Majer et al., 2008).

2.2.2. Mood and Neurocognitive Syndrome

There is a wealth of literature illustrating a co-morbidity of mood and cognitive disorders in patients treated with IFN- α . Raison (2005) further describes three main syndromes affecting neurocognition: 1) an acute confusional state; 2) a depressive syndrome and, less common, 3) manic conditions (Raison et al., 2005). The acute confusional state is often observed in patients taking high doses of IFN- α via i.v. or

i.c.v. route. An excellent review is provided by Raison on these specific syndromes. The perspective we will take is to focus on depression and anxiety related symptoms. Specific cognitive disorders will be included alongside a discussion into the involvement of specific anatomical regions.

IFN- α and Depression

The majority of research assessing adverse events in IFN- α treatment indicates depression as a significant contender. The DSM-IV criteria categorise IFN α -induced depression as a “substance-induced mood disorder”, the symptoms associated with this category reflect those of major depression (Raison et al., 2005). Additionally, the criteria include symptoms that are comorbid with depression, i.e. anxiety and fatigue. A recent study conducted by Capuron et al (2009), investigated cytokine-induced depression and idiopathic depression and found a high degree of overlap between these two types of depression (Capuron et al., 2009). However, much confusion surrounds the incidence of IFN- α -induced depression, spurred from the inconsistencies in diagnoses. Interestingly, it was determined that medically healthy depressed patients experienced more pronounced feelings of guilt. The IFN- α patients however, did not express such emotion; this was comparable to patients suffering from depression as a consequence of bereavement. The origin of the distress experienced may explain why IFN- α patients experience less guilt, the depression was seen as outside of their locus of control, therefore more comparable to reactive depression (Kendler, Myers, & Zisook, 2008). The sample size in this experiment was small (20 medically ill and 28 medically healthy patients); however the results appear consistent with the literature.

Neurovegetative symptoms are included in the DSM-IV classification and are also common-place in sickness behaviour in animals and humans as a consequence of cytokine therapy/immune challenge. However, Capuron found that treatment (selective serotonin reuptake inhibitor (SSRI) paroxetine) of patients presenting with IFN- α adverse events resulted in recovery from the mood/neurocognitive effects but not from the neurovegetative syndrome (Capuron & Miller, 2004). This suggests that neurovegetative adverse events although manifesting alongside depression, may also be experienced in isolation. Bonaccorso et al (2002) conducted a study in hepatitis C patients determining that 40.7% of patients experienced major depression by the end of treatment (Bonaccorso, Marino, Biondi et al., 2002). Preclinical studies are vital to

unravel the complexities of the underlying mechanisms of depression associated with IFN- α . Depressive adverse events have been successfully confirmed in rodent models of IFN- α using the forced swim test (Fahey, Hickey, Kelleher, O'Dwyer, & O'Mara, 2007; Makino, Kitano, Komiyama, & Takasuna, 2000).

Suicidal Ideation

A study conducted by Dieperink and colleagues (2004), reports the incidence of suicidal ideation (SI) in patients not receiving IFN- α -therapy as 27% and those receiving IFN- α 43% (Dieperink et al., 2004). This high incidence of suicidal ideation in patients taking IFN- α therapy underscores the gravity of the adverse effects experienced as a consequence of this therapy.

2.2.3. Risk Factors and IFN- α -induced depression

There appears to be a susceptibility to neuropsychiatric disorders in patients treated with IFN- α , while not all patients exhibit severe adverse events.

Predisposition to Neuropsychiatric disorders

Early life stress, personal or family exposure to depression may be precipitating factors for developing neuropsychiatric deficits as a consequence of IFN- α (Heim, Newport, Miller, & Nemeroff, 2000; Holscher et al., 2000; Musselman et al., 2001). IFN- α may be withheld when psychiatric predisposition has been noted (Dieperink, Willenbring, & Ho, 2000). Studies in preclinical models have confirmed that the detrimental effect of IFN- α on affect can be augmented by previous stressors (Anisman & Merali, 2003; Anisman, Poulter, Gandhi, Merali, & Hayley, 2007). Additionally, Capuron and colleagues (2003) determined that there is a specific vulnerability that may be predicted by hyperactivity of the HPA axis and the presentation of IFN- α adverse events (Capuron & Dantzer, 2003).

Demographics

IFN- α appears to have a more pronounced effect with age, considering the altered immune system with age this may be responsible for hypersensitivity to depressive episodes.

Treatment Dose and Administration

It is apparent that the severity of the adverse events experienced by patients treated with IFN- α therapy is dictated by variations in treatment dose and method of administration. More severe side effects may be evident with administration of IFN α via i.v/i.c.v methods (Schaefer et al., 2002). Discontinuation is often correlated with an increased dose, indicating that dose has a significant impact on tolerability. One study compared the rate of depression between those treated with the more classical IFN- α monotherapy or the contemporary PEG-IFN- α -treatment (Kraus, Schafer, Faller, Csef, & Scheurlen, 2003), and found the rates of depression did not differ in these methodological administrations.

Could the Hepatitis disease itself infer vulnerability?

It is well established that genotypic variations in the population of patients suffering from the disease can affect treatment efficacy. In addition, the adverse events experienced by Hepatitis C sufferers appear to be more pronounced (at the same dose) than those treated for other malignancies. Hepatitis C patients are immune challenged, upon HCV infection the immune system engages to try and ameliorate this attack. However, HCV evades the innate immune response, dampening down the production of IFN- α .

Alterations in the brain metabolite levels (choline-containing compounds and N-acetyl aspartate) in HCV have been established, which may be associated with cognitive impairment. Recently, Forton and colleagues (2008) found an increase in numbers of activated microglia in the brain of HCV patients in the absence of IFN- α treatment (Forton et al., 2008). This suggests that there is a putative stress activated backdrop in HCV patients that may prime vulnerability to IFN α . Although it is important to note, HCV patients have a higher incidence of depression prior to IFN- α -treatment.

3. Anatomical and molecular mechanisms involved in IFN- α Deficits

We now move on to discuss the brain regions and molecular mechanisms that may be involved in IFN- α -deficits. The hippocampus, prefrontal cortex and amygdala are anatomical regions associated with the pathophysiology of depression (Drevets, 2001).

We will focus on the hippocampus and prefrontal cortex for the rest of this review but note where appropriate the importance of the amygdala.

3.1. Sites of Putative Anatomical Dysfunction

3.1.1. The Hippocampus

The hippocampus is a major structure of the medial temporal lobe; this is depicted in figure 5 below. A seminal study reported by Scoville and Milner (1957) concerning a destructive surgery of the hippocampus was pivotal in revealing the function of this structure (Scoville & Milner, 1957). The surgery on the patient H.M. for intractable epilepsy included the bilateral removal of his medial temporal lobe, including two thirds of the hippocampus, the hippocampal gyrus, amygdala and periamygdaloid cortex. The remaining third of his hippocampus was atrophied and the major afferent (entorhinal cortex) was also destroyed resulting in a complete ablation of this structure. H.M. presented with severe anterograde amnesia on recovery, he could no longer maintain long term memories (declarative memory) however working memory and procedural memory remained intact (Neylan, 2000).

The study of H.M. revolutionised theories on memory formation, providing insight into the different forms of memory and their underlying substrates. It was a direct result of H.M.'s spared memory which revealed that the anatomical basis of memory was a result of a concerted effort of several brain regions. It was determined from this influential case study that hippocampal integrity is vital for declarative memory (Lupien et al., 1997; Squire & Zola, 1996). Squire (1992) demonstrated that the involvement of the hippocampus in memory was only temporary and specified that long term multimodal memories were stored in other regions, such as the neocortex (Squire et al., 1992)

The hippocampus plays a major role in long term memory and is one of the fundamental regions affected in Alzheimer's disease (Huesgen, Burger, Crain, & Johnson, 1993). It is a highly plastic structure, with plasticity associated processes such as Long Term Potentiation (LTP) widely recognised as the substrate of learning and memory (Bliss & Lomo, 1973).

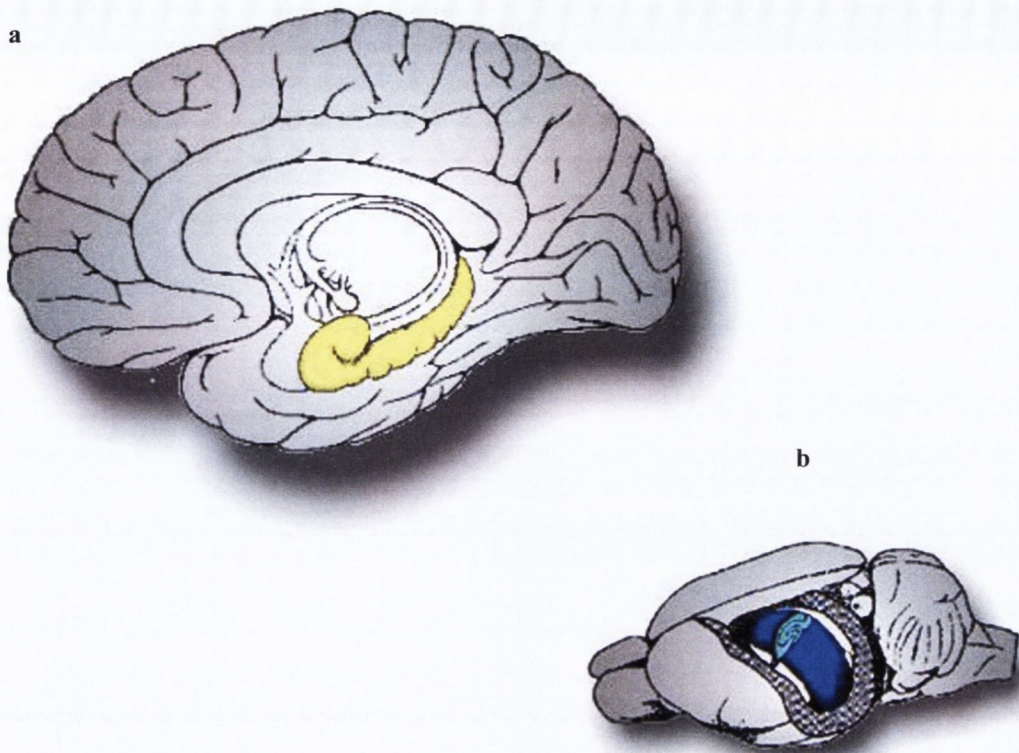


Fig.5. Caricatures illustrating the size and position of the hippocampus in **a**) humans (in yellow) and **b**) rats (in blue, coronal section in aqua). Pictures from O'Keefe and Burgess research group.

The hippocampus is vulnerable to many diseases and may also be affected by many external factors such as pharmacological interventions, stress, and sleep. However, this structure is one of the few structures where neurogenesis (the birth of newborn neurons) arises. The role of the hippocampus in memory is well established, it is only recently that the association has been made between hippocampal deficits and the presentation of mood disorders (E. S. Brown, Rush, & McEwen, 1999; Duman, 2004). Morphological changes including atrophy and retraction of dendrites and cells of the CA3 region may result from stressful situations. Moreover, stressful events can also result in an arrest in neurogenesis and a decrease in volume (McEwen, 1999).

Assessment of hippocampal function may utilise spatial memory paradigms and LTP protocols to determine if dysfunction arises. The role of the hippocampus in spatial memory and navigation is pivotal in animal literature (O'Keefe & Nadel, 1978).

Involvement in IFN- α -treatment

Hippocampal involvement in IFN- α -induced deficits has been investigated previously in our laboratory (Fahey 2005). Fahey (2005), found rats treated with IFN- α exhibited delayed acquisition in the Morris water maze and were unable to perform object displacement tasks. Additionally, the induction of LTP in the perforant pathway was also blocked.

3.1.2. The Prefrontal Cortex

The Prefrontal cortex is widely accepted as a structure responsible for higher cognitive function (Fuster, 1997). Phylogenetically this is the region that develops much later than the rest of the brain. Goldberg (2002) elegantly describes the PFC as the seat of the civilised mind. The PFC has often been referred to as the central executive and behaviour that emerges from this brain region is appropriately termed executive function. The PFC orchestrates finely-tuned activity in many of the other cortical and subcortical regions and is the most richly connected cortical region (E. K. Miller & Cohen, 2001). It has many functions that are often revealed from lesion studies. Historically, the most famous case indicating specific roles for PFC in behaviour was exemplified by the case of Phineas Gage. In 1848, Phineas Gage, a foreman on a railroad construction site, was involved in an accident resulting in a metal tamping rod piercing his skull and through his PFC (figure 6). Initially Phineas appeared cognisant and remained conscious, miraculously it was thought that the accident had caused no ill effects. However, in the coming weeks, colleagues noticed that his personality had changed dramatically, leading co-workers reporting he was “no longer Gage”. Phineas had turned from a reliable, hard working, religious man to one who was angry, promiscuous, and unable to follow plans and directions (Stuss & Knight, 2002). This account illustrates the drastic effects damage to the PFC can have on both personality and executive function in everyday society. Further revelations surrounding the functions of the PFC were determined via dysfunction resulting from several different diseases such as schizophrenia and Attention Deficit Hyperactivity Disorder (ADHD).

Fig.6. Sketch depicting the passage of the tamping rod through the skull of Phineas Gage, as reconstructed by his doctor, Harlow in 1868. Taken from (Stuss & Knight, 2002)



Subregions of the PFC

In humans the prefrontal cortex is clearly demarcated anterior to the precentral gyrus. However, much controversy surrounds the delineation of structural and function domains of the prefrontal cortex in rodents. The prefrontal cortex exhibits significant anatomical variation between species. This cross-species diversity has led some researchers to debate the existence of PFC in rodents entirely and question the significance of studying PFC in rodents who may not share homologous anatomy with humans and non-human primates (Preuss, 1995). Uylings (2003) challenged this argument in an elegant review examining the anatomy and connectivity of the rodent prefrontal cortex. It is now widely accepted that rodents do possess PFC, however discrepancies still remain in the anatomical characterisation of these cortical territories that need to be reconciled (Ongur & Price, 2000).

The prefrontal cortex in the rat can be subdivided into three well-defined topographical regions, the medial, lateral and ventral PFC. The medial PFC (mPFC) can be further subdivided into dorsal and ventral regions, comprising the precentral (PrC/Fr2) and anterior cingulate (ACg) cortices in the former region and the prelimbic (PrL), infralimbic (IL) and medial orbital cortices in the latter (Figure 7) (Dalley et al., 2004; Uylings, Groenewegen, & Kolb, 2003). Of these divisions the mPFC and vPFC have received most attention in rodent models (Dalley et al., 2004). Historically the first defining feature of the possession of prefrontal cortex was implicated by the receipt of a reciprocal connection with the mediodorsal thalamus (MD) (Rose & Woolsey, 1948).

Thus far there is a paucity of research investigating prefrontal function in IFN- α -treatment. Four key studies document potential prefrontal deficits including alterations in anterior cingulate processing (Capuron et al., 2005), decrease in glucose metabolism (Capuron et al., 2007), hypometabolism (Juengling et al., 2000b) and finally decreases in the density of monoaminergic axons (Ishikawa, Ishikawa, & Nakamura, 2007). These will be discussed in a later section.

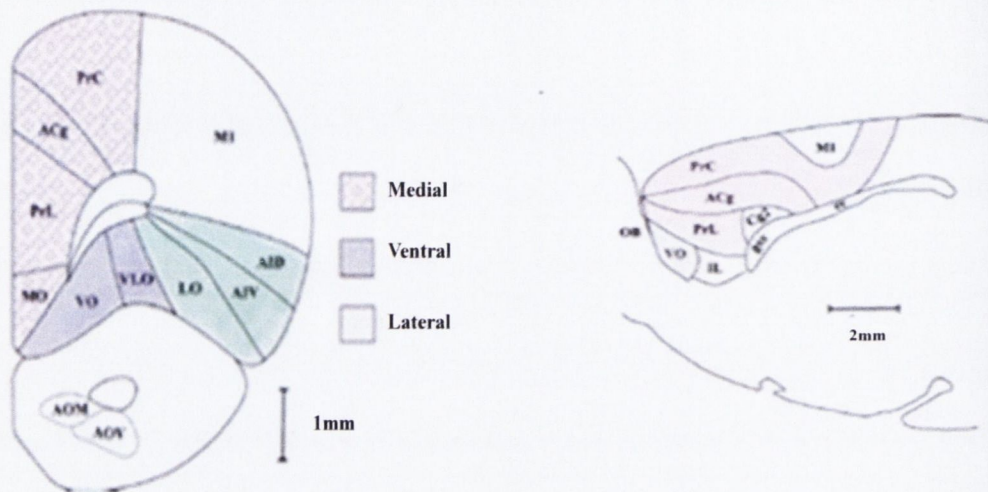


Fig.7. Diagram of the prefrontal cortex in the rodent, a) coronal view b) lateral view, depicting the three major subregions, medial, ventral and lateral PFC. Abbreviations: ACg, anterior cingulate cortex, AID, dorsal agranular insular cortex; AIV, ventral agranular insular cortex; AOM, medial anterior olfactory nucleus, AOV, ventral anterior olfactory nucleus, cc, corpus callosum; Cg2, cingulate cortex area 2; gcc, genu of corpus callosum, IL, infralimbic cortex, LO, lateral orbital cortex; MI, primary motor area; MO, medial orbital cortex; OB, olfactory bulb; PrL, prelimbic cortex; PrC, Precentral cortex; VLO, ventrolateral orbital cortex; VO, ventral orbital cortex. Adapted from (Dalley, Cardinal, & Robbins, 2004)

There is however, widespread evidence suggesting a pivotal role of PFC hypometabolism and subsequent monoaminergic deficits in the pathophysiology of affective disorders (Delgado, 2000). It is surprising given the well-documented affective adverse events associated with IFN- α , that the PFC has not been investigated further in this clinical population.

3.1.3. The Hippocampo-PFC (HC-PFC) Connectivity

We now move on to discuss the interplay of these two regions. There is a direct Hippocampo-PFC (HC-PFC) connection, arising from the ventral CA1/subiculum (Jay

& Witter, 1991; Swanson, 1981). HC-PFC fibres pass through the fimbria/fornix, progress through the nucleus accumbens, the infralimbic area and finally find their endpoint in the prelimbic and medial orbital regions (Thierry, Gioanni, Degenetais, & Glowinski, 2000). This pathway is able to sustain an LTP (Laroche, Jay, & Thierry, 1990), which is suggested to be N-methyl-D-aspartic acid) NMDA dependent (Jay, Burette, & Laroche, 1995). It has been ascertained that this pathway is vital for the organisation of behaviours (Floresco, Seamans, & Phillips, 1997) and dysfunction in plasticity in this pathway is also implicated as a major contributor to neuropsychiatric diseases (Jay et al., 2004).

IFN- α -induced deficits may be mediated via this pathway, previous research in our lab demonstrates hippocampal impairments in IFN- α -treated animals (Fahey, 2005). Although these impairments are subserved by the dorsal hippocampus, the full extent of hippocampal damage is unknown; therefore there could be detrimental effects on the ventral hippocampus. A loss of integrity in the ventral hippocampus would infer the potential for a dysregulation in this pathway.

3.1.4. Hypothalamic-Pituitary-Adrenal (HPA) Axis

Hyperactivity of the HPA axis is implicated in the induction of mood disorders (Gould et al. 1999). The literature implicating the HPA axis in cytokine-induced adverse events is for the most part contradictory. Wichers and colleagues (2007), demonstrate alterations in circulating cytokines (IL-2 and IL-6) and TNF α in Hepatitis C patients but an absence of altered cortisol concentrations (Wichers et al., 2007). A study that contradicts these findings determined a raised peripheral cortisol level immediately after IFN α injection however, and no alteration in IL-6 (Capuron, Hauser, Hinze-Selch, Miller, & Neveu, 2002). Additionally Capuron (2002) and colleagues determined a correlation between this early activation of cortisol in IFN- α -treatment and the susceptibility to depression in long-term treatment. It may be suggested that cortisol levels are not the source of dysfunction *per se* but an indicator of a stress background and vulnerability of individuals to IFN- α treatment (Capuron, 2004).

Other experiments elucidated a structural and functional relationship between IFN- α and adrenocorticotrophin hormone (ACTH) and found that IFN- α can stimulate the release of cortisol in humans (Menzies et al., 1996). Muller et al (1991) found that cortisol levels could increase as much as 300% with subcutaneous IFN α injections (R.

Muller, 1991). However, this may be a short term phenomenon with increased cortisol levels no longer apparent after 3 weeks of treatment (Gisslinger et al., 1993). IFN- α may act directly on the hypothalamus inducing the increase of cortisol which may have a detrimental effect on many anatomical regions. Circulating glucocorticoids have been demonstrated to produce detrimental effects on the hippocampus, resulting in dendrite retraction and a decrease in neurogenesis (Gould & Tanapat, 1999; McEwen, 1999).

3.2. Putative Molecular Mechanisms Underlying IFN- α Pathophysiology

There are many theories underlying the pathophysiology of IFN- α -induced affective disorders. The main theories will be briefly discussed.

3.2.1 Monoamine Balance

Serotonin/5-Hydroxytryptamine (5-HT)

The prominent role of serotonin (5-HT) in the treatment of mood disorders (Baldwin & Rudge, 1995; Meltzer, 1990) is well established. This is also emphasized by the wide-scale use of selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine (Dierick, 1996), for the treatment of depression. In preclinical studies it has been demonstrated that the serotonin transporter (5HTT) is up-regulated, therefore up-regulating 5-HT uptake. The mechanism underlying this uptake was found to be modulated by elevated levels of phospho-p38 MAPK, phospho-ERK and phospho JNK (Tsao et al. 2008).

Lotrich et al (2008) recently found an interaction between the serotonin reuptake transporter promoter (5HTTLPR), inflammatory activation and the coincidence of depression in IFN- α -treated patients. They also suggested that the depression experienced may be a consequence of poor sleep quality (Lotrich, Ferrell, Rabinovitz, & Pollock, 2009). Decreases in serotonin levels have been demonstrated in previous studies (Kamata, Higuchi, Yoshimoto, Yoshida, & Shimizu, 2000), both serotonin and 5-hydroxyindoleacetic acid are depleted in rats administered with IFN α intracerebroventricular (I.C.V) (Kamata et al., 2000).

The precursor of 5-HT, (Tryptophan (Trp) has also been linked to the pathophysiology of depression. The kynurenine shunt theory emphasizes the importance of Trp metabolism for the maintenance of 5-HT levels (Mangoni, 1974). In the kynurenine (Kyn) shunt, Trp is degraded to kynurenine by the enzyme indoleamine-2,3

dioxygenase (Uda, Ishido, Kami, & Masuhara, 2006) instead of synthesizing 5-HT. This results in kynurenine production effectively, ‘stealing’ the Trp resources and in turn depleting 5-HT levels. IFN- α , and other pro-inflammatory cytokines and TNF- α stimulate the induction of IDO, which then can precipitate the decrease in 5-HT (Loftis & Hauser, 2003).

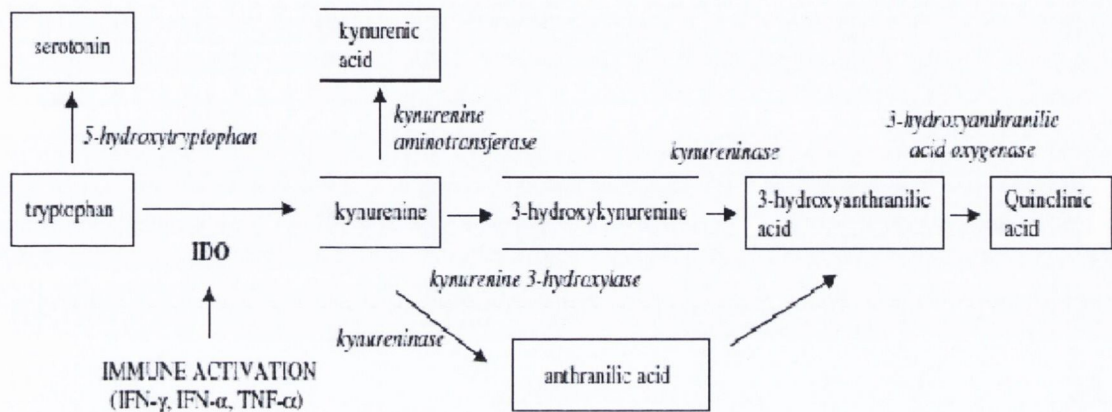


Fig.8. Diagram of the Kynurenine shunt, demonstrating the modulation of tryptophan metabolism and the induction of the kynurenine pathway with IDO. Note IFN- α activates IDO, (Wichers & Maes, 2004).

Another byproduct of this Kyn shunt results in the catabolism of Kyn to 3hydroxykynurenine (3OHK) which has oxidative stress properties and can stimulate apoptotic events in neurons (figure 8). Furthermore, quinolinic acid is also a product of catabolism and has been demonstrated as apoptotic to astrocytes in humans and result in neurodegeneration (Guillemin, Smythe, Takikawa, & Brew, 2005). Several studies confirmed a link between the levels of peripheral Kyn and level of depression and consequential decrease in 5-HT levels in Hepatitis patients treated with IFN- α (Bonaccorso, Marino, Biondi et al., 2002; Capuron & Dantzer, 2003; Wichers, Koek, Robaey, Praamstra, & Maes, 2005).

Dopamine

The dopamine system is suppressed following chronic IFN- α -treatment. Bethus et al (2003) demonstrated a potentiated latent inhibition response in rats treated with IFN- α ;

this is a classical test of dopaminergic activity and indicates a suppression of dopamine in the brain (Bethus, Stinus, & Goodall, 2003). This is consistent with another study in mice demonstrating a decrease in dopaminergic neuronal activity with the onset of IFN- α -treatment (Shuto et al., 1997). However it is apparent that 5-HT can modulate dopamine levels, therefore it is unknown if IFN- α is directly affecting dopamine levels or if this is a secondary effect (Gonzalez-Burgos & Feria-Velasco, 2008). Additionally the HC-PFC pathway requires dopamine for efficient LTP. A global decrease in dopamine levels may have deleterious effects on plasticity in this pathway (Jay et al., 1995).

Noradrenaline

Kamata et al (2000) administered a single dose of IFN- α i.c.v. and measured monoamine levels and found that noradrenaline levels were decreased in the prefrontal cortex (Kamata et al., 2000). Additionally, a decrease in noradrenergic axons in the ventral PFC and dorsal PFC in IFN- α -treated animals has been reported (Ishikawa et al., 2007). It therefore appears that noradrenaline levels are central to IFN- α -induced effects.

3.2.2. IFN- α and opiates

IFN- α , pro-opiomelanocortin and β -endorphin all share structural similarities. Lymphocytes stimulate IFN- α inducers to produce ACTH and endorphin-like substances. IFN- α shares many pharmacological properties with opiates and is able to bind to the opiate receptors, therefore acting like an endogenous opioid (Blalock & Smith, 1981). Blalock and Smith (1981) further revealed that IFN- α was 320 times more potent than morphine. This may lead to adverse events such as anxiety and aggressive behaviour.

Interestingly another beneficial application of IFN- α may be in decreasing the symptoms of opiate abstinence withdrawal symptoms. IFN- α may reduce the dependency and tolerability to opiates. IFN- α given before morphine treatment reduces the withdrawal severity (Dafny, 1998).

3.2.3. Plasticity

Electroencephalographic (EEG) abnormalities were evident in recordings from rats treated with IFN- α for 3 weeks resulting in a dampened EEG response. These animals

exhibited behavioural changes, such as agitation and aggression (Dafny, 1998). Additionally, plasticity-related changes have been demonstrated previously in our laboratory (Fahey 2005). IFN- α -treated animals possess an inability to express LTP in the perforant pathway. This impairment in the induction of LTP is however restored with the co-administration of the SSRI, Fluoxetine. Similarly Mendoza-Fernandez et al. (2000) found that IFN- α inhibits LTP in hippocampal slices but also unmasked Long-term depression (LTD). Using APV to block NMDA channels it was demonstrated that this effect was independent of NMDA channels. The blockade of LTP and induction of LTD is hypothesised to occur as a result of non-NMDA glutamate synaptic potentials, involving the postsynaptic action of tyrosine kinases (Mendoza-Fernandez, Andrew, & Barajas-Lopez, 2000)

4. Prophylaxis of Neuropsychiatric Adverse Events

We approach this section by examining the behavioural modification routes of treatment first. Many pharmacological treatments for affective disorders are symptom suppressive rather than curative (DeRubeis, Siegle, & Hollon, 2008), therefore behavioural implementations, attempting to modify the dysregulation in the long term are advantageous.

4.1. Behavioural Modification

Behavioural modification strategies for the treatment of mood disorders are becoming increasingly popular. The treatment not only precludes the requirement for pharmacological intervention but also imparts a sense of ownership to the patient's own well-being.

4.1.1. Cognitive Behavioural Therapy

Cognitive behavioural therapy may be an alternative to the mainstay pharmacological treatments to alleviate depression and anxiety. Cognitive behavioural therapy was recently found to be as efficacious as antidepressant medication and also reduced the risk of relapse (DeRubeis et al., 2008). A strategy that implements a life change is preferable over pharmacological treatment which, although effective, aims to suppress the symptoms rather than provide a cure. Schaefer et al (2002), recognises the importance of psychotherapeutic strategies for IFN- α patients. He suggests education to ensure the patient and family are informed of the transient nature of the

neuropsychiatric effects and of the treatment strategies (Schaefer et al., 2002). Additionally, it is suggested that relaxation therapy may have positive implications for immune-modulated side effects, one study indicates a reduction in interleukin 6 (IL-6) and tumour necrosis factor (TNF- α) with relaxation therapy (Koh, Lee, Beyn, Chu, & Kim, 2008).

4.1.2. Exercise

For the purpose of this thesis exercise is placed under the umbrella of behavioural modification. However, exercise plays an integral part in many people's lives; therefore behavioural modification in this context implies lifestyle change.

'Mens sana in corpora sano'

This memorable Latin quotation is particularly poignant when discussing the positive benefits of exercise for physiology and is translated to 'A healthy mind in a healthy body'. An explosive literature in this area emphasises the positive effects of exercise on general physiology, cognition and psychological well-being. Inversely, a sedentary lifestyle has been shown to produce a negative impact on cognitive function and memory performance (Weuve et al., 2004). Therefore, sedentary lifestyles may be a precipitating factor for several diseases. Exercise may be beneficial in counteracting cognitive decline and for enhancing psychological welfare. It may therefore be beneficial for stress resistance, acting as an external coping mechanism (Collins et al., 2009).

It is well established that exercise has a positive effects on synaptic plasticity, resulting in the stimulation of LTP (O'Callaghan, Ohle, & Kelly, 2007) and activation of neurogenesis (van Praag, 2008). Exercise may positively impact on general cognitive aging (Erickson et al., 2009) and diverse pathological states such as Alzheimer's disease (Jee et al., 2008; Nichol, Parachikova, & Cotman, 2007) and Traumatic Brain Injury (Griesbach, Hovda, Molteni, Wu, & Gomez-Pinilla, 2004).

Several lines of evidence point to the likelihood of the positive benefit of exercise being mediated by nerve growth factors. Circulating IGF-1 levels are responsible for many of the positive effects of learning and memory in animals (Trejo, Llorens-Martin, & Torres-Aleman, 2008). Exercise may prime a molecular memory for BDNF protein induction in the hippocampus (Berchtold, Chinn, Chou, Kessler, & Cotman, 2005). It

has also been ascertained that intact hippocampal neurogenesis is required for the enhancement of spatial memory when induced by exercise (P. J. Clark et al., 2008; Uda et al., 2006); furthermore, exercise can reverse the suppressive effects of LPS on neurogenesis (Wu et al., 2007). VEGF is also necessary for exercise induced hippocampal neurogenesis (Fabel et al., 2003).

We have previously found that exercise can have a positive impact on IFN- α induced deficits in novel object recognition (Fahey, Barlow, Day, & O'Mara, 2008). We demonstrated that the implementation of a chronic exercise regime restores object recognition; however, there is no restoration in reactivity to spatial change.

However a major caveat in using exercise to modulate IFN- α -treatment must be noted here. It has been addressed at an earlier stage in this review that fatigue is one of the most widely reported adverse events in IFN- α treatment. This begs the question of whether IFN- α -treatment result in a non-compliance to exercise treatment. If a regime is initiated prior to treatment the compliance with an exercise regime may not be futile. There is a paucity of research on the implementation of exercise routines in HCV. One study addressed self confidence and body perception in HCV patients treated with pegylated IFN- α and ribavirin and found a positive impact on the SF36 quality of life assessment (Payen et al., 2009). This therefore suggests the implementation of an exercise regime to patients receiving IFN- α therapy could provide a positive benefit.

4.2. Concomitant Pharmacological Treatments

4.2.1. Antidepressants

Antidepressants are currently the gold standard treatment for any depression associated alongside disease. The positive benefits of antidepressants have been elucidated in clinical treatment of Hepatitis C patients. Capuron found that patients co-treated with IFN- α and the SSRI Paroxetine demonstrated a 4-fold decrease in depression (from an incidence of 45% to 11%) (Capuron & Miller, 2004). This led to a significant increase in adherence to the treatment regime with patients ensuring that they would be more likely to finish the course.

Preclinical studies also support the use of antidepressants to alleviate adverse events; Sammut et al (2002) found a reversal in IFN- α induced anhedonia with desipramine

and fluoxetine pre-treatment. Additionally, previous research in our laboratory, demonstrated the positive restoration of object displacement, Morris water-maze and LTP with co-administration of fluoxetine in IFN- α -treated animals (Fahey, 2005).

4.2.2. Anti-inflammatory Agents

The implication of non-steroidal anti-inflammatory drugs (NSAIDs) for treatment of depression stemmed from the revitalisation of the immune-depression theory. De La Garza and Asnis (2003) reported that the NSAID diclofenac sodium altered the monoamine turnover in the brain with acute IFN- α exposure. Serotonin turnover in the PFC and dopamine turnover in the hippocampus were increased with an I.C.V administration of IFN- α . Pre-treatment with diclofenac prevented the increase in monoamine turnover (De La Garza & Asnis, 2003).

The tetracycline analogue minocycline may also be beneficial for the treatment of IFN- α - treatment in decreasing the action of pro-inflammatory cytokines in the brain. Minocycline is known to down-regulate brain macrophages and microglia (Fan et al., 2007). There are specific receptors to microglia in the brain which can lead to activation of pro-inflammatory cytokines (Yamada & Yamanaka, 1995) therefore the functional significance of minocycline is revealed.

It is important to note here that the use of anti-inflammatory agents hold some caveats for treatment, although treating the inflammation they can also expose patients to an increased sensitivity to infection. Any agents that act on the inhibition of proinflammatory cytokines may result in an ineffective treatment of Hepatitis C, therefore any anti-inflammatory treatment regimes need to be monitored closely to regulate the delicate balance between immune injury and repair.

4.2.3. Nootropic Drugs – Modafinil

One study to date implicates the use of Modafinil as a beneficial co-treatment for IFN- α (Martin, Krahn, Balan, & Rosati, 2007). Modafinil is thought to increase dopamine availability by decreasing the presynaptic dopamine reuptake. It is similar to amphetamines in action but holds none of the negative side effects. Martin et al (2006) found that Modafinil aided in slightly reducing fatigue in IFN- α - treated patients. Unpublished findings from our group suggest clinically relevant levels of Modafinil

can have a positive effect on mnemonic and affective tasks, suggesting this may be an interesting avenue to explore.

5. Rationale and Aims of the Thesis

The work documented in this thesis is directed at investigating the neuropsychiatric side effects of IFN- α -therapy. An assimilation of different experimental methods were used to approach investigation into this area. Primarily, behavioural experiments were used to assess both prefrontal function and neuropsychiatric side effects of IFN- α -treatment. Immunohistochemistry was used to assess neurogenesis in IFN- α -treatment and additionally with SSRI co-treatment and pharmacological MRI was used to assess further related changes in plasticity in the form of *in-vivo* structural changes. In addition, a preliminary microarray study was conducted to assess differentially expressed genes and illuminate potential future targets for investigation. Discussed in this review there is a wealth of literature surrounding the appearance of neuropsychiatric deficits in patients treated with IFN- α . However, there are many key questions that remain unanswered. There are several key molecular mechanisms that have been discovered to be involved in the appearance of IFN- α -induced adverse events however much confusion remains. We attempt to decipher these mechanisms further by using novel approaches in the context of IFN- α research. This literature review demonstrates the implication of both prefrontal and hippocampal deficits in IFN- α -treated patients. Here we aim to assess the prefrontal cortex using behavioural tasks and the hippocampus using immunohistochemistry and small animal MRI. This may provide more insight into the level of involvement that these two key regions have in the adverse events that occur as a consequence of IFN- α -treatment.

Chapters II and III

Uncertainty remains concerning the anatomical regions involved in the production of sickness behaviour and the appearance of mood and neurocognitive syndromes associated with IFN- α -treatment. There is a paucity of research investigating the impact of IFN- α on the prefrontal cortex. To our knowledge animal model studies of IFN- α have not been used thus far to assess prefrontal function. This is surprising considering many of the neuropsychiatric adverse events associated with IFN- α rely heavily on the integrity of the PFC. We hypothesised that IFN- α significantly impacts on the PFC, producing executive function disorders. Here we aimed to use behavioural paradigms

to assess the integrity of the prefrontal cortex and further delineate the anatomical regions that may be dysregulated.

Chapters IV and V

The link between inflammation and the incidence of depression is gaining momentum, the overlap between the characteristics of sickness behaviours and depressions are glaringly apparent. However, little is known regarding the underlying molecular mechanisms. One avenue of research that has been ill-defined is the presence of structural changes in the brain of IFN- α -treated individuals. As discussed earlier there is a wealth of literature that indicates alterations in plasticity and mood disorders.

Here we investigate structural remodelling by addressing the impact IFN- α may have on neurogenesis and volume of the hippocampus. Several lines of evidence link depressive disorders with alterations in the integrity of the hippocampus; here we examine this with both immunohistochemistry and volumetrics using magnetic resonance imaging (Fuller, Hemrick-Luecke, & Snoddy, 1994). We aimed to delineate if any plasticity-related or structural changes were apparent in the hippocampus after chronic IFN- α -treatment.

Chapter VI – Microarray and IFN- α -treatment (a preliminary study)

The aim in this study was to assess differential gene expression in IFN- α -treated animals and with SSRI co-treatment. Microarray analysis is a high-throughput technique that can assess thousands of genes in parallel.

Collectively it is hoped that these results will provide some clear focus on the loci of dysfunction in IFN- α deficits. Additionally, we hope to reveal some underlying mechanisms that may be of central importance in the pathophysiology of IFN- α induced deficits.

Chapter II

Prefrontal Deficits in IFN- α -treatment

Mild behavioural Stress and IFN- α treatment (systemic stress) disrupt Temporal Order Memory.

Function is restored with the introduction of a forced Exercise regime.

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Summary

The source of dysfunction that leads to adverse events in IFN- α -treated patients is ill-defined. However, it has been proposed that the hippocampus and the prefrontal cortex (PFC) are strong candidates for the origin of the maladaptive symptoms. To date, hippocampal deficits have been well-characterised; nevertheless, there remains a lack of insight into the nature of prefrontal participation. Here, we explicitly assess the effect IFN- α -treatment has on the integrity of the PFC using a Temporal Order Memory task. Temporal Order Memory is a form of working memory vital for keeping information on-line in ordered sequences by utilising recency discriminations. Prior to investigation of a pharmacological treatment on temporal order memory, a behavioural modification of prefrontal function was implemented. There is a long established link between mild behavioural stress and prefrontal deficits. An initial examination of the effect of mild behavioural stress on prefrontal function was performed. In line with previous data indicating the negative impact of stress on prefrontal function a reduction in temporal order memory proficiency was determined.

Moreover, IFN- α -treatment abolished temporal order memory distinctions completely, leaving performance to chance levels. Recognition memory was spared in these animals, reflecting a dissociation between recognition and temporal order memory processing. Expanding on these initial observations, a longitudinal assessment of temporal order deficits following the cessation of IFN- α -treatment was conducted to determine if symptoms persist. The data herein indicate there are long-term effects post-treatment. Another avenue of enquiry investigates the potential for functional recovery of temporal order memory in IFN- α -treated animals. We reveal that the implementation of a convenient forced exercise regime reverses deficits in temporal order memory. Here we present a direct measure of prefrontal deficits in rats treated with IFN- α therapy and demonstrate that exercise has mnemonic benefit in this mnemonic task. The temporal order memory task is a robust task that can be incorporated into behavioural batteries to assess the effects of pharmacological interventions on higher order memory.

1. Introduction

1.1. The Prefrontal Cortex and Interferon- α -treatment

The nature of the adverse events resulting from Interferon- α -treatment and the anatomical regions underlying these symptoms suggest that treatment has a negative impact on prefrontal function. Patients receiving treatment experience deficits in working memory (Kraus, Schafer, Wissmann, Reimer, & Scheurlen, 2005; Pawelczyk, Pawelczyk, Strzelecki, & Rabe-Jablonska, 2008) attentional processing (Capuron et al., 2005; Pawelczyk, Pawelczyk et al., 2008) and general cognitive impairment (Capuron & Miller, 2004; Lieb et al., 2006; Pavol et al., 1995; Poutiainen et al., 1993). The aetiological basis for these prefrontal deficits remains unknown however, recent studies verify a decrease in serotonergic and noradrenergic axons in the ventromedial and dorsomedial prefrontal cortex (Ishikawa et al., 2007). In addition, prefrontal hypometabolism has been indicated using PET scanning studies (Juengling et al., 2000b). Here our primary aim is to investigate the impact of IFN- α on the integrity of the prefrontal cortex in a rodent model.

Several lines of research suggest that there may be a combined dysfunction of the hippocampus and the prefrontal cortex in IFN- α -treated individuals. The anatomical connectivity of these distinct regions may support the notion that a dysregulation occurs in the neuronal pathway between these two structures.

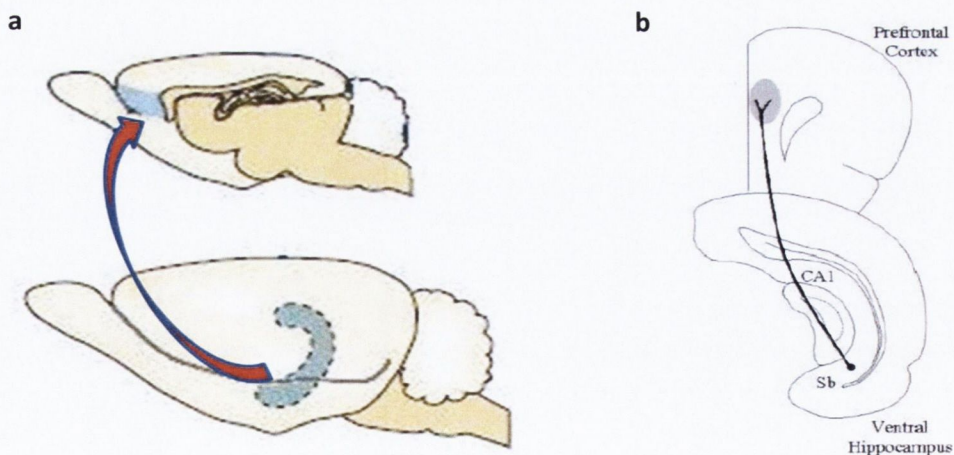


Fig.1. a) Schematic showing the lateral perspective of the direct anatomical connectivity between the ventral hippocampus and the prelimbic cortex in the rat brain. Adapted from (Eichenbaum, 2000). **b)** Coronal sections demonstrating the unidirectional projection from the ventral hippocampus to the prelimbic cortex (Rocher, Spedding, Munoz, & Jay, 2004).

A direct unidirectional, monosynaptic projection relays from the ventral hippocampus CA1 to the prelimbic cortex (see fig 1) (Jay, Burette, & Laroche, 1996; Swanson, Sawchenko, & Cowan, 1981; Thierry et al., 2000). This would suggest that hippocampal deficits may have a subsequent secondary effect on the prefrontal cortex. The reciprocal connectivity between these brain regions is poor with the lack of a direct efferent projection from the prefrontal cortex back towards the hippocampus (Laroche, Davis, & Jay, 2000). A dysfunction in this pathway may underlie deficits that arise; however it is evident that prefrontal damage might have comparable effects. It is therefore of benefit to determine the status of the prefrontal cortex in IFN- α -treated animals to extend the framework of this animal model.

1.2. Examining the Integrity of the Prefrontal Cortex

The prefrontal cortex has received extensive interest in recent years due to its pivotal role in the neural basis of higher cognitive functions. Executive function, a synonym for higher cognitive processes, facilitates the assimilation of plans in specific sequences for goal direction. Despite the interest in this anatomical region the assessment of prefrontal function in a rodent model has been problematic to date. For a review see (Dalley et al., 2004; Dudchenko, 2004; Pontecorvo, Sahgal, & Steckler, 1996; Robbins, 2005). This difficulty is based on two fundamental reasons. Firstly, the tasks that have been validated thus far often have limitations, requiring the use of negative reinforcement, rule learning or long training schedules. Secondly, the prefrontal cortex is the most highly connected of cortical regions; (E. K. Miller & Cohen, 2001; Uylings & Smit, 1974), and has rich connectivity with subcortical regions. When regarding memory processes it is evident that the hippocampus and prefrontal cortex are closely related. Therefore, it is often difficult to investigate one anatomically distinct region in isolation. A task that weighs heavily on the integrity of the PFC and would preclude the need for lesion studies is invaluable. This would help to pinpoint the source of anatomical dysfunction when assessing effects of pharmacological interventions.

Rodents have a natural tendency to explore their environment and are particularly interested in novel objects and experiences (Ennaceur & Delacour, 1988; O'Keefe & Nadel, 1978; Whishaw, 2005). Mitchell and Laiacona (1998) developed a temporal order memory task for rodents utilising a spontaneous exploration paradigm, which capitalises on the natural tendencies of rat behaviour (Mitchell & Laiacona, 1998). The

employment of spontaneous exploration tasks has been adopted freely since the original incorporation of them into behavioural paradigms (Ennaceur & Delacour, 1988). They are not only favoured for their naturalistic basis but also for their ease of administration. The non-invasive nature of these tasks enables reproducibility without practise effects for repeated testing schedules, facilitating the assessments of longitudinal drug profiles. Also of advantage in spontaneous exploration paradigms is the lack of requirement for external motivators reducing confounds caused by physiological limitations (i.e. appetite suppression due to sickness behaviour).

1.3. Temporal Order Memory

Temporal-order memory is a form of memory that utilises the ability to temporally sequence past experiences in order to plan for prospective goals and actions (Fuster, 2001). In essence, it is the recollection of time-stamps that enable us to distinguish new experiences from previous ones in a chronological order. This type of memory is subserved by the prefrontal cortex and is therefore classified as a higher-order mnemonic task (Barker, Bird, Alexander, & Warburton, 2007). Furthermore, it is one of the defining features of episodic memory, representing the 'when' element. Once partnered with the other elements of the triad, 'what' and 'where', a full picture of the episode can be formulated enabling revisits of a past experience (Suddendorf & Corballis, 2007). Temporal-order discriminations are based on recency judgements, signifying that an experience that was encountered remotely in time regains an element of novelty. This may be exemplified by the poetic quote below

"We shall not cease from exploration and the end of all our exploring will be to arrive where we started... and know the place for the first time" T.S. Eliot 1888-1965

In parallel with behavioural data, electrophysiological recordings of order-sequencing in the primate PFC denotes its vital importance to plan future motor behaviour (Ninokura, Mushiake, & Tanji, 2003). Ninokura and colleagues (2004) trained monkeys on a three object visual sequencing task and subsequently identified task related cells in the lateral PFC (Ninokura et al., 2003). 26% of the active cells responded to stimulus properties, whereas 44% responded to rank order. To perform a temporally sequenced discrimination task both stimulus identity and rank are required, this was supported by 30% of the cells selective for both properties (Ninokura,

Mushiake, & Tanji, 2004). Given the positive properties of spontaneous exploration tasks and the pivotal role of the PFC in temporal order memory it was decided that a temporal order memory task would be adopted in this series of experiments.

1.4. Temporal-Order Memory Deficits

It is well established that individuals with prefrontal lesions and dysexecutive syndrome often demonstrate deficits in temporal order representations (B. Milner, McAndrews, & Leonard, 1990); (D'Esposito, Cooney, Gazzaley, Gibbs, & Postle, 2006; Shimamura, Janowsky, & Squire, 1990); (Kesner, Hopkins, & Fineman, 1994; Mangels, 1997). These deficits can disrupt planning and decision making and subsequent behavioural sequencing. This impairment in the executive functions is the most characteristic behaviour observed in patients with damage to the PFC (Fuster, 1995). Despite the literature pertaining to the loss of temporal order memory with traumatic brain injury there are instances where this type of memory remains intact after closed head injury (Schmitter-Edgecombe & Wright, 2003). The prefrontal cortex is thought to be one of the earliest areas to be compromised with ageing although temporal order memory proficiency is conserved in ageing (Sekuler, McLaughlin, Kahana, Wingfield, & Yotsumoto, 2006). However, there do appear to be age related differences in processing this type of memory, which may correspond to compensatory mechanisms (Cabeza, Anderson, Houle, Mangels, & Nyberg, 2000; Fabiani, Kazmerski, Cykowicz, & Friedman, 1996; Grady & Craik, 2000); (Mangels, 1997)). Other pathologies can present with temporal order memory deficits: hippocampal lesions (Mayes & Montaldi, 2001). Alzheimer's disease (Madsen & Kesner, 1995), amnesia (Yasuno et al., 1999), schizophrenia (Dreher et al., 2001; Elvevag, Egan, & Goldberg, 2000; B. L. Schwartz, Deutsch, Cohen, Warden, & Deutsch, 1991), and Korsakoffs syndrome (Downes, Mayes, MacDonald, & Hunkin, 2002). Temporal order memory impairments are evident in a large clinical population signifying the importance of preclinical investigation into this mnemonic function. An important preclinical study in rodents was conducted by Barker et al (2007) examining the anatomical loci of temporal order memory using an elegant lesion design. They demonstrated a dissociable effect of the PRH and mPFC lesions on recognition memory. Rats could not discriminate temporal order memory if the mPFC, PRH or PRH-mPFC lesion groups. However, only the PRH lesion group was impaired in novel object recognition. Therefore one determining factor that is required to assess the type

of dysfunction is to determine if the deficit is a pure recognition deficit or can be attributed to a more complex disorder.

1.5. Stress and Temporal Order Memory

It has been well documented that the prefrontal cortex is particularly vulnerable to the effects of stress (De Kloet, 2004; McEwen, 1998b). In this regard, stress can produce pronounced structural remodelling of the PFC via dendrite retraction (Radley et al., 2005) and also disrupt LTP in the hippocampo-prefrontal pathway (HC-PFC) (Jay, Glowinski, & Thierry, 1989; Rocher et al., 2004). There are also direct reciprocal connections between the PFC and hypothalamus which infers activation of the HPA axis in response to PFC dysfunction (Diorio, Viau, & Meaney, 1993; Sullivan & Gratton, 2002). Accordingly, IFN- α -treatment has been characterised as a systemic stress with reports of the activation of the hypothalamic-pituitary-adrenal (HPA) axis and proinflammatory cytokines (A. H. Miller, Maletic, & Raison, 2009). In light of these commonalities between stress and IFN- α treatment, it would be noteworthy to hypothesize that a putative mechanism underlying the neuropsychiatric and cognitive side effects may be a result of dysregulation in the HC-PFC axis, the HPA axis or structures pertaining to these axes.

1.6. Reversal of Temporal Order Deficits

Prolonged episodes of stress or acute stressful events may have a negative impact on higher cognitive function, in particular temporal order memory processing. There is an increasing incidence of stress-based disorders, general anxiety disorder, and depression (Kendler et al., 2008). Strategies that may ameliorate this stress activation and aid in reparative benefit in regards to specifically temporal order memory await determination.

There is a paucity of research into the potential for a reversal of deficits in this higher-order memory task. One study using a pharmacological intervention indicates a potential promnestic benefit of the selective dopamine D1 agonist, SKF 81297 on retrieval stage temporal order memory judgements (Hotte, Naudon, & Jay, 2005). Other factors that may have a positive impact on temporal order memory have been ascribed to the enhancing effects of sleep (Drosopoulos, Windau, Wagner, & Born, 2007). However, there is a well established body of evidence that indicate exercise has a positive benefit for memory and general cognition (van Praag, Christie, Sejnowski, &

Gage, 1999). Notably, exercise has also been implicated in alleviating mood disorders (Byrne & Salter, 1983). Therefore, the implementation of an exercise regime may be advantageous in more than one domain. Exercise has positive effects on the hippocampus; can this extend to prefrontal function also?

1.7. Rationale for Temporal order Experiments

The experiments presented here were designed to investigate the integrity of the prefrontal cortex under conditions of behavioural and systemic stress. The experiments were conducted in several parts. First, a preliminary experiment was performed in order to determine any delay dependent effects on the consolidation of temporal order memory. We hypothesise that with longer time intervals temporal order memory discriminations will be deficient. Second, mild behavioural stress was used as a probe to assess the sensitivity of the task to prefrontal deficits. Would a behavioural mediator of the PFC (stress), which is far less severe than a lesion, disrupt temporal order memory? Third, the pharmacological impact of IFN- α -treatment on temporal order memory was assessed after four weeks and after treatment cessation. Alongside this, recognition memory was examined to differentiate a temporal order deficit from a pure recognition memory failure. Finally, the potential for the amelioration of temporal order deficits was examined using the implementation of a forced exercise regime. We propose that both behavioural stress and IFN- α -treatment will result in deficits in temporal order memory and that these deficits may be recovered with the exercise. The main aim of this chapter was to further characterise the anatomical basis underlying the adverse events associated with IFN- α therapy.

2. Experiment 1

Validation of a Temporal Order Memory Task

The purpose of this experiment was to assess the ability of rats to discriminate the order of object presentation of two objects by using recency judgements. A further aim was to assess the time frame in which this distinction of object presentation remains intact using delay periods of varying lengths.

2.1. Animals

8 male Wistar rats (BioResources Unit, Trinity College Dublin) weighing between 215-285g were used for this experiment. Animals were housed two/three to a cage within a controlled environment (Laminar airflow unit, 12h light/dark schedule with lights on at 08:00-20:00). Rats received food and water *ad libitum*. Behavioural testing was conducted during the light phase of the schedule. Prior to behavioural testing all rats were handled at regular intervals to acclimatise them to the experimenter. All rats were naïve to injection stress. All experiments were carried out in accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experimentation.

2.2. Materials and methods

Rats were tested in an open grey plastic packing crate with the following dimensions L69 x W45 x H70 cm (length x width x height). The testing room was dimly lit with four lights pointing downwards and placed at the corner of the arena. The arena was surrounded by floor to ceiling length black curtains to prevent any extrinsic cues interfering with the testing sessions. The objects used for exploration consisted of different conformations of children's Duplo® lego bricks. The objects were affixed to the floor of the arena with blu-tack® to prevent displacement during the exploratory phases. Two stopwatches were used to measure the time spent exploring the objects in the exploration trials. The operational definition for exploration was defined as active exploration consisting of head directed movements towards the object and direct sniffing or snout within ≤ 2 cm away from the object face. If excessive climbing, on the object occurred (defined as sitting on the object for several seconds), this was discounted as exploration.

2.2.1. Behavioural Procedure

The behavioural procedure consisted of three distinct elements; two sample phases and a test phase, a schematic is provided in figure 2. Duration of each phase was 5 minutes, varying delay periods between sample 2 and the test phase were selected; 1 hour, 6 hour or 24 hours inter-trial interval. These intervals were counterbalanced for repeated measures so that all the rats were exposed to the three different intervals in a different order. Animals were given a 7 day break between each testing session and new sets of objects were used for each week.

Prior to the commencement of behavioural testing rats were habituated to the empty arena for 3 minutes a day for four consecutive days. On the fifth day behavioural testing began. The objects presented in sample phase one and sample phase two were constructed with distinct conformations.

In sample 1, rats were placed in the arena in facing and in the middle of the long wall; animals were always placed into the arena in the same position. The arena contained two identical copies of objects ('Old' objects) located 10cm away from the walls in the middle of the arena. Following this sample phase the animal was removed from the arena and placed in the home cage for a 1 hour inter-trial interval before re-entry back into the arena for the second sample phase. In sample phase 2, the rat was again placed in the company of two new identical objects ('New' objects). Following a delay period (1 hour, 6 hour or 24 hours) the rat was reintroduced to the arena for the test phase. The test phase consisted of a third copy of each object from the sample phases. The location of the object in the arena was counterbalanced in order to account for any bias towards exploration of the left or right portion of the arena. Additionally the identities of the objects for sample trials were counterbalanced in order to prevent object bias. If temporal order was considered intact the animal would spend more time exploring the object from the first sample phase ('Old' object) than the object from sample 2 (the 'New' object). This distinction in exploration is based on a recency judgement i.e. the object from sample two was seen more recently therefore, the object in sample 1 regains an element of novelty. After all exposures to exploration the arena and objects were cleaned thoroughly with Savlon® and duplicates were used to prevent the detection of odour traces. All behavioural sessions were recorded using the Canopus Mediacruise® system for subsequent analysis.

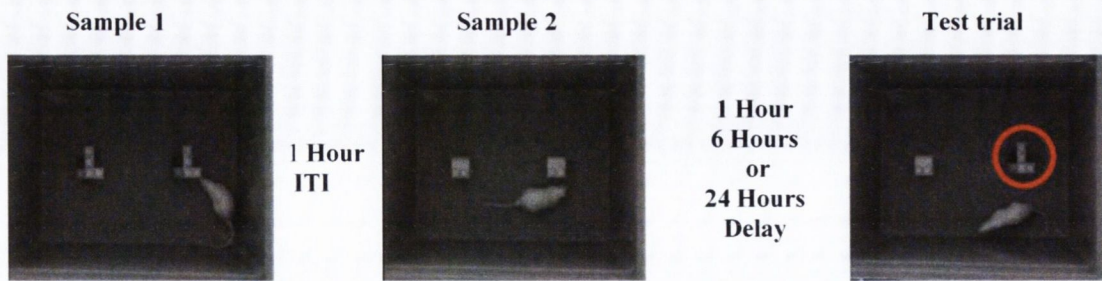


Fig.2. Representation of the experimental design in the Temporal Order Memory task. Two 5 minute sample phases were used followed by a 5 minute test phase. Sample Phase 1 and Sample Phase 2 were separated by a 1 hour inter-trial interval. The second sample phase and the test phase were separated by varying delays of 1 hour, 6 hours or 24 hours. The red ring ('old' object) indicates the target for explorative attention if temporal order memory proficiency is intact. ITI, Inter trial interval

2.2.2. Experimental Measures

Exploration was assessed using the *Discrimination ratio*, calculated by the difference in exploration of sample 1 (old object) and sample 2 (new object) divided by the total time spent exploring the test phase.

2.3. Statistics

All data was analysed using SPSS version 14. Normality was assessed prior to analyses using the Shapiro-Wilk's test. Independent t-tests, repeated measures ANOVA or one-way ANOVA were used where appropriate, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Repeated ANOVAs may provide limitations in interpretation of the data, including the potential for Type I errors.

2.4. Results

One rat was excluded from the analysis due to abnormal levels of exploration (>2 S.D of the mean). Abnormal exploration included less than 10 seconds exploring the objects in the sample trials.

Temporal Order Memory is delay-dependent, impairments occur after a long delay of 24 hours

During the test phase the animals explored the old familiar object more than the new object when a 1 hour or a 6 hour delay period was imposed. In contrast, when a 24 hour delay elapsed the exploration was comparable between old and new objects, therefore no discrimination was present. The performance of the animals at these three test delays can be seen in figure 3. A repeated-measures ANOVA revealed a significant main effect of stimulus object (old versus new) ($F_{(1, 12)} = 6.763$; $p=0.023$). There was no significant effect of test delay, ($F_{(2,24)} = 3.206$; $p= 0.058$), although the p values denotes an approaching significance. There was a stimulus object by test delay interaction, ($F_{(2, 24)} = 3.576$; $p=0.044$) which would account for the converging points on at the 24 hour period. Due to the marginal significance of stimulus, subsequent one-way ANOVA's were conducted and reveal significant differences in exploration of the old and new object after one hour; ($F_{(1, 12)} = 6.543$, $p<0.05$) and six hour delays ($F_{(1, 12)} = 24.179$, $p <0.001$). Exploration of the old and new object were comparable at a 24 hour delay ($F_{(1, 12)} = 2.313$, $p = 0.850$).

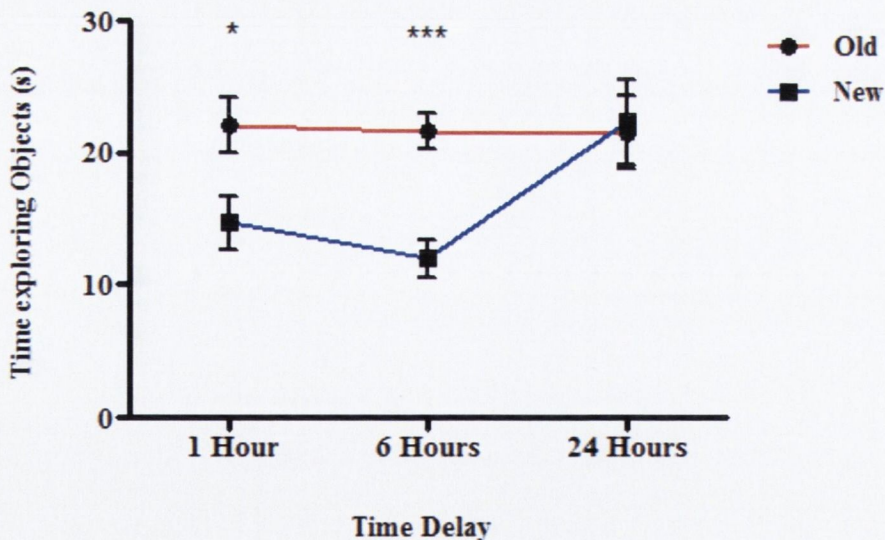


Fig.3. Naïve control animals distinguish temporal order at 1 hour and 6 hour intervals but not at a 24 hour delay. Data presented as absolute mean time \pm SEM spent exploring the old and new objects at the varied delay periods between the sample phases and the test phase (1 hour, 6 hour or 24 hours). * $p<0.05$, *** $p<001$ ($n=7$)

During the test phase the animals explored the old object more than the new object at the 1 hour and 6 hour delay period. The discrimination index in figure 4 demonstrates that at the 24 hour delay period the rats were unable to discriminate the old from the new object. A repeated measures ANOVA revealed a significant main effect of delay ($F_{(2, 18)} = 5.420$; $p < 0.05$). The within factors, were old and new object and the delay period. *Post Hoc* LSD analysis demonstrate animals discriminate objects based on temporal sequencing more proficiently when a 1 ($p < 0.05$) or 6 hour ($p < 0.01$) delay is imposed than a 24 hour delay.

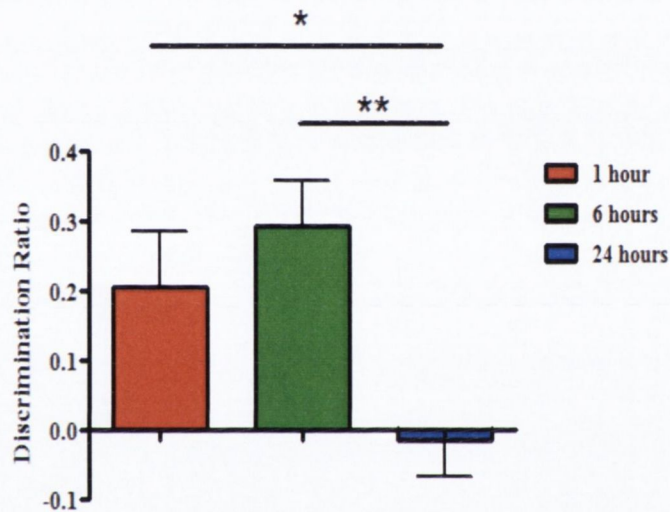


Fig.4. Discrimination index demonstrating naïve control animals performance at different delay periods (n=7).

Exploration during the sample phases and test phase

Table.1. Duration of time exploring the objects in the sample phases and test phase at different delay periods between the sample phases and the test phase

Delay	Expl. In Sample Phase 1 (s)	Expl. In Sample Phase 2 (s)	Expl. In Test Phase (s)
1 Hour	34.3 ± 6.8	31.6 ± 4.7	36.8 ± 2.5
6 Hours	38.2 ± 5.0	30.1 ± 6.6	33.6 ± 1.9
24 Hours	42.0 ± 8.1	47.8 ± 8.1	43.8 ± 5.4

Expl. = Exploration

Raw exploration times of the objects in the sample trials and the test trial are included in Table 1. In general, rats explored the objects actively for between 30 and 50 seconds; this exploration remained consistent between trials. One-way ANOVA's were used to determine this: 1 hour delay: $F_{(2, 20)} = 0.5900$, $p=0.5647$; 6 hour delay: $F_{(2, 17)} = 1.168$, $p= 0.3377$; and 24 hour delay: $F_{(2, 20)} = 0.4077$, $p= 0.6712$. Overall, the above data suggest that there is no effect of repeated exposure on the time spent exploring in the sample phases.

Figure 5 depicts the total duration of exploration during the test phase, this was analysed using a repeated-measures ANOVA. There were two within factors, exploration and test delay. There was no significant effect of group: $F_{(2, 20)} = 2.959$, $p=0.0902$, although it appears that there may be slightly more exploration in the test phase at 24 hours, the trend did not reach significance.

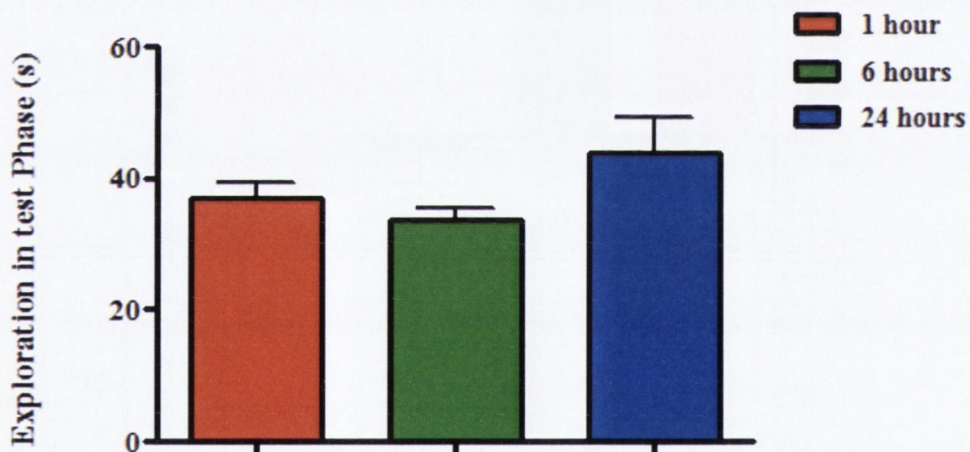


Fig.5. Total exploration of the objects in the test phase at the varying delay periods. Data is presented as Mean + SEM, $n = 7$

2.5. Discussion

This experiment was conducted as a preliminary study to investigate delay-dependent effects that may occur when making temporal order memory judgements. We found that naïve rats are able to discriminate the temporal order of object presentation at a 1

hour and 6 hour delay period (fig 3), replicating the initial findings of Mitchell and Laiacona (1998) (Mitchell & Laiacona, 1998). However, a discrepancy arises when a 24 hour delay is used. Our results conflict with those of the aforementioned study (Mitchell & Laiacona, 1998) in demonstrating an inability to discriminate temporal order at a 24 hour delay period. This finding is also substantiated in the discrimination index in the test phase; at 24 hours animals could not discriminate between the old and the new object (figure 4). A simple explanation for this inconsistency may reside in an effect of strain. A strain difference is evident in the two studies; Mitchell and Laiacona used Long-Evans rats in their experiment, whereas in the present experiment Wistar rats were used. Andrews et al (1995) found a difference in object discrimination learning in Long-Evans and Wistar rats with the pigmented strains performing at an enhanced level (Andrews, Jansen, Linders, Princen, & Broekkamp, 1995). Long-Evans rat are most related to wild rats on spatial memory processing in the Morris Water Maze, this may also be true of other tasks (Harker & Whishaw, 2002). Wistar rats are well known for their poor visual acuity (Hupfeld & Hoffmann, 2006), which may interfere with object recognition and consequently modify the encoding of memory. Therefore, it is plausible that the consolidated strength of the encounter with the objects is weaker in this strain leading to the encoding of a more transient memory trace. Contrary to this argument, the sole use of visual discrimination in spontaneous exploration tasks has been disputed. A predominant exploratory sense in rats is the use of the vibrissae for tactile discrimination of objects (Brecht, Preilowski, & Merzenich, 1997; Carvell & Simons, 1990; Mumby, Tremblay, Lecluse, & Lehmann, 2005).

Alternatively, it is possible that a cognitive explanation may underlie this difference. When delay periods increase in an object exploration task the demand on memory also increases. Fuster (2001) suggests that temporal integration requires that elements of working memory are active for the retention of the information needed for guiding prospective actions. This may be a possible explanation for the inability to discriminate at the 24 hour time point, indicating that the animals are no longer buffering elements of working memory to monitor the objects encountered recently with those that occurred remotely. Although this is a passive exploration experiment with no explicit rule learning required there is a monitoring system in place to maintain an active representation of the last object encountered. The internal representation of the objects degrades over the course of a long delay period. However, it is long

established that animals can successfully perform a recognition memory task with long retention intervals far exceeding a 24 hour delay period (Forwood, Winters, & Bussey, 2005; Mumby et al., 2005). Therefore, it is unlikely that a pure delay dependent effect reflects the inability to recollect temporal order in this case. Task complexity is a likely explanation for deficits in discrimination at a 24 hour delay in temporal order memory but a sparing of recognition memory at this delay timepoint.

It is noteworthy to emphasise there were no significant difference in exploration of objects in the sample phases (table 1). This infers that there was no preferential bias towards discriminanda and therefore an equal salience for the objects presented. This also indicates an absence of habituation to the objects signifying that the objects in the two sample phases are recognised as distinct entities, therefore stimuli fatigue was not apparent.

The data represented here may be used to guide further experiments using the temporal order memory task. It is evident that strong temporal order distinctions are made when imposing a 1 hour and 6 hour delay period between sample 2 and the test phase. A 6 hour time point is the more attractive delay period, both for practical reasons as this enables block testing of animals and also cognitively by designating a challenge in recollection.

3. Experiment 2

Temporal Order Memory and Mild Behavioural Stress

Prefrontal lesion studies have been used to determine the specific localisation of function of temporal order memory (Barker et al., 2007; Hannesson, Howland, & Phillips, 2004; Howland, Harrison, Hannesson, & Phillips, 2008; Mitchell & Laiacona, 1998). It was our intention to confirm the predictive validity of the temporal order memory task and determine the effects of mild behavioural stress on subsequent judgements. It has been ascertained by many research groups that behavioural stress challenges the integrity of PFC function (Cerqueira, Almeida, & Sousa, 2008; de Kloet, Joels, & Holsboer, 2005; Holmes & Wellman, 2008; McEwen, 1998a). A behavioural stress paradigm is a valuable alternative to the invasive nature of experimental lesion studies but also was sought to predicate the impact of IFN- α which is characterised as a systemic stress modulator (Capuron & Miller, 2004). In addition, an assessment of the delay-dependent effects was conducted to determine if they remain robust with the inclusion of an experimental group.

3.1. Animals

Fifteen male Wistar rats (BioResources Unit, Trinity College Dublin) weighing between 300-350g were housed two/three to a cage within a controlled environment (Laminar airflow unit, 12h light/dark schedule with lights on at 08:00-20.00). Rats received food and water *ad libitum*. All behavioural testing was conducted during the light phase of the schedule. Prior to behavioural testing all rats were handled to acclimatise them to the experimenter. All experiments were carried out in accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experiments.

3.2. Materials and Methods

The testing arena and objects were the same as those described in the previous experiment (Section 2.2). Delay periods of 6 and 24 hours were adopted between the second sample and the test phase. Light stress (photophobia) was utilised to induce mild behavioural stress. Photophobia is a natural response in rats and produces a reliable stressful response. Plastic buckets were used as holding containers during the

light exposure period. Angle poise lamps with 100 watt bulbs were used to implement this stress protocol.

3.3. Behavioural Procedure

Rats were placed in a plastic bucket with a bright light angled directly above and shone for a period of 30 minutes. Stress was implemented as an acute induction of mild behavioural stress. Common signs of stress such as piloerection and defecation were noted by the experimenter. Control animals were also placed in the bucket for a period of 30 minutes with the light turned off in order to experience comparable conditions. Rats were placed in the testing apparatus immediately after the light exposure and given time to explore during the sample phases. The behavioural testing protocol was the same as that described in the previous experiment (Section 2.2.1.) however the discrimination measured differed. Frequency measures were taken due to inadequate tracking equipment available at the time of this experiment. This measure was defined as the number of contacts of the animal's nose with the object.

3.4. Results

Discrimination of temporal order memory is attenuated in stressed animals

Figure 6 shows control rats spent a greater proportion of time exploring the old object than the stressed rats at the 6 hour time point. A repeated-measures ANOVA revealed a significant main effect of group (control vs. stressed) ($F_{(1, 13)} = 4.786$; $p=0.049$). There was a significant effect of test delay, ($F_{(1, 13)} = 11.073$; $p= 0.006$). There was no group by test delay interaction, ($F_{(1, 13)} = 1.559$; $p=0.236$). Subsequent one-way ANOVA's reveal significant differences in exploration of the groups at six hours ($F_{(1, 13)} = 4.724$, $p=0.50$) however, exploration of the objects were comparable at a 24 hour delay ($F_{(1, 12)} = 0.674$, $p = 0.428$). Additionally, paired sampled t-tests, reveal that the control animals discriminate more at 6 hours than at a 24 hour delay ($t=4.178$), $df = 6$, $p = 0.006$). Stressed animals did not however differ between discrimination at the 6 hour delay and the 24 hour delay ($t = 1.242$, $df = 6$, $p = 0.260$).

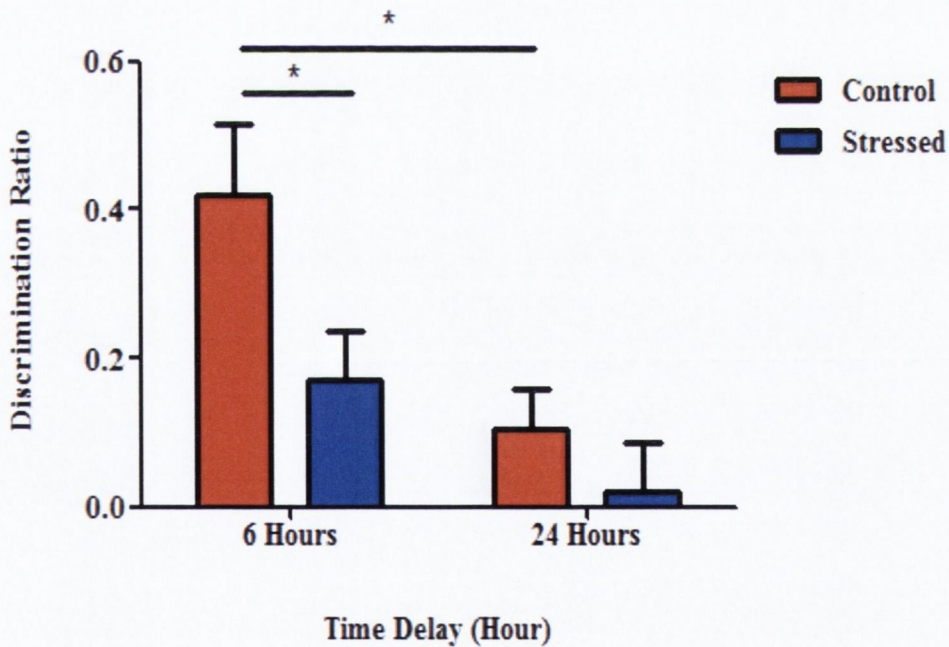


Fig. 6. Discrimination ratio of the exploration of the old object. Data is presented as mean + SEM. Saline ($n=8$), stressed ($n=7$)

Control and stressed rats explore the objects more at a 24 hour delay period in the test phase

Figure 7 shows the total time spent exploring the objects in the test phase at both delay timepoints. A repeated-measures ANOVA revealed no significant main effect of group (control vs. stressed) ($F_{(1, 13)} = 0.607$; $p=0.451$). There was a significant effect of test delay, ($F_{(1,13)} = 55.298$; $p=0.000$). There was no group by test delay interaction, ($F_{(1,13)} = 0.765$; $p=0.399$). Additionally, paired sampled t-tests, reveal that the control animals ($t = -16.806$, $df = 6$, $p = 0.000$) and stressed animals ($t = -4.743$, $df = 6$, $p = 0.006$) spend more time exploring the objects in the test phase at 24 hours than at a 6 hour delay.

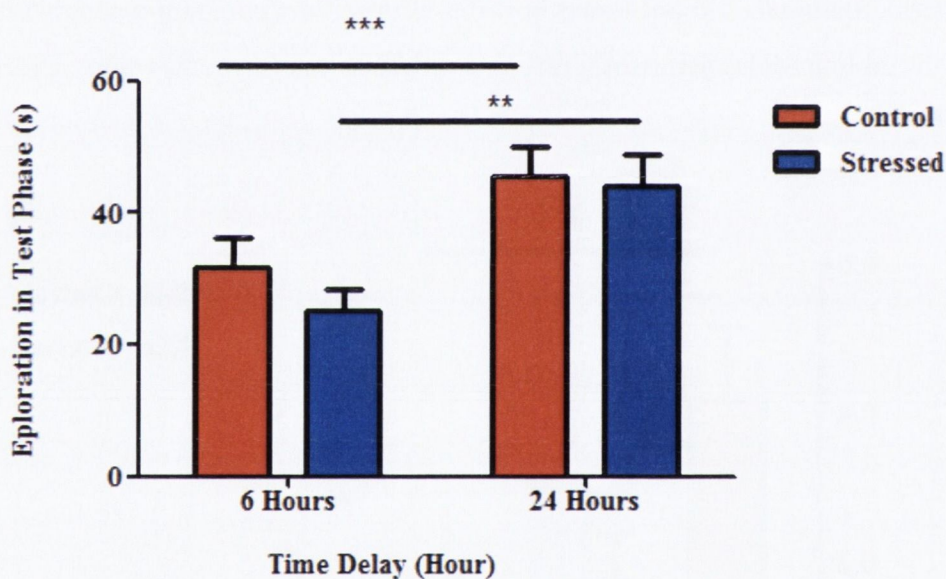


Fig.7. Total time spent exploring the objects in the test phase at the two delay periods, Data presented as mean + SEM. Saline (n=8), stressed (n=7)

Exploration during the sample phases

Table.2. Exploration of the objects in the sample phases for control and stressed rats. Data presented as mean frequency visits to the object.

Condition	Expl. Sample 1	Expl. Sample 2
<i>6 Hours</i>		
Control	10.6 ± 1.32	7.6 ± 1.25
Stressed	11.0 ± 0.87	9.4 ± 1.00
<i>24 Hours</i>		
Control	9.3 ± 1.23	8.0 ± 1.38
Stressed	10.3 ± 1.11	9.4 ± 1.56

Expl. Exploration

Frequency measurements of exploration of the objects in the sample trials are included in Table 2. In general, rats actively approached the objects between 7 and 11 times in the space of the trial; this exploration remained consistent between trials. Paired sample t-tests determine that there are no differences between sample 1 and 2 in both delay periods, control, 6 hours ($t=2.324$, $df=6$, $p=0.059$); control, 24 hours ($t=1.229$, $df=7$, $p=0.259$); stressed, 6 hours ($t=0.497$, $df=6$, $p=0.637$); stressed, 24 hours ($t=1.721$, $df=6$, $p=0.136$).

3.5. Discussion

This series of experiments used acute mild behavioural stress as a probe for prefrontal function providing further validation for the use of a spontaneous exploration task to examine temporal order memory. Verifying the results from the previous experiment, control rats were able to distinguish remote from recently presented objects at a 6 hour delay but not at a 24 hour delay (Figure 6). Furthermore, it is evident that stress disrupts temporal order memory significantly when compared with control animals. Mild behavioural stress does not however abolish the discrimination of temporal order memory entirely. The animals remain able to distinguish the order of object presentation but to a lesser extent than control animals thereby signifying a decline in temporal order proficiency. This may be an indication of a functional depression of the PFC which has been reviewed as particularly vulnerable to stressful encounters (de Kloet et al., 2005; McEwen, 1998a).

One consideration worth noting is the potential for an inadvertent physiological impact of the light stressor on the exposure to the first sample phase. The light exposure prior to sample 1 may have caused disruption in exploration in the sample phase resulting in a reduction in the saliency of the object experienced at that timepoint. This would be reflected by an inordinate amount of attention paid to the object in the test phase due to its under-exposure or inattention to the object in the sample phase and would obscure any cognitive temporal sequencing effects. However, this was not the case, the two sample phases were explored equally implying equal interest and attention to the objects (table 2). However, it is of note, to report that both albino and pigmented rats are susceptible to the adverse effects of light and this may result in damage to the retina if exposed to light for prolonged periods (Wasowicz, Morice, Ferrari, Callebert, & Versaux-Botteri, 2002).

There are suggestions that under certain conditions the exposure to a stressor may enhance the consolidation of memory (Roozendaal, 2002). This depends on a multitude of factors: habituation routines, severity, duration and placement of the stressor (Maroun & Akirav, 2008). The Yerkes-Dodson law (1908) demonstrates an inverted U-shaped function of stress, stating that mild stress may be facilitatory and conversely, moderate to high levels of stress disadvantageous. The stress employed in this study may be defined as a mild to moderate acute stress. Unfortunately, a limitation in this

study was the omission of a recognition memory examination. Therefore it remains unknown if the maintenance of temporal order memory albeit to a lesser extent than controls is a result of cognitive processing or is mediated by a state dependent change in the motivation/identification of familiar objects. Moreover, reports in the literature suggest that long-delay object recognition memory (≥ 3 hours) may be affected by acute stress (Baker & Kim, 2002). However, this study utilised a more extreme stress induction protocol using tail-shock. Impairment in recognition memory is unlikely in the animals in this experiment, considering recognition memory is a key component in temporal order memory processing. This remains intact but to a lower extent than unstressed animals. To emphasise, if recognition memory was deficient discrimination of temporal order would diminish entirely. A further limitation of this study lies in the lack of complementary corticosterone analysis; this analysis would provide a surrogate marker of the induction of stress. This would shed some light on where in the continuum of stress this particular stress lies indicating if there is assumed facilitation or inhibition of memory processing. The reason underlying the lack of corticosterone assay in this experiment lies in the loss of integrity of the plasma samples due to a technical fault with a laboratory freezer.

The stress induction in the present experiment was implemented prior to encoding the objects in the sample phases. Therefore any detriment to the mnemonic processing can be concluded to result from an effect of encoding and consolidation rather than an inability to retrieve memory traces. An alternative line of study would be to assess the effect of stress on retrieval in temporal order memory by imposing the stressor prior to the test session. This would further clarify the relationship between stressful events and the integrity of the PFC.

An interesting pattern emerged in the total exploration time in the test phase with a disparity in the amount of time spent exploring the objects at 6 hours and 24 hours (Figure 7). It is apparent in both experimental conditions that animals appear to spend more time exploring the objects after a longer delay period is implemented. A simple explanation for this is that the short-term memory buffer does not remain active over long delay periods therefore more explicit exploration is reinstated as both of the objects regain novelty.

It is evident from the data presented here that stress may have a negative impact on the prefrontal cortex. The stress used in this experiment was of a mild/moderate and of an acute nature. If chronic mild stress (CMS) was employed it may be predicted that temporal order deficiencies may be more pronounced, indicating support for a dose effect of stress. However, there are reports that a single acute encounter of stress produces long-lasting inhibition of LTP in the HC-PFC neuronal pathway (Jay et al., 2004; Rocher et al., 2004). Synaptic plasticity in this pathway is dependent upon dopamine therefore deficiencies in the mesocortical dopamine input may be key to the impairment. Moreover, Impairments in the HC-PFC pathway be alleviated with antidepressants; Rocher et al (2004), used an unsteady elevated platform stress as a stress paradigm and demonstrated that Tianeptine and Fluoxetine aid in the recovery of LTP in the HC-PFC pathway (Rocher et al., 2004). Tianeptine has been shown to modulate the HC-PFC pathway via an increase in dopaminergic tone. Further studies in this lab report the apparent reversal of LTP impairment in the HC-PFC pathway with blockade of glucocorticoid receptors (Mailliet et al., 2008). Additionally an acute 30 minute stress induction can decrease neurogenesis in the hippocampus by around 50% (Malberg) which suggests a possible source of the HC-PFC impairment. These studies highlight the HC-PFC pathway as an area of dysregulation in neuropsychiatric conditions which may be precipitated by exposure to stressful stimuli. It is also important to note the effect that stress has on the hippocampus. Animals subjected to restraint stress may result in a decrease in hippocampal volume when induced at a chronic level (McEwen, 1998a).

However, when discussing the effect of stress on memory formation it is also vital to consider amygdala involvement. Fundamental research also points to impairment of LTP induction in the amygdala-prefrontal pathway with induction of stress protocols (Akirav & Richter-Levin, 1999; Maroun & Richter-Levin, 2002). The above-mentioned results indicate a deficit in temporal order memory processing with acute mild behavioural stress. This may be mediated by different axes, the HC-PFC, or the amygdala-PFC. Studies performed using a similar behavioural stress paradigm signify impairment of LTP in both of these limbic-cortical axes. Modulation of these regions may occur by glucocorticoid regulation via the activation of PFC-hypothalamic axis. Thereby, it may be speculated that stressful events may be modulated by an extended dysregulation of the limbic-PFC circuit.

4. Experiment 3

The effect of IFN- α -treatment on Temporal Order Memory

The Previous experiment examined behavioural stress and temporal order memory and demonstrated a negative effect of stress on this type of memory. It was our intention here to examine this further using a systemic stress with pharmacological treatment using IFN- α .

4.1. Animals

Sixteen male Wistar rats (BioResources unit, Trinity College Dublin) weighing between 300-350g were housed two/three to a cage within a controlled environment (Laminar airflow unit, 12h light/dark schedule with lights on at 08:00-20.00). Rats received food and water *ad libitum*. All behavioural testing was conducted during the light phase of the schedule. Prior to behavioural testing all rats were handled to acclimatise them to the experimenter. All experiments were carried out in accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experiments.

4.2. IFN- α -Treatment

Roferon-A (human recombinant interferon-alpha 2a, Roche Pharmaceuticals, USA) (~170,000 IU/kg, 0.2mL, diluted in saline, s.c.), or vehicle (0.2mL 0.9% NaCl, s.c.) was administered once a day, three times a week for four weeks prior to the commencement of behavioural testing The dose was adjusted once a week to compensate for any increase in weight gain.

4.3. Behavioural Procedure

Temporal Order Memory Task

The behavioural procedure used in this experiment was the same as that described in section 2.2.1. The inter-trial delay period between the second sample and the test phase was 6 hours. This procedure included a repeated measures protocol with temporal order testing performed at a 4 week time point and 3 timepoints after cessation of treatment (figure 8). Eight different sets of objects were used for this experiment with three copies in each set.

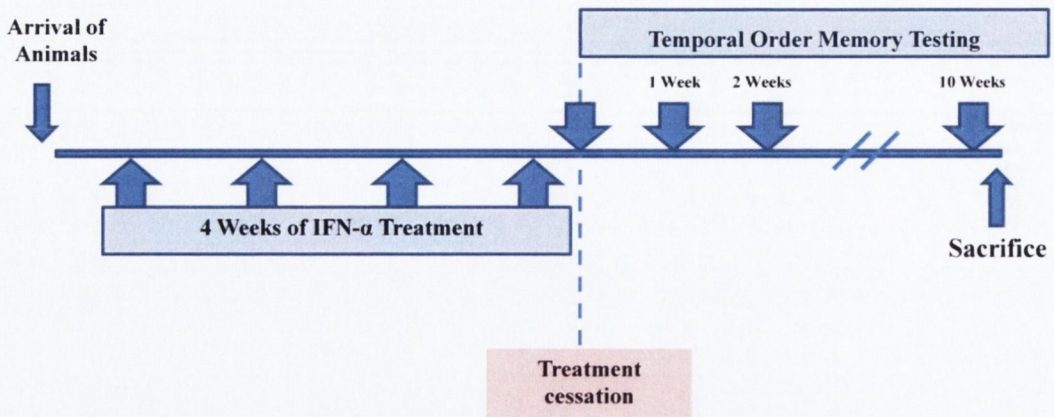


Fig.8. Schematic illustrating the experimental procedure employed, providing details of drug delivery and testing schedule. Rats received IFN- α for 4 weeks thrice weekly. Four testing sessions were conducted, at 4 weeks of treatment, then at 1, 2 and 10 weeks off treatment.

Recognition Memory Task

This experiment was completed to verify that the animals did not have a problem with recognition memory *per se* and that the problem was based specifically in the sequencing of the object presentations. The arena is the same as described previously in the temporal order memory task. The arena was placed inside a curtained-off enclosure in a dimly-lit room. Objects for this experiment were constructed from general laboratory equipment. Each object had a distinct conformation but with equivalent dimensions and interest. Following 4 weeks of IFN- α -treatment the animals were exposed to the arena for 4 consecutive days for 3 minute habituation sessions. The test phases consisted of exposure to three five minute trials with two different objects (objects A and B) with an inter-trial interval of 5 minutes (figure 9). After each exposure, the objects and the arena were cleaned with Savlon® to remove any odour cues. The final stage (choice phase) of the test session involved the substitution of one of the objects with a novel object (object C) and a duplicate of object A or B. Recognition memory was defined as more exploration of the novel object (Object C).

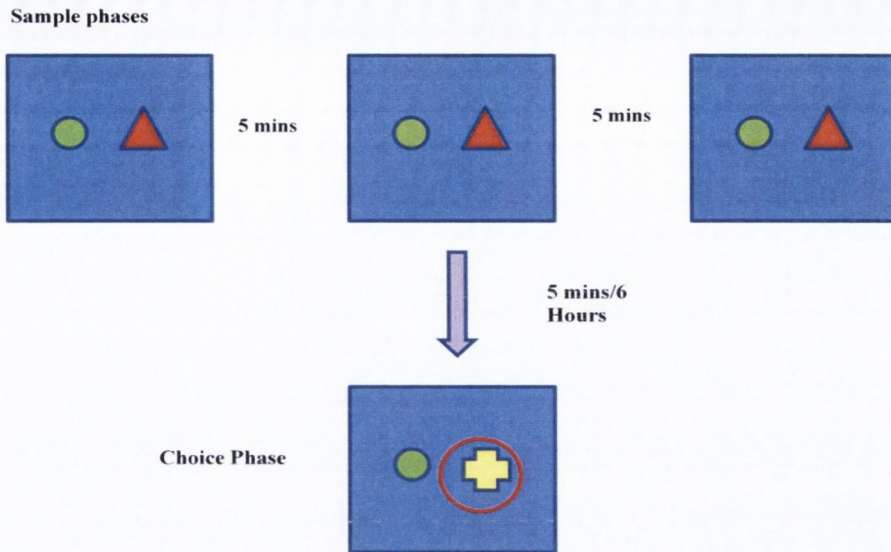


Fig.9. Schematic illustrating the novel object recognition task. Three exposures of 5 minute duration trials of two different objects. A choice phase with one old familiar object and a novel object. The red ring around the cross depicts the object that should be of interest to the animal.

Two delay periods (either 5 minutes or 6 hours) were used between the third sample phase and the test phase. These time periods were chosen to reflect the time period used in the temporal order task and to determine that there was no delay-dependent effect. Recognition memory task was performed after 4 weeks of IFN- α treatment.

4.4. Results

Temporal Order Memory

Temporal Order Memory is impaired in IFN- α -treated animals

An independent samples t-test revealed that IFN- α animals do not discriminate the old object from the new object however to saline-treated counterparts spend more time exploring the old object. Levene's test for equality of variance determines that equal variance was not assumed. $t = -3.138$, $df = 8.337$, $p = 0.013$).

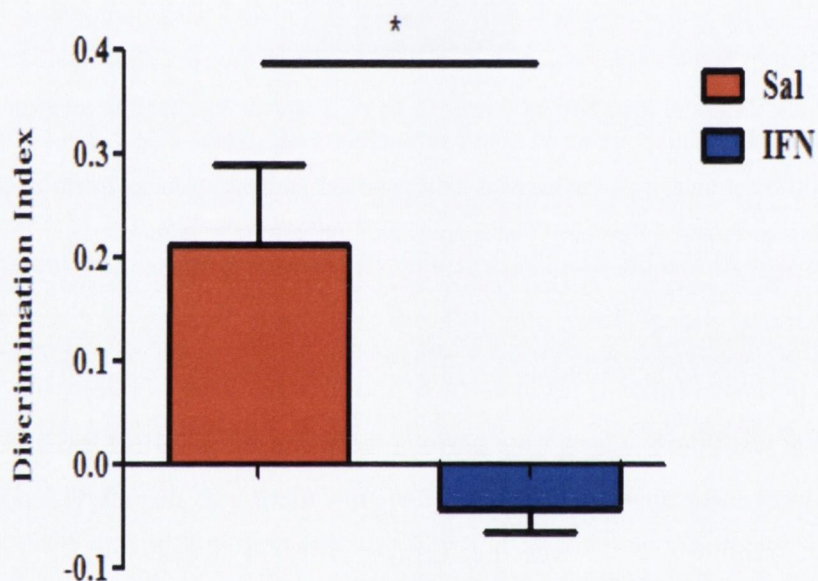


Fig.10. Performance of saline- and IFN- α -treated animals on the temporal order memory task. Data presented as. Mean discrimination ratio + SEM * $p < 0.05$, $n = 8$ for both groups.

Exploration during the sample phases and test phase

Table.3. Duration of exploration of the objects in the sample phases and test phase with IFN- α or saline-treated animals

Treatment	Expl. Sample 1 (s)	Expl. Sample 2 (s)	Expl. Test Phase (s)
Saline	48.4 \pm 6.3	40.1 \pm 5.3	36.4 \pm 3.7
IFN- α	40.5 \pm 2.4	33.0 \pm 3.9	30.4 \pm 5.1

Expl. = Exploration

Table 3 presents the raw exploration times in the sample phases and test phase in the temporal order memory task. Rats in all groups generally spent between 30 and 50 seconds actively exploring the objects in the trials. Explorations across trials were, on the most part consistent, examination of trial effects was conducted: Saline group: $F_{(2, 21)} = 1.400$, $p = 0.269$. IFN group: $F_{(2, 21)} = 1.669$, $p = 0.212$. Overall, the data suggest that there is no impairment in exploration and no bias of exploration of either of the sample phases occurring as a consequence of IFN- α -treatment. Analysis using an independent t-test of the amount of time spent exploring in the test phase suggests there was no difference in the amount of exploration ($t = 0.943$, $df = 14$, $p = 0.362$) therefore a suggestion that there is no alteration or loss of motivation in exploration with pharmacological treatment.

Recognition Memory

Exploration in the test phase

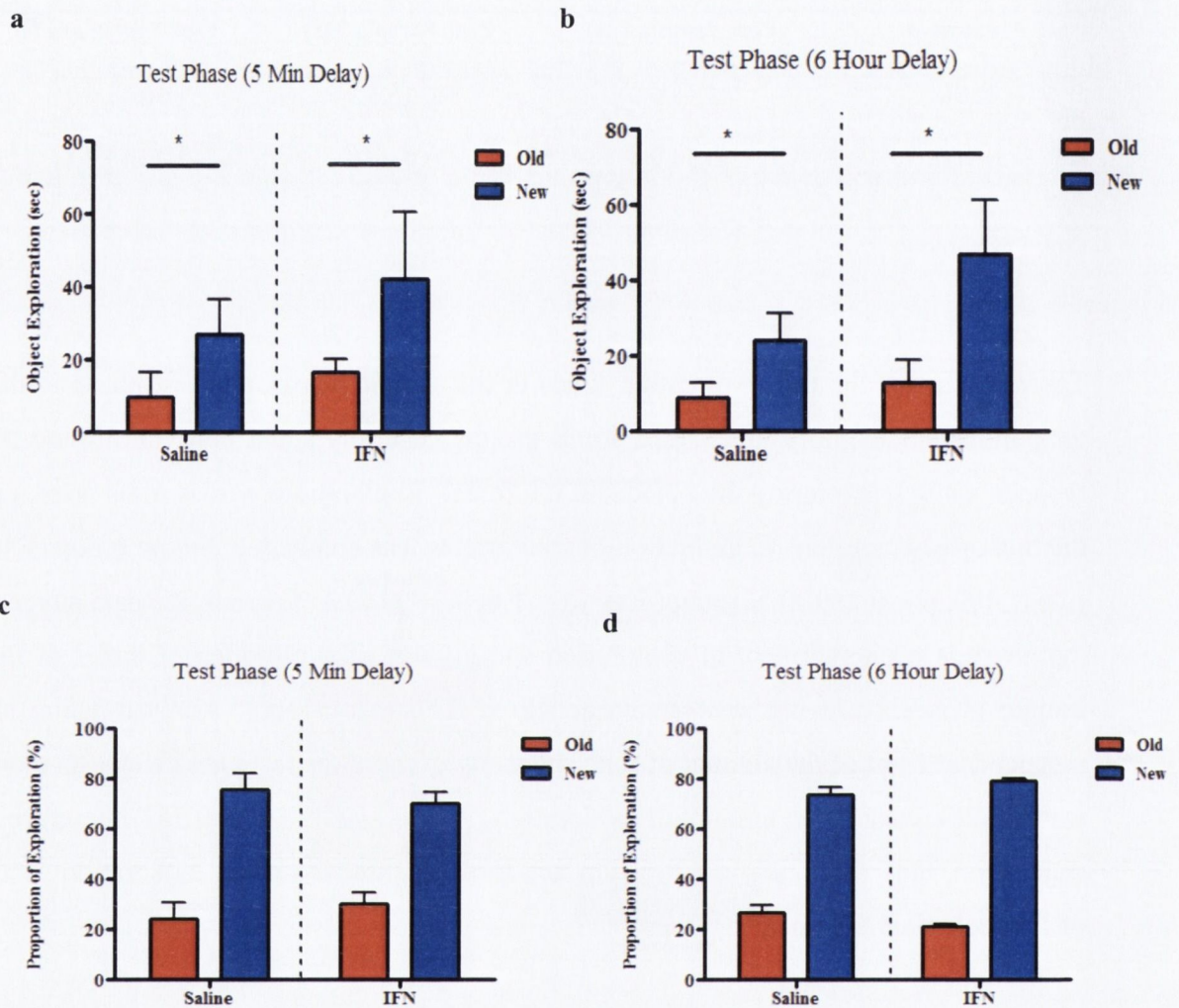


Fig.11. Time spent exploring the old and new object in the choice phase of the object recognition task; a) absolute time spent exploring objects after a 5 min delay b) absolute time exploring objects at a 6 hour delay c) relative time spent exploring objects at a 5 min delay, d) relative time spent exploring objects at 24 hours. Data presented as mean + SEM, n= 5 per group, *p < 0.05

Figure 11 illustrates that IFN- α - treatment did not affect object-recognition memory with a 5 min delay or a 6 hour delay between the sample and test phases; Non-parametric Wilcoxon matched paired sample t-tests were completed for all groups, control-5min (T=3.5, p=0.028), control 6 hour (T=3.5, p=0.28) IFN- α 5 mins (T=3, p=0.043) and IFN- α 6 hours (T=3.0, p=0.043) spent more time exploring the new object than the old object.

Additional Mann Whitney U tests were conducted to determine any differences between the treatment groups in exploring the novel object (figure 4c, d). At both delay periods no significant differences were determined, 5 min delay; ($U = 13$, $p = 0.715$); 6 hours ($U = 9$, $p = 0.273$).

Temporal Order Memory at 5 minutes

This experiment was completed to support the previous experiment, determining that when a short delay is used the complexity of the task is reduced. The task relies on a recognition-based memory rather than temporal order memory when a short delay is used.

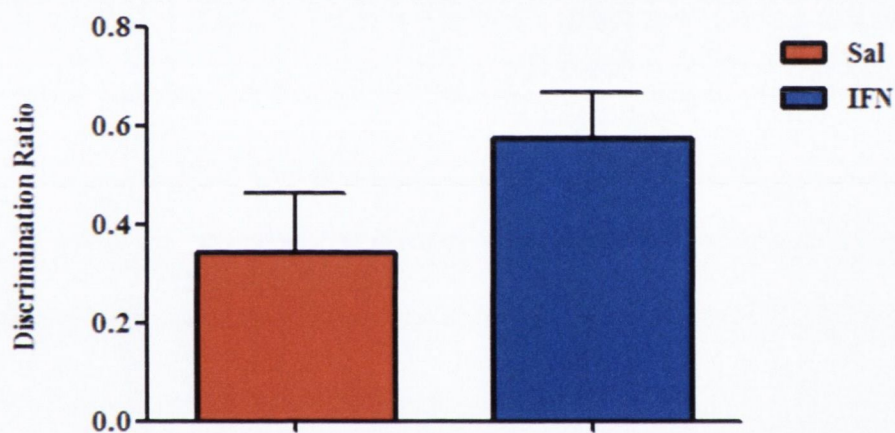


Fig.12. Performance of saline- and IFN- α -treated animals on the temporal order memory task. Data presented as mean + SEM, $n = 6$

Figure 12 above illustrates the performance of rats on the temporal order memory when a 5 minute delay is imposed between the sample 2 and the test phase. Mann Whitney U tests demonstrate that the treatment groups performed comparably on this task ($U = 8.0$, $p = 0.201$). Both groups are able to discriminate temporal order at a 5 minute delay period.

Table.4. Duration of exploration of the objects in the sample phases and test phase in IFN- α or saline-treated animals

Treatment	Expl. Sample 1 (s)	Expl. Sample 2 (s)	Expl. Test Trial (s)
Saline	51.6 \pm 5.32	52.41 \pm 8.76	51.07 \pm 9.64
IFN- α	59.2 \pm 13.04	79.44 \pm 12.17	54.2 \pm 6.92

Expl. = Exploration

Table 4 presents the raw exploration times in the sample phases and test phase in the temporal order memory task. Rats in all groups generally spent between 50 and 80 seconds actively exploring the objects in the trials. Explorations across trials were, on the most part consistent, examination of trial effects was conducted: Saline group : $\chi = 0.047$, $df=2$, $p= 0.977$, IFN group: $\chi= 5.064$, $df=2$, $p=0.079$. Overall, the data suggest that there is no impairment in exploration and no bias of exploration of either of the sample phases occurring as a consequence of IFN- α -treatment. Analysis using an independent t-test of the amount of time spent exploring in the test phase suggests there was no difference in the amount of exploration ($U = 14.0$, $p= 0.855$) therefore a suggestion that there is no alteration or loss of motivation in exploration with pharmacological treatment.

Cessation of treatment

Figure 13 below demonstrates the temporal order memory task over a longitudinal testing schedule. A repeated-measures ANOVA revealed a significant main effect of group ($F_{(1, 14)} = 7.984$; $p=0.013$). There was no significant effect of testing session, ($F_{(3,42)} = 0.643$; $p= 0.643$). There was a group by test delay interaction, ($F_{(3, 42)} = 3.139$; $p=0.035$). Subsequent one-way ANOVA's reveal significant differences in exploration of the groups after 4 weeks of treatment ($F_{(1, 14)} = 9.849$, $p=0.007$), exploration after 1 week off treatment ($F_{(1, 14)} = 1.804$, $p=0.201$). However, 2 weeks off treatment ($F_{(1, 14)} = 0.116$, $p=0.116$) did not differ between groups. In contrast after 10 weeks it was found IFN- α animals performed worse on the task ($F_{(1, 14)} = 0.674$, $p = 0.030$).

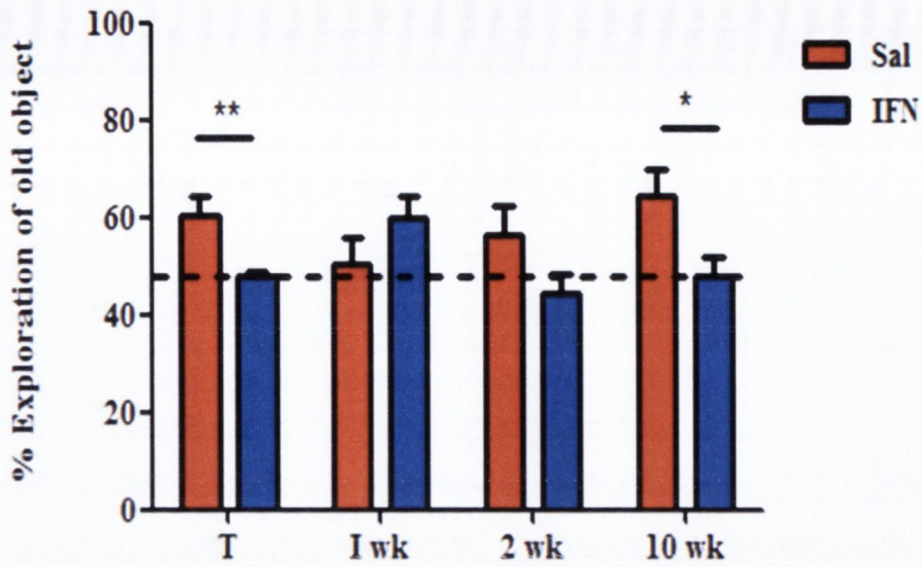


Fig.13. Longitudinal assessment of temporal order after the cessation of treatment in IFN α and saline treated animals. Abbreviation: T, test. Data presented as mean + SEM, n= 8 per group. Dashed line denotes 50% chance level exploration

Table.5. Duration of exploration of the objects in the sample phases and test phase in IFN- α or saline-treated animals

Treatment	Expl. Sample 1 (s)	Expl. Sample 2 (s)	Expl. Test Trial (s)
<i>1 Week off treatment</i>			
Saline	43.8 \pm 6.36	37.5 \pm 5.45	39.1 \pm 7.50
IFN	51.6 \pm 6.36	53.3 \pm 7.78	40.9 \pm 7.30
<i>2 weeks off treatment</i>			
Saline	33.9 \pm 4.24	30.2 \pm 5.04	29.1 \pm 4.27
IFN	35.5 \pm 3.35	37.0 \pm 9.37	25.5 \pm 4.29
Saline	41.0 \pm 2.71	46.5 \pm 3.44	36.8 \pm 6.06
IFN	38.5 \pm 4.71	38.5 \pm 4.20	37.7 \pm 3.97

Expl. Exploration

Table 5 presents the raw exploration times in the sample phases and test phase in the temporal order memory task. Rats in all groups generally spent between 30 and 55 seconds actively exploring the objects in the trials. Explorations across trials were, on the most part consistent, examination of trial effects were conducted, 1 week off treatment; Saline group: $F_{(2, 18)} = 0.067$, $p = 0.935$. IFN group: $F_{(2, 15)} = 0.621$, $p = 0.551$), 2 weeks after cessation: Saline group: $F_{(2, 18)} = 0.067$, $p = 0.935$. IFN group: $F_{(2, 15)} = 0.621$, $p = 0.551$), 10 weeks after cessation: Saline group: $F_{(2, 21)} = 1.273$, $p = 0.301$. IFN group: $F_{(2, 21)} = 0.014$, $p = 0.986$). Overall, the data suggest that there is no impairment in exploration and no bias of exploration of either of the sample phases occurring as a consequence of IFN- α -treatment. Analysis of the amount of time spent exploring in the test phase using independent t-tests demonstrates there was no difference in the amount of exploration between the two groups, 1 week off, ($t = 0.5561$, $df = 14$ $p = 0.5869$) 2 weeks off, ($t = 0.6006$, $df = 14$, $p = 0.5577$), and 10 weeks off ($t = 0.1185$, $df = 14$, $p = 0.9074$) therefore this illustrates no alteration or loss of motivation in exploration with pharmacological treatment.

4.5. Discussion

The experiments presented here examined the effects of IFN- α -treatment on temporal order memory and recognition memory. The results suggest that there is a failure to perform temporal order discriminations in rats treated with IFN- α . Although, IFN- α -treated animals were impaired in the temporal order memory task, they performed normally in the recognition memory task (Figure 11), sparing this mnemonic function.

IFN- α -treated animals could not discriminate between objects based on the sequence of presentation (Figure 10). Therefore, they were unable to distinguish events that occur remotely compared to those that occurred more recently. The basis for this impaired temporal order proficiency cannot be ascribed to an inability to make familiarity judgements but is a result of deficits in recency discriminations. In other words, the selectivity of task performance and sparing of recognition memory ensure it is unlikely that the basis of discrimination impairments reside in the inability to identify the objects. This is in line with experiments conducted previously (Barker et al., 2007; Ennaceur, Neave, & Aggleton, 1997; Hannesson, Vacca, Howland, & Phillips, 2004), which demonstrate dissociation between these two mnemonic tasks with prefrontal impairments. It also reflects selective impairments in patients with select PFC lesions (B. Milner et al., 1990; Shimamura et al., 1990). Temporal order memory when conducted using a 5 minute delay between the sample phase and the test phase can be completed by IFN- α -treated animals (Figure 12). It is possible that this task is less complex, the information is retained online for a shorter duration therefore heavily relies on recognition memory.

The data presented here are consistent with experiments producing either reversible (Hannesson, Vacca et al., 2004) or permanent (Barker et al., 2007; Mitchell & Laiacona, 1998) lesions of the prefrontal cortex. The prefrontal cortex integrity is required for the completion of temporal order memory tasks, although there is evidence that this region may not be the sole neural correlate (Barker et al., 2007; Eichenbaum, Fagan, & Cohen, 1986; Hannesson, Howland et al., 2004). This is the first time known to the author that the systemic administration of a pharmacological treatment has been used to examine deficits in the temporal sequencing behaviour of objects in rats. Extensive disconnection studies completed to investigate the neural correlates of

temporal order memory have precluded the need for lesion studies in this series of experiments.

The anatomical basis for deficits in the animals in this experiment may be deconstructed using a systems approach. Comparisons of the data presented here and those from Barker's elegant disconnection study suggest that the impairment in temporal order memory but sparing of recognition memory lend support to preserved perirhinal cortices in IFN- α -treated animals.

It is true that the hippocampus has been implicated in temporal order memory (Eichenbaum et al., 1986; Kesner, Gilbert, & Barua, 2002). However, the aforementioned disconnection studies assist in shedding light on this, lesions of the PFC sparing the hippocampus result in impairments in temporal order memory. This suggests that an intact hippocampus but deficits in PFC results in impairments in this mnemonic task, weakening the notion that the task depends on the hippocampus. These distinct brain regions may however, both be required and therefore act in concert to complete some versions of temporal order memory tasks. It is important to note that the experiments examining the role of the hippocampus in temporal order memory incorporate elements of spatial memory in the task completion requirements; this may infer the role for the hippocampus (Eichenbaum et al., 1986; Kesner et al., 2002). However, Eichenbaum formulated an experiment absent of any spatial elements and still proposed a significant role for the hippocampus in temporal order memory processing. This task involved the temporal sequencing of olfactory stimuli, using reward for motivation. This reward may result in alternative memory processing via stimulus response associations, and may impact on a different axis. The clinical evidence mainly resides around frontal damage resulting in recency discrimination problems (Shimamura, 2000). More clarification may be provided for the anatomical basis by a recent study conducted by Howland et al 2007. They find that the ventral hippocampus is required for spatial versions of the temporal order memory task (Howland, Hannesson, Barnes, & Phillips, 2007). This reveals why temporal order memory is lost with prefrontal damage (Barker et al., 2007 ; Mitchell & Laiacina, 1998), but remains when the hippocampus is intact. Prefrontal damage would prevent the cross talk between the ventral hippocampus and prefrontal cortex. Therefore, in the

IFN- α -treated animals dysfunction which results in an inability to perform this task is anatomically located in either the ventral hippocampus, HC-PFC pathway or the PFC.

It is important to note that a four trial (distributed) object recognition task was utilised in this study instead of the mainstay procedure of a one-trial massed approach (Ennaceur & Delacour, 1988). The recognition memory task that was used in this experimental utilised a spaced initial stimulus familiarisation as opposed to a massed familiarisation. There is evidence that distributing the familiarisation sessions over a spaced procedure may enhance the recognition ability (Anderson, Jablonski, & Klimas, 2008). Performance was spared on this recognition memory task with a short-delay and long-delay period.

Exploration in the sample phases and test phases were comparable over the period of the testing session negating stimulus bias and habituation effects of the task.

It is interesting to note that the discrimination indices differ in rats naive to injection stress and saline-treated control rats. The discrimination ration is lower in saline-treated rats (experiment 3) than naive rats (experiment 2). Rationalisation of this discrepancy may be ascribed to the relative vulnerability of the prefrontal cortex to stress. In experiment 2 the animals were naive to injection stress, whereas in the present experiment they were exposed to s.c. injections thrice weekly. Dere et al., (2005) found that mice could not perform an episodic-like memory task which incorporates the temporal order memory paradigm (“when” element) after subcutaneous injections (Dere, Huston, & De Souza Silva, 2005). Additionally, Hotte (2005) reports that temporal order proficiency was not intact in their control treated rats; however this may be explained by the procedural administration in the form of a stressful i.p injection (Hotte et al., 2005). Therefore, it is notable that temporal order memory distinctions are sensitive to stressful episodes within the context of the behavioural testing and may be modulated by these events.

Cessation of treatment

Of central interest to this study is the enduring nature of the deficit in temporal order memory. A longitudinal examination of temporal order proficiency was conducted after

the cessation of treatment to determine the persistence of cognitive symptoms. Fig 13 demonstrates that one week after treatment cessation there appears to be a reflexive increase in the ability of IFN- α -treated animals to perform this task, with performance comparable to saline-treated counterparts. It may be speculated that the Neurovegetative symptoms, i.e. the flu-like symptoms may be temporarily be alleviated, therefore improving performance on the task. However, the performance of the saline-treated animals decreased on this session, the reason for this is unknown, although it is important to note the performance remained within consistency across the testing sessions. Two weeks after IFN- α -treatment the performance falls once again to chance level exploration. This poor performance persists for 10 weeks post-treatment. The persistence of deficits after cessation of treatment is in line with a self rated report study conducted by Reichenberg et al., (2005). Patients in this study reported cognitive deficits persisting 2 months after the discontinuation of treatment when treated with peg-IFN. Some caution is directed here to the limitations in the population assessed; all patients were classified as treatment resistant. A further constraint resides in the subjective nature of the self report indicating a requirement for an objective study to confirm the incidence of the post-treatment deficiencies. Interestingly, Poutiainen et al., (1994) determined that cognitive decline can be reversed after cessation of treatment. However, Meyers et al., 1991 find that symptoms of neurotoxicity on cognition are not reversible in some cases. The debate on the persistence of IFN- α induced deficits remains active; however the data included here support a persistence of prefrontal deficits. It may be speculated that persistence of the deficits may stem from structural reorganisation or a specific hypometabolism (Juengling et al., 2000b) associated with potentially a volume decrease in this area.

The implication of this persistence in cognitive deficits is an important indication that there is a requirement for extended monitoring of patients with regular follow-ups after the cessation of treatment.

5. Experiment 4

Recovery of Temporal Order Memory Deficits with the implementation of a forced exercise regime

The purpose of this experiment was to synthesize the data obtained from the previous experiments and determine if the implementation of a forced exercise regime alongside IFN- α -treatment administration would recover deficits in temporal order memory. There are many papers that indicate a positive effect of exercise for the preservation or recovery of memory deficits (O'Callaghan et al., 2007).

5.1. Animals

32 male Wistar rats (BioResources Unit, Trinity College Dublin) weighing between 215-330g at the start of the experiment were housed two/three to a cage within a controlled environment (Laminar airflow unit, 12h light/dark schedule with lights on at 08:00-20.00). Rats received food and water *ad libitum*. All behavioural testing was conducted during the light phase of the schedule. Prior to behavioural testing all rats were handled to acclimatise them to the experimenter. All experiments were carried out in accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experiments. The animals used in this experiment are the same animals used in a larger-scale experiment (Fahey et al., 2007)

5.2. IFN- α -Treatment

IFN- α treatment was administered as described earlier on in this chapter (Section 4.2)

5.3. Behavioural Procedures

Exercise Protocol

The exercise component is the same as that described by (Fahey et al., 2008) and a modified version of that used by (O'Callaghan et al., 2007). Rats were habituated to the training equipment (Exer 3/6 treadmill, Columbus instruments) for a 2 day period. Animals were placed on the belt of the treadmill running at a slow moving pace (0.78 km/h), the belt speed was increased to 1.02 km/h over a 15-30 minute period.

Reinforcement to run was provided by the placement of wire loops at the base of the treadmill belt which would provide a mild electric shock to the animal should they stop running. The shock was set at a low level of intensity (2 on a 0-10 scale, representing 0.7mA with an inter-pulse interval of 2 seconds). During the habituation period qualitative details of signs of distress or fatigue were noted in order to select good runners for the prospective experiment.

Animals were assigned to the four treatment groups on the basis of their performance on the 2 habituation days: Saline sedentary, Sal; Saline exercised, SalEx; IFN- α Sedentary, IFN; IFN- α exercised, IFNEx, n=8 for each group, however 2 animals from the Saline exercised group were removed due to ill health. The exercise procedure design was based on the performance on the habituation trials. Observations during this initial introduction to the treadmills determined that the rats tired after 20 minutes running. Therefore, rats were trained every other day for a period of 6 weeks, with each training session day consisting of two 20-minute running sessions (belt speed 1.02km/h), with a 20-minute rest interval between run sessions. The exercise sessions were conducted at the end of the light period (18.30-20.00), in order to reduce interruptions in the sleep pattern of the animals. Rats were monitored throughout the exercise sessions and promoted to run when tired by attracting their attention to the end of the treadmill. Sedentary rats were placed on a stationary treadmill for the same duration, with shock loops activated.

Animals were exercised for two weeks prior to commencement of IFN- α -treatment to prevent negative associations between exercise and IFN- α administration.

Temporal Order Memory Task

The temporal order memory behavioural procedure using a 6 hour intertrial interval between the second sample and test phase was employed, as described in the previous experiments (section 2.2.1). Rats were tested at a 2 week and 4 weeks after the commencement of IFN- α treatment, reflecting 4 weeks and 6 weeks of exercise respectively (Figure 14). New objects were used for the two testing sessions. The other behavioural experiments that were run alongside this experiment were conducted after these temporal order experiments. Temporal order is sensitive to stress therefore

it was vital that no aversive tasks were implemented prior to this procedure that may interact with behavioural performance.

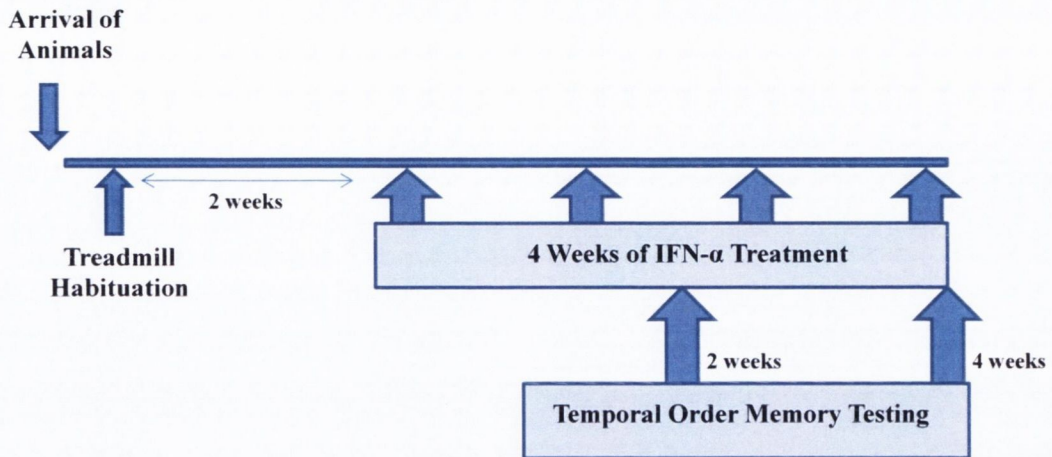


Fig.14. Schematic illustrating the experimental procedure employed, providing details of drug delivery, exercise regime and testing schedule. Rats received IFN- α for 4 weeks thrice weekly. Two testing sessions were conducted, 2 weeks after the beginning of treatment and 4 weeks after treatment.

5.4. Results

Exploration during the Test Phase

Figure 2a represents the proportion of time exploring the old object in the test phase after 2 weeks of IFN- α -treatment and 4 weeks of exercise. A one-way ANOVA revealed no significant effect of drug treatment on exploration of the old object ($F_{(3, 25)} = 1.864$, $p = 0.1616$). *Post-hoc* Newman Keul's multiple comparisons tests showed demonstrated no significant differences between treatment groups. Figure 2b represents the proportion of time exploring the old object in the test phase after 4 weeks of IFN- α -treatment and 6 weeks of exercise. A one-way ANOVA revealed a significant effect of drug treatment on exploration of the old object ($F_{(3, 25)} = 7.813$, $p = 0.0008$). *Post-hoc* Newman Keuls multiple comparisons tests showed that Sal ($p < 0.01$), SalEx ($p < 0.01$) and IFNEx ($p < 0.01$) explored the old object for significantly longer than the IFN- α group. IFNEx animals did not perform any differently from the saline-treated counterparts.

In both treatments time points the IFN- α -treated animals perform at or below the 50% chance level of exploration for the two object discrimination task.

Figure 15 represents the discrimination ratio for exploration of the old object over the new object. Statistics are expressed as those appearing above. It appears in this figure that there is a loss of discrimination ability of IFN- α -treated animals and the comparable discrimination proficiencies of the other groups.

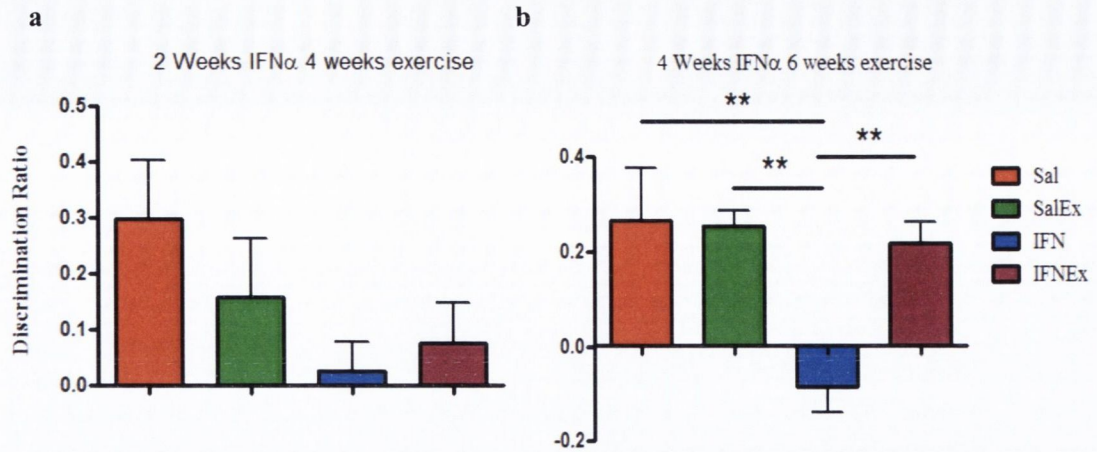


Fig.15. Performance of the groups expressed as discrimination ratios, demonstrating discrimination between the old object and new object in the temporal order memory task at; a) 2 weeks of IFN- α -treatment and 4 weeks of exercise, b) 4 weeks of IFN- α -treatment and 6 weeks of exercise Discrimination ratio ($p < 0.01$ in all cases); $n=8$ for IFN and IFNEx group, $n=6$ for Sal and SalEx. Data are expressed as mean + SEM.

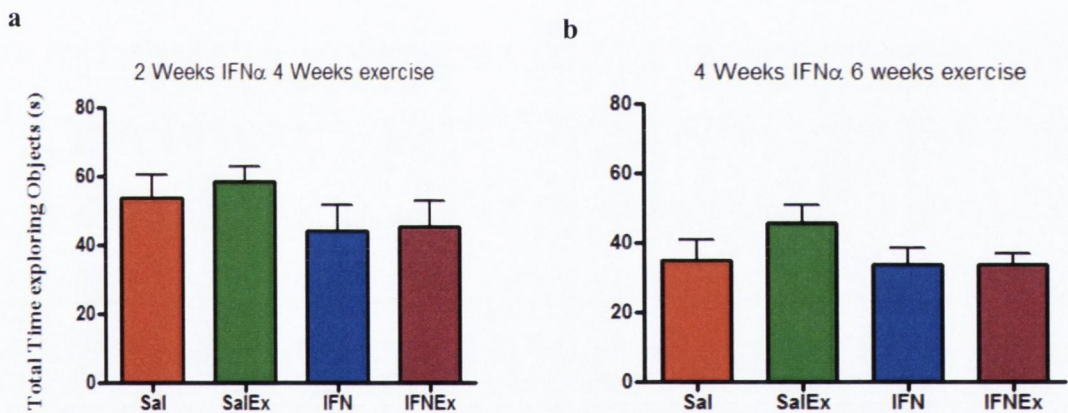


Fig.16. Performance of the four treatment groups total exploration of objects in the test phase of the temporal order memory task at; a) 2 weeks of IFN- α -treatment and 4 weeks of exercise, b) 4 weeks of IFN- α -treatment and 6 weeks of exercise ($p < 0.01$ in all cases); $n=8$ for IFN and IFNEx group, $n=6$ for Sal and $n=7$ for SalEx. Data are expressed as mean + SEM.

Figure 16a represents the total time spent exploring both objects in the test phase after 2 weeks of IFN- α -treatment and 4 weeks of exercise. A one-way ANOVA revealed no significant effect of drug treatment on exploration of the old object ($F(3, 25) = 1.006$, $p = 0.4065$). *Post-hoc* Newman Keuls multiple comparisons tests showed no significant differences between treatment groups. Figure 4b represents the total time spent exploring both objects in the test phase after 4 weeks of IFN- α -treatment and 6 weeks

of exercise. A one-way ANOVA revealed no significant effect of drug treatment on exploration of the old object ($F(3, 25) = 1.569, p = 0.2218$). *Post-hoc* Newman Keuls multiple comparisons tests showed no significant differences between treatment groups.

Exploration during the sample phases

Table 6 represents the raw exploration times in the sample phases and test phase in the temporal order memory task. Rats in all groups generally spent between 30 and 60 seconds actively exploring the objects in the trials. Exploration across trials was on the most part consistent, examination of trial effects was conducted for the 2 week exercise time point: Saline group: $F(2, 15) = 6.091, p = 0.0116$ *Post Hoc* Newman-Keuls multiple comparisons analysis suggest significantly more exploration spent in the test trial, compared to sample 1 or sample 2 ($p < 0.05$). However, no significant difference was found between sample 1 and 2 and the test trial determining equal exploration of these sample phases. SalEx group: $F(2, 21) = 0.6615, p = 0.5339$; IFN group: $F(2, 21) = 0.7468, p = 0.4860$; IFNEx group: $F(2, 21) = 2.840, p = 0.0810$. Overall, the data suggest that there is no impairment in exploration occurring as a consequence of the exercise regime or IFN- α -treatment at the 2 week time point.

Examination of trial effects was conducted for the 4 week exercise time point: Saline group: $F(2, 15) = 0.2269, p = 0.7997$; SalEx group: $F(2, 18) = 3.209, p = 0.0643$; IFN group: $F(2, 18) = 0.01262, p = 0.9875$; IFNEx group: $F(2, 21) = 0.6789, p = 0.5180$. Overall, the data suggest that there is no impairment in exploration occurring as a consequence of the exercise regime or IFN- α -treatment at the 4 week time point.

Table.6. Exploration times (in seconds; mean \pm SEM) of the objects in the sample phases and test phase in the temporal order discrimination task.

Treatment	Expl. Sample 1 (s)	Expl. Sample 2 (s)	Expl. Test Trial (s)
<i>2 Week Treatment</i>			
Saline	35.2 \pm 7.6	38.4 \pm 6.7	53.7 \pm 6.7 *
Saline + Exercise	47.8 \pm 11.6	47.6 \pm 8.1	58.4 \pm 4.9
IFN- α	38.8 \pm 4.3	34.3 \pm 3.7	44.0 \pm 7.8
IFN- α + Exercise	28.1 \pm 4.6	42.6 \pm 3.4	45.2 \pm 7.6
<i>4 Week Treatment</i>			
Saline	30.5 \pm 6.3	29.6 \pm 6.9	35.0 \pm 5.8
Saline + Exercise	34.6 \pm 7.2	30.9 \pm 1.8	42.5 \pm 4.5
IFN- α	35.7 \pm 6.9	31.3 \pm 6.2	33.5 \pm 4.8
IFN- α + Exercise	28.6 \pm 6.7	37.0 \pm 4.9	33.8 \pm 3.1

* = $p < 0.05$

5.5 Discussion

A number of interesting findings were produced from this series of experiments. First, the temporal order deficits induced by IFN- α -treatment demonstrated in experiment 3 are confirmed, indicating a further validation of the deficit.

The present findings suggest that there is a reinstatement of temporal order memory function with the implementation of an exercise regime in IFN- α -treated rats. Exercise, when implemented prior to the initiation and throughout the course of IFN- α -treatment, restores the ability to differentiate the serial order of object presentation. The recovery is dependent on the time points that were used regarding length of drug treatment (time receiving IFN- α). There are no differences ascertained on the temporal order task when animals were tested only 2 weeks into the treatment regime. However, a trend does appear in the proportion of exploration of the old object, the sedentary IFN- α treated

animals and the exercised IFN treated animals perform around the chance level, whereas the saline-treated counterparts perform better. There are no differences apparent between the sedentary and exercised IFN- α -treated animals therefore indicating that any positive benefit of exercise is not in place at this stage. However, with 4 weeks of IFN- α treatment there is a further loss of temporal order discriminative ability. Further, there is an increase in discriminative capacity in the exercised IFN- α -treated animals to the level of discrimination that the Saline treated animals perform. A basis behind the lag in rescue of temporal order memory is two-fold, first, it has been documented in experiments in our laboratory (Fahey et al., 2007) and other research that the neurovegetative symptoms appear initially with the cognitive side effects appearing later after 3 weeks (Capuron & Miller, 2004). Second, there are indications that exercise may have modulatory effects on neurogenesis and that the neurogenic process is of 3-4 weeks in timescale (Duman, 2004). A ceiling effect is apparent with the task, SalEx animals perform comparably to Saline alone. However, exercise does appear to be of great importance when deficits are evident providing a reinstatement of ability to complete this task.

This experiment was conducted as part of a larger scale experiment, in which recognition memory was assessed and found to be deficient in rats treated with IFN- α treatment. This may compromise the results included herein, signifying that there is a pure recognition memory problem with the task, however, it has been validated in the previous experiment (Figure 11) that using the two object protocol the IFN- α -treated rats can recognise objects. The recognition task that was used as part of this large scale experiment using exercise utilised a 4 object exploration paradigm with the use of external cues. Forwood et al (2005) express that when there is a lack of context and the task includes two objects there is no requirement for hippocampus (Forwood et al., 2005). The spatial navigation theory dictates that the relationship among a constellation of stimuli is required rather than just the use of one cue. It is possible that when an object is included in a crowded environment and may include some spatial element there is more of a hippocampal element. The saliency of the objects as separate entities may be reduced compared to the saliency that may occur when objects are presented in a more impoverished environment (Dere, Kart-Teke, Huston, & De Souza Silva, 2006). Additionally, the size of the arena is important as a small arena is seen as an object whereas a larger arena are treated as spaces engaging spatial and relational encoding

(Andersen, 2007). The arena used in Fahey et al (2008) was much larger than the one used in this experiment and the use of more objects may indicate a more complex environment, including extrinsic elements which would make the task more contextual (Fahey et al., 2008).

Few studies have investigated the effect of exercise on the PFC, one study found that there is an improvement in a prefrontal task; spatial working memory task and go no go paradigm in joggers (Harada, Okagawa, & Kubota, 2004). Additionally, oxyhaemoglobin levels have been measured and found to be increased in the prefrontal cortex when running (Suzuki et al., 2004). However conflicting theories have been postulated, such as the transient hypofrontality hypothesis, which suggests there is a decrease in PFC activity during physical exercise (Dietrich & Sparling, 2004). This decrease may aid in the restorative benefit of exercise to mood disorders. Increased activity returns following exercise, therefore providing a potential dissociable effect of exercise on mood and cognition.

How is exercise recovering temporal order deficits?

There are several putative mechanisms for this recovery of temporal order memory. These may be centred around recovery of the hippocampus or direct impact on the PFC. It is well known that exercise increases neurogenesis in the hippocampus and that this may be a result of the protective effects of increased neurotrophins (van Praag et al., 1999) such as BDNF and VEGF. This is a very plausible reason for the return of function. This explanation also fits in well with the timeline of neurogenic development of 3 weeks. If the hippocampus recovers it may be postulated that the HC-PFC pathway may strengthen and the PFC function returns.

6. Concluding remarks

The temporal order memory task is sensitive to assess amnesic (IFN- α) and promnesic manipulations (Exercise regime). Implementing an exercise routine will be of considerable value for the treatment of memory deficits. The present study reveals a number of distinct factors that may influence temporal order memory. Both mild behavioural stress (Experiment 2) and IFN- α -treatment (Experiment 3) have a negative impact on this mnemonic task; however there was a sparing of novel object recognition

memory. Additionally, the implementation of an exercise regime (Experiment 4) appears to act as a positive modulator of this type of memory and restores function.

The deficits that arise in temporal order processing in IFN- α -treated rodents may reflect some of the adverse events seen in the clinic (Capuron et al., 2005; Kraus et al., 2005; Pawelczyk, Bialkowska et al., 2008). These deficits also share common signs of stress and anxiety disorders. The primary brain regions involved in temporal order memory will be examined and translated to implications for patients receiving IFN- α -therapy.

There are many theories surrounding the basis of the temporal organisation of behaviour, one of the most well known is the perception-action cycle advocated by Fuster (2001). This dictates that the prefrontal cortex is essentially a 'doer' or 'action' cortical region which receives sensory input from the perceptual brain (posterior brain - occipital, parietal and temporal cortices) and temporally integrates the information to guide behaviour in a 'top-down' fashion (Fuster, 2001). Alongside this integration there is a profound influence of environment on the cognitive processing. The prefrontal cortex is a richly innervated structure with many reciprocal pathways connecting cortical and subcortical regions (Uylings, Parnavelas, & Walg, 1981). Owing to the affluent connectivity, it is evident that an extensive network of anatomically connected brain regions is required for the production of behavioural sequencing. In light of this fact, this series of experiments sought to clarify and untangle the specific roles of these regions in this higher order mnemonic task in the context of IFN- α -treatment.

It is evident from these experiments that the PFC appears to be compromised with IFN- α -treatment. What remains to be established is the anatomical location of the PFC affliction. The direct afferent connection from the ventral hippocampus to the prelimbic cortex may suggest that this deficit could arise from a dysregulation in this pathway. Although, it is plausible that there may be a dysregulation in the amygdala-PFC pathway, indicating a general limbic-cortical dysregulation as the source of neurocognitive effects. Additionally, we have determined that exercise is beneficial for recovery of this temporal order memory deficit. One may speculate that exercise may be beneficial for hippocampal strengthening and that this additionally strengthens the connection along this HC-PFC axis. This experiment provides important information regarding ameliorative strategies for prefrontal impairments. Not only patients

receiving IFN- α -treatment would benefit from an intervention that may restore significant deficits in temporal sequencing of behaviour, but also those patients with dysexecutive syndrome. Although this study assesses temporal order memory in animals the significant species specific homology ensures relevance of this data to the clinic. The temporal order memory task would be a useful paradigm to use in preclinical testing to determine effects of pharmacological agents on the PFC.

Chapter III

Variable-Goal Location Task

Impairments in Behavioural Flexibility arising from IFN- α -Treatment

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Summary

This experiment builds on the previous chapter investigating deficits in the prefrontal cortex that may arise from systemic stress (IFN- α -treatment). A paradigm that assesses task switching and behavioural flexibility is used here to determine if there are deficits in perseverative tendencies of the IFN- α -treated animals. This experiment employs a water plus-maze (WPM) protocol to assess both hippocampal- and prefrontal-dependent behaviours. The task utilises a place learning phase and subsequent reversal learning phases to dissociate specific impairments.

IFN- α animals exhibited delayed acquisition in the variable start phase. It was also elucidated that there are impairments in behavioural flexibility in IFN- α -treated animals. IFN- α -treated animals were not impaired on the acquisition of goal changes however they exhibited more perseverative errors on initial goal reversal trials.

This experiment further underlines the prefrontal deficits that occur as a consequence of IFN- α -treatment. The underlying functional prefrontal correlates of the behaviour we hypothesise are attributed to impairments in the prelimbic cortex. This provides elucidation of a specific focal PFC vulnerability, although PFC impairments may remain global.

1. Introduction

1.1. Goal Directed Behaviour

Goal directed behaviour is a fundamental executive function vital for guiding decision making and choice selection. It is purposive and is often reinforced by associations with prospective rewards (Ridderinkhof, Ullsperger, Crone, & Nieuwenhuis, 2004). Decisions can be graded ranging from simple decisions to more complex ones which may have life-changing consequences. Goal directed behaviour essentially characterises the higher cognitive function capacity in humans, although rudimentary goal direction is evident in rodents. When animals are faced with a problem they do not respond randomly but test, hypothesise, and strategise (O'Keefe & Nadel, 1978) thus inferring the use of goal directive strategies.

1.2. Behavioural Flexibility and Perseveration

Behavioural flexibility is a dynamic function of the prefrontal cortex, which enables the animal to update experiences and adjust behaviour according to environmental demands. Flexibility requires the inhibition of previously learned associations in order to direct focus to the new strategy. The inverse behaviour, perseveration, consists of repeated actions with the inability to inhibit irrelevant information and attend to novel relevant input (M. D. Hauser, 1999). Heightened perseveration is a characteristic of many psychiatric disorders including, obsessive-compulsive disorder, schizophrenia and generalised frontal disorders. The Wisconsin Card sorting task is used to assess prefrontal flexibility in humans and is based on rule changes involving the use of categories. Patients with damage to the frontal lobes are unable to shift strategies and tend to perseverate over the rule already learned (Rolls, 2004).

1.3. Neural Correlates of goal direction

The PFC regions may differentially contribute to learning switches in strategies (Birrell & Brown, 2000; Granon & Poucet, 1995). The prelimbic cortex subserves strategy switching behavioural responses. Inactivation of the prelimbic cortex results in an increase in errors to the previous goal/strategy, indicating perseverative behaviour (Ragozzino, Kim, Hassert, Minniti, & Kiang, 2003). Intradimensional shifts (shifts within the same domain) such as reversal learning, often involving reward require the

integrity of the orbitofrontal cortex (Dias, Robbins, & Roberts, 1996; Murray & Izquierdo, 2007).

Added support for the importance of the prefrontal cortex in goal location tasks comes from the identification of spatial place fields specifically firing in the prelimbic region (Hok, Save, Lenck-Santini, & Poucet, 2005). These 'goal' cells were also found to be specifically sensitive to reward, indicating a role for the PFC in spatial goal direction. Also, hippocampal place cell firing is augmented with damage to the PFC suggesting an interactive role for both the hippocampus and PFC in goal directed strategies (Kyd & Bilkey, 2003). The HC-PFC pathway (Jay et al., 1995) may be the portal for the coordinated organisation of future planned paths in a spatial goal directed task.

1.4. Water Plus-Maze

The pioneering studies utilising an eight radial arm maze design enable the study of both reference memory and working memory deficits using food reward (Olton & Samuelson, 1976). The strategies that the animals choose to adopt to successfully complete the task are limited due to the predetermined pathways that constrain the animals to find the goal (Hodges, 1996). Adaptation of this task took place in 1985 with the modification of the maze to a wet maze design (Buresova, Bures, Oitzl, & Zahalka, 1985). The incentive to motivate learning in this hybrid (radial arm maze and water maze) maze changed from an appetitive motivation to a desire to escape from an aversive experience. The task produces less procedural burden on training, requiring little training for task completion and therefore is more suitable for pharmacological investigations.

The water plus-maze is superior to the dry-maze paradigm for the investigation of behavioural flexibility due to several reasons. The mode of reward for task completion in this case is aversive which expedites the learning process and no food deprivation is required. Additionally the wet maze precludes the use of odours to complete the task. A dry maze was used in an experiment conducted by Gemmell and O'Mara (1999); a similar protocol is used here, only a wet maze is employed (Gemmell & O'Mara, 1999).

1.5. Rationale for experiments

The rationale for examining the behavioural flexibility of IFN- α -treated rats was to further delineate prefrontal deficits occurring as a consequence of this treatment. The

previous chapter elucidated prefrontal functioning impairments in IFN- α -treated animals in a behavioural sequencing domain. Here, we examine behavioural flexibility and perseverative behaviour in order to dissociate regional localisation of prefrontal impairment with this pharmacological treatment. Prior to the water plus maze an elevated plus maze will be used to probe for anxiogenic symptoms of IFN- α -treatment. We predict that IFN- α -treated animals will have deficits in behavioural flexibility further strengthening the evidence for deficits in prefrontal functioning.

2. Materials and Methods

2.1. Animals

Fifteen male Wistar rats (BioResources Unit, Trinity College Dublin) weighing between 300-335g at the beginning of the experiment were used. Animals were housed two or three to a cage within a controlled environment (Scantainer (Scanbur®) airflow unit, 20-22°C, and 12h light/dark schedule with lights on at 08:00-20.00). Behavioural testing was conducted during the light phase of the schedule. Rats received food and water *ad libitum*. Prior to behavioural testing all rats were handled to acclimatise them to the experimenter. All experiments were carried out in accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experimentation.

2.2. IFN- α -Treatment

Roferon-A (human recombinant interferon-alpha 2a, Roche Pharmaceuticals, USA) (~170,000 IU/kg, 0.2mL, diluted in saline, s.c.), or vehicle (0.2mL 0.9% NaCl, s.c.) was administered once a day, three times a week for four weeks prior to the commencement of behavioural testing to ensure that treatment was at the chronic dose level. The dose was adjusted once a week to compensate for any increase in weight gain.

2.3. Behavioural Procedures

Figure 1 below shows an annotation of the testing schedule, two behavioural paradigms were included in this experiment, the elevated plus maze to probe for the drug effect (demonstrated previously (Fahey et al., 2007)) and a water plus-maze to assess behavioural flexibility.

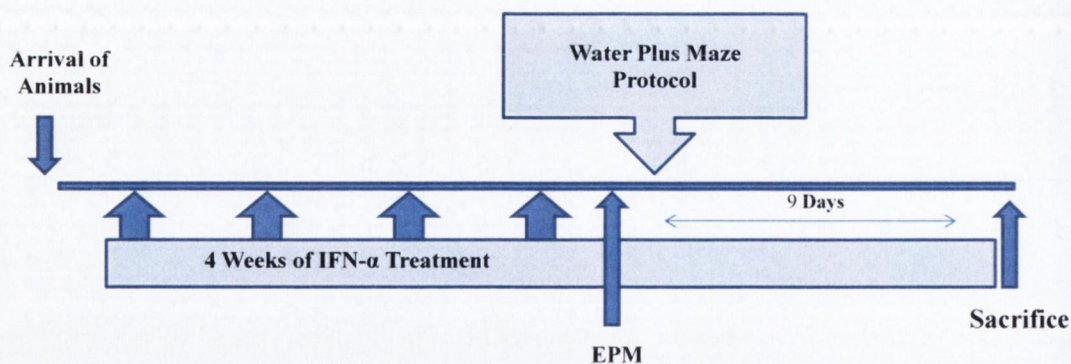


Fig.1. A schematic illustrating the testing schedule demonstrating the IFN- α -treatment period and commencement of behavioural testing. IFN- α -treatment continued throughout the course of the experiment. EPM: Elevated Plus-maze

2.3.1. Elevated Plus-Maze

Apparatus

Rats were tested in an elevated plus-maze, consisting of two open arms and two closed arms 41.5cm x 59 cm (height x width). The closed and open arms were opposing with a hexagonal central region 38cm x 16cm (diameter x sides). The maze was elevated 76.5cm above the ground. The maze was placed within a curtained testing environment to exclude any extrinsic environmental cues. Two lamps were placed around the maze (40 watt bulbs) at regular intervals to ensure that the lighting was homogenous throughout the testing environment.

Behavioural Procedure

Rats were placed on the maze opposite an open arm to encourage entry into the arm. They were allowed free exploration of the maze for a period of 10 minutes. The trial was recorded using an overhead camera and the Mediacruise® recording software. The time spent in the open and closed arms and the frequencies of entries into the arms were scored online during the testing session. Further ethological measurements were analysed *post hoc* to further define the pharmacological profile. Rats were returned to the home-cage after the testing session and the maze was thoroughly cleaned with Savlon® to remove odour cues before the next animal was tested.

2.3.2. Water Plus-Maze

Apparatus

Two days after the elevated plus maze rats began training on the water plus maze. A custom-made polypropylene water plus-maze was used for this test. Each arm (50cm) extended from the central decision region and could be blocked with the use of a divider. A concealed platform was placed at the end of the selected arm with the water level (3cm above the platform) the maze was placed in a dimly lit behavioural testing room, with four (40 watt bulbs) lamps. One large cue (consisting of a cardboard striped flag) was placed in the north east corner of the room. The maze was surrounded by thick-lined black curtains spanning from floor to ceiling to ensure that only the intrinsic cues were available for observation. The experimenter stood within the testing environment at a fixed position the south west corner for the whole length of the testing period. All testing sessions were recorded using an overhead camera and the Mediacruise® software.

Behavioural Procedure

All rats were trained to find the escape platform in the water plus-maze. Rats were placed into the maze facing the wall and allowed to explore the possible arms of the maze. The start points were counterbalanced to prevent any possible spatial bias. If the rat found the escape platform he was left on the platform for 20 seconds in order to reinforce the escape location and to allow for the observation of the spatial cues in the environment. Entry into the wrong arm resulted in blocking the rat in the arm for 15 seconds. This acted as a negative reinforcement to expedite the training process. Following an incorrect response or a correct response the rat was taken out of the maze and placed in a holding cage next to the experimenter for a period of 30 seconds. After this inter-trial interval the animal was placed back into the maze for the next trial. A maximum of 30 trials were given each day. The position of the goal location was counterbalanced between the rats and the water in the maze mixed with a ruler at the end of each rat's testing session in order to minimize odour traces. The training consisted of three distinct stages; these are annotated in figure 2 below.

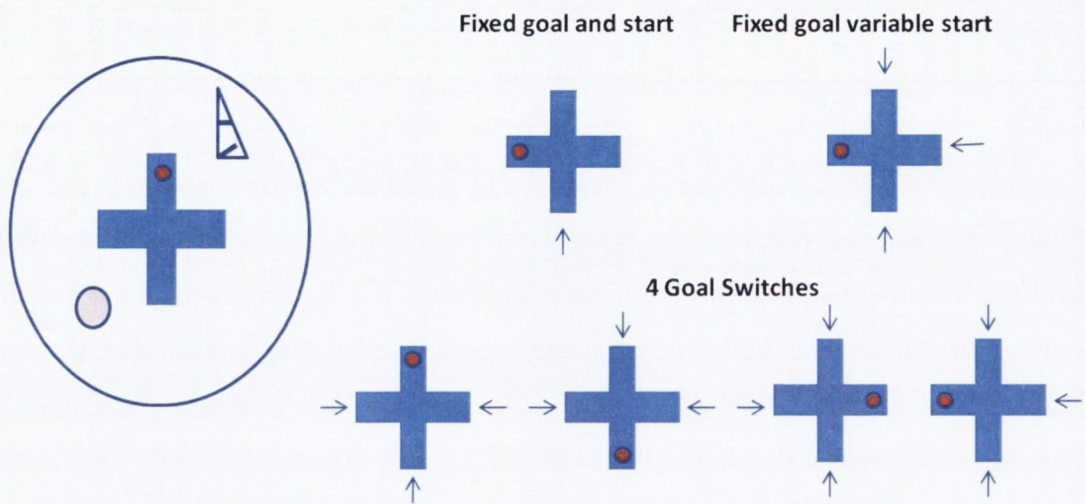


Fig.2. Annotation of the Water Plus-Maze testing schedule. The schedule incorporates three distinct stages. Each trial session was trained to asymptotic level of 80% criterion before moving onto the next session. Arrows indicate possible entry points. The red circle represents the submerged platform. The Pink circle represents the experimenter and the triangle depicts the cue.

Fixed Goal Location session: The goal location and the entry point of the rat into the maze remained the same for the whole testing period. This was a shaping procedure that was used to train the rats on how to perform in order to successfully complete the task. Each rat was given consecutive trials until they performed at an 80% criterion (i.e. they swam to the arm with the escape platform 8 times out of 10 consecutive trials). If criterion was not reached within the 30 trials allocated for that day the same procedure was run the following day until the criterion was met. Most rats reached criterion within the 30 trials of this session. Once the criterion was achieved the rats were allowed to commence onto the next stage of behavioural testing.

Variable Start Task: This stage differs from the original stage by introducing a variable start location of the rat. The start location was pseudorandomly selected however the training parameters remain the same as the fixed goal location session. The previous stage depended on the self movement cues of the animals however this stage requires a spatial processing of the goal in the maze. By using the external cue the rat has to determine where the escape platform is.

Switch Task: This stage involves the movement of the goal location. The goal is moved through a multiple of 90 degrees and the start location remains pseudorandomly selected. This stage consisted of four switches in the goal location, once the criterion was reached on one goal the animal was allowed to progress to the next switch. The final switch acted as a reacquisition of the original goal location.

After each animal's testing session of the day (maximum 30 trials), the animal was dried off with a towel and placed in an empty cage in front of a fan heater to dry fully and groom before returning to the home cage.

2.4. Statistics

All data was analysed using SPSS version 14. Normality was assessed prior to analyses using the Shapiro-Wilk's test. Independent t-tests, repeated measures ANOVA or one-way ANOVA were used where appropriate, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3. Results

3.1. Elevated Plus-Maze (EPM)

IFN- α -treated animals exhibit anxiogenic traits in the elevated plus-maze

The time saline- and IFN- α -treated animals spent exploring the open and closed arms of the elevated plus maze can be seen in figure 3a and b. Independent t-tests demonstrate that there is a significant difference in time spent in the open arms with saline-treated animals (M=16.90, SEM = 4.620) spending more time in the open arms than IFN- α -treated animals (M=3.963, SEM = 2.338), $t=2.599$, $df = 9$, $p<0.05$ ($p= 0.02$, two-tailed). There is also a significant difference in time spent in the closed arms with saline-treated animals spending less time in the closed arms than the (M=424.3, SEM = 9.097) IFN- α -treated animals (M=491.0, SEM = 16.56), $t=3.385$, $df =13$, $p<0.01$ ($p= 0.0049$, two-tailed).

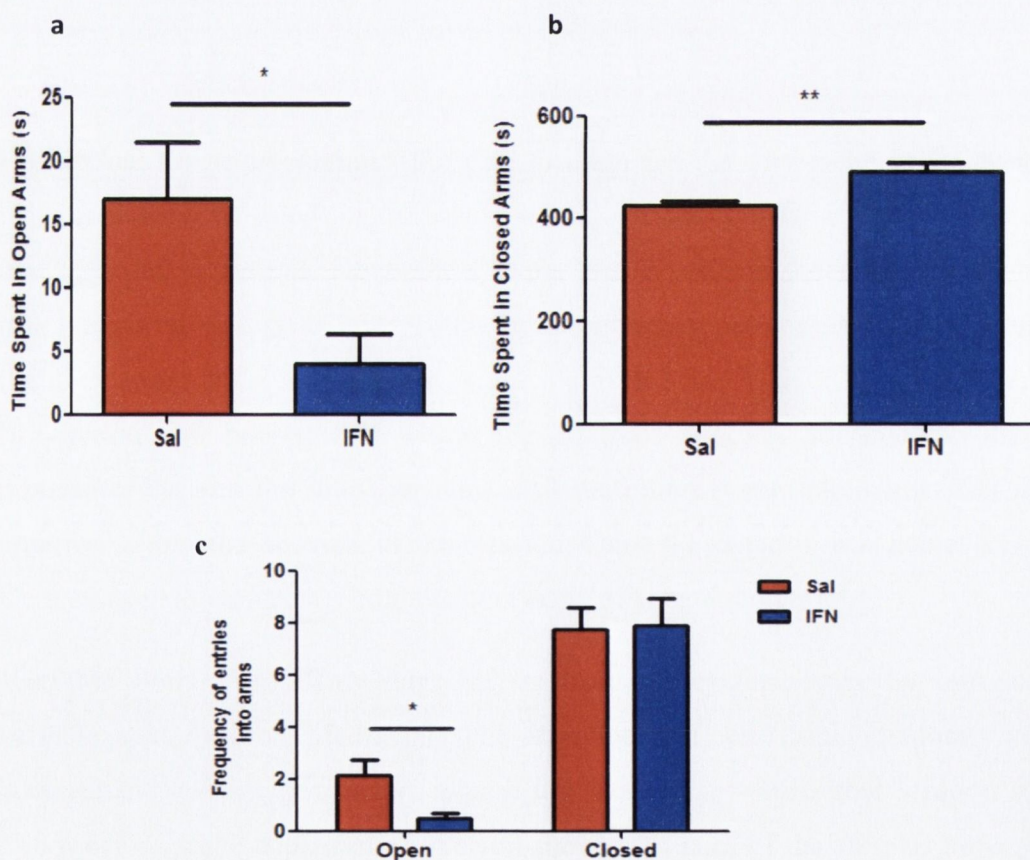


Fig.3. Time spent exploring the open and closed arms of the elevated plus maze a) time spent exploring the open arms b) time spent exploring the closed arms, c) frequency of entries into the open and closed arms. Data presented as mean + SEM, $n=6$ (IFN) and $n=8$ (Sal).

Additionally, figure 3c shows a significant difference in the frequency of entries into the open arms for saline treated animals ($M= 2.143$, $SEM = 0.5948$) and IFN- α -treated ($M=0.500$, $SEM = 0.1890$), $t=2.788$, $df = 13$, $p<0.05$ ($p= 0.0154$, two tailed). IFN- α -treated animals make fewer visits to the open arms. However, there are no significant differences apparent between the exploration of the closed arms for saline treated ($M= 7.714$, $SEM = 0.8650$) and IFN- α -treated animals ($M= 7.875$, $SEM = 1.076$), $t= 0.1141$, $df = 13$, $p>0.05$ ($p=0.9109$, two-tailed).

3.2. Water Plus-Maze

IFN- α animals require more days and more trials to complete the water plus-maze paradigm

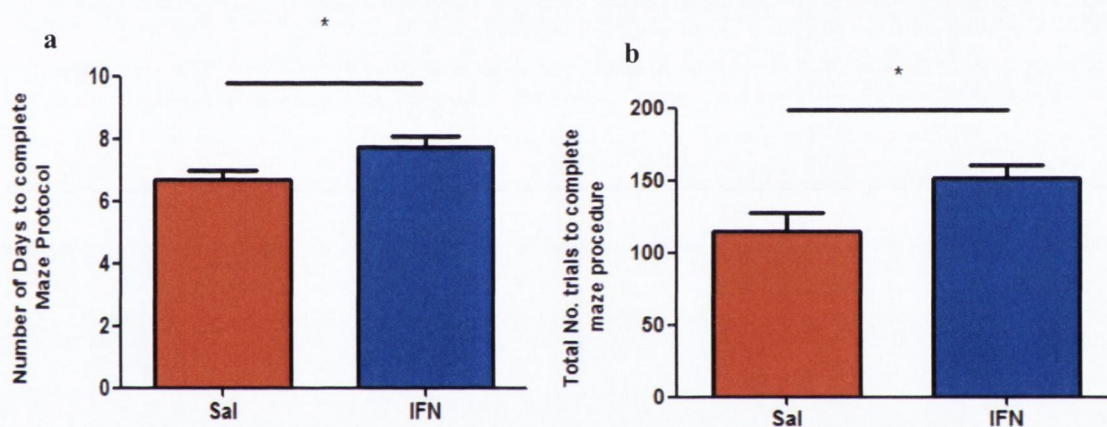


Fig.4. Parameters for task completion. a) Number of days taken to complete the water plus maze protocol and b) number of trials to complete the protocol. Data presented as mean + SEM, IFN- α (n=8) and Saline (n=6), * $p<0.05$

Figure 4 above depicts the number of days and trials required to complete the water plus-maze protocol. Independent t-tests demonstrate that there is a significant difference between the number of days required to complete the water plus-maze protocol for IFN- α -treated ($M = 7.750$, $SEM = 0.3333$) and saline rats ($M = 6.667$, $SEM = 0.3134$). IFN- α rats required more days in order to complete the water plus-maze procedure, Fig 4a ($t = 2.338$, $df = 12$, $p<0.05$ ($p=0.0375$, two-tailed). Consequently, the total number of trials required to acquire the task differ between the saline ($M = 114.8$, $SEM = 13.14$) and IFN- α animals ($M= 151.9$, $SEM = 8.766$). IFN- α animals require

more trials in total ($t = 2.440$, $df = 12$, $p < 0.05$ ($p = 0.0312$, two-tailed) to complete the water plus maze protocol.

IFN- α animals require more trials to reach criterion on the variable start session

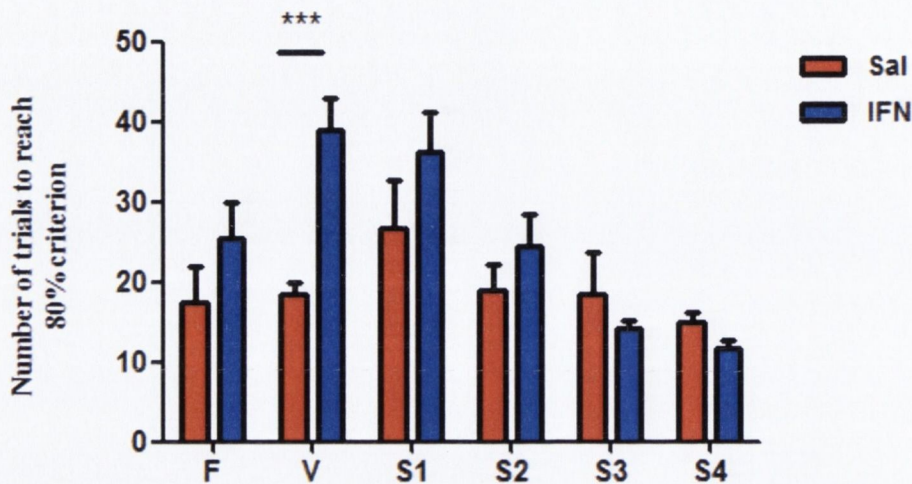


Fig.5. The number of trials required to reach 80% criterion for the different testing sessions. Abbreviations: F, Fixed goal location; V, Variable start task; S1, goal switch 1; S2, goal switch 2; S3, goal switch 3; S4, goal switch 4. Data presented as mean + SEM, IFN α ($n=8$), Sal ($n=6$), *** $p < 0.001$

Figure 5 shows the number of trials required to reach 80% criterion over the testing sessions. A two-way repeated-measures ANOVA revealed a significant effect of testing session ($F_{(5, 72)} = 6.80$, $p < 0.0001$), a significant interaction between the testing session and group ($F_{(5, 72)} = 2.93$, $p = 0.0216$), and a significant effect of group ($F_{(1, 72)} = 5.98$, $p = 0.031$). Subsequent one-way ANOVA's performed for each testing session demonstrate that there was a significant difference in the number of trials required to reach 80% criterion in the variable start session for the IFN- α and saline-treated animals, with the IFN- α animals requiring more trials to acquire the task rule ($F_{(1, 12)} = 18.886$, $p < 0.001$). However, significant differences were not apparent in the other testing phases. Nonetheless, there is a significant trend in the final goal switch (S4) for IFN- α animals reaching the 80% criterion with less trials than the saline controls, however this did not quite reach significance ($F_{(1, 12)} = 3.896$, $p = 0.072$).

On initial examination of figure 5 there is no significant difference in the number of trials required to reach 80% criterion in the goal switch sessions. However, the acquisition of the task rule does not discount performance deficits within the trial session. Further analyses of the behavioural performance within these switch phases will further clarify the evolution of the errors within these phases and elucidate underlying performance strategies.

3.2.1. Evolution of the errors within the distinct phases

Self-movement behaviours are comparable in the experimental groups

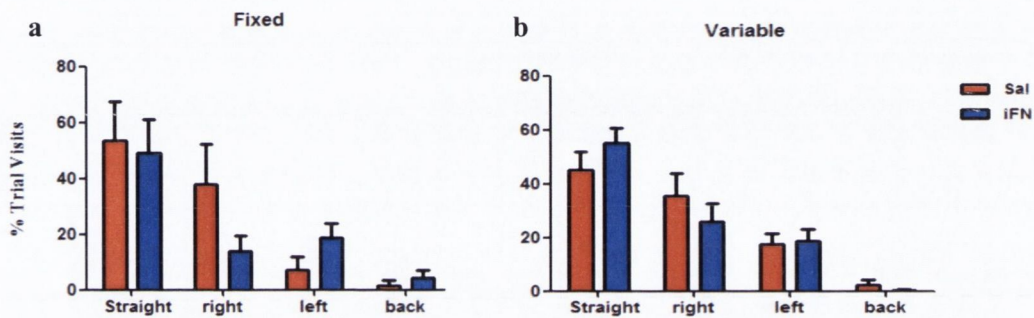


Fig.6. Breakdown of the intrinsic body movements in the trials leading up to reaching criterion in a) the fixed trial and b) variable start sessions. Data presented as mean + SEM, IFN- α (n=8) and Saline (n=6).

To establish if there were any locomotor effects (i.e. vestibular self-movement patterns) an analysis of the directional movement of the animal in the incorrect trials of the fixed goal and variable start phase was completed and can be seen in figure 6 above. A χ^2 test reveals no significant differences in directed movements in the fixed trial between the saline and IFN- α treated animals ($\chi^2 = 2.53$, $df = 6$, $p = 0.86$). Likewise in the variable start phase there are no differences in directed movements in the fixed trial between the saline and IFN- α treated animals ($\chi^2 = 2.28$, $df = 6$, $p = 0.89$) demonstrating comparable motor performances of the treatment groups when making an incorrect arm entry

Variable Start Session

Figure 7a below illustrates the number of correct visits to the goal location in the variable start condition. The IFN- α (M = 21.38, SEM = 1.1413) and saline- (M = 10.17, SEM 1.167) treated rats differ in the number of visits to the correct goal arm in this phase. The IFN- α -treated rats make significantly more correct visits to the goal arm

($t=5.818$, $df = 12$, $p<0.0001$). This corresponds with the increased trials that are required in order to acquire the 80% criterion performance on this phase. However, the relative proportion of correctly performed trials does not differ between saline ($M = 54.89$, $SEM = 3.875$) and IFN- α - ($M = 57.02$, $SEM = 3.465$) treated animals ($t = 0.4071$, $df = 12$, $p = 0.6911$).

IFN- α animals visit the correct arm more, however proportion of visits is comparable

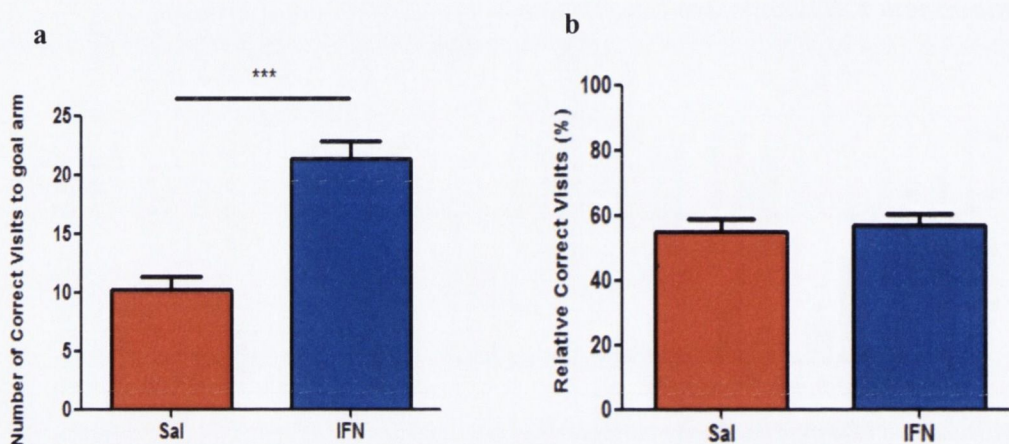


Fig.7. Examination of the correct trials to the goal location in the variable start session a) total number of correct trials to the goal location in the testing session b) Relative proportion of visits to the correct arm. Data presented as mean + SEM, IFN- α (n=8) and Saline (n=6), *** $p<0.001$

Analysis of the number of correct arm entries and number of left turns in the first 10 trials of the variable start phase is illustrated in figure 8a and b. The IFN- α ($M = 5.000$, $SEM = 0.3780$) and saline ($M = 4.167$, $SEM = 0.7032$) treated rats make comparable visits to the correct goal arm in the first 10 trials of this phase ($t=1.119$, $df = 12$, $p = 0.2852$). The number of self-movements of the animal to the left independent of the start position in the first 10 trials does not differ between the saline ($M = 3.167$, $SEM = 0.6009$) and IFN- α ($M = 2.875$, $SEM = 0.7892$) animals ($t = 0.2767$, $df = 12$, $p = 0.7867$).

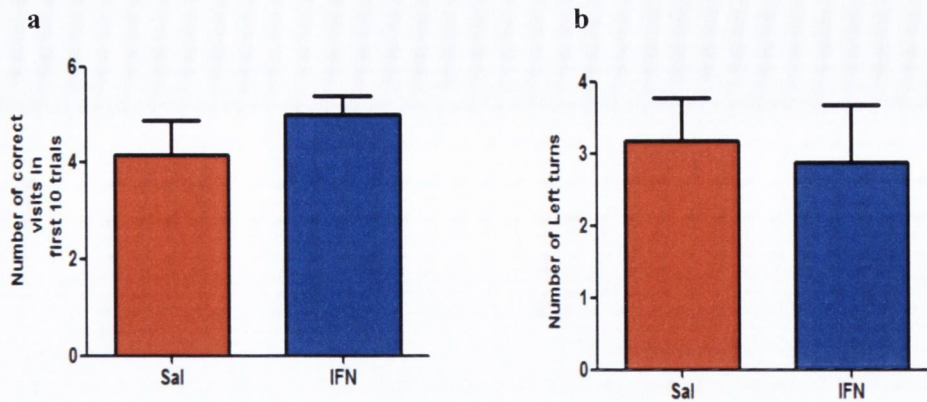


Fig.8. Examination of the first 10 trials of the variable start session a) Number of correct trials b) Number of left turns. Data presented as mean + SEM, IFN- α (n=8) and Saline (n=6).

Goal Switch Trials

IFN- α animals make more perseverative errors to the previous goal in the first switch session

Figure 9 below depicts the number of visits to the previous goal location as a percentage of the total number of incorrect trial visits made by saline and IFN- α animals. A two-way repeated-measures ANOVA revealed no significant effect of testing session ($F_{(3, 48)} = 1.105, p=0.360$), a significant interaction between the testing session and group ($F_{(3, 48)} = 4.980, p < 0.01$), however no significant effect of group ($F_{(1, 12)} = 1.909, p = 0.1992$) was demonstrated. Subsequent one-way ANOVA's performed for each testing session demonstrate a significant difference in the number of previous goal location visits in switch 1 that the IFN- α and saline treated animals make; IFN- α animals make more perseverative visits to the previous goal location ($F_{(1, 12)} = 8.894, p < 0.05$). However, significant differences were not apparent in the second and third goal switch phase. Nonetheless, a significant difference is revealed in the errors made in the final switch (when the goal returns back to the first goal location) with the saline controls performing at chance level visiting the previous goal arm a third of the time. However, the IFN- α animals made incorrect visits to the previous goal location at below chance levels ($F_{(1, 12)} = 5.414, p < 0.05$).

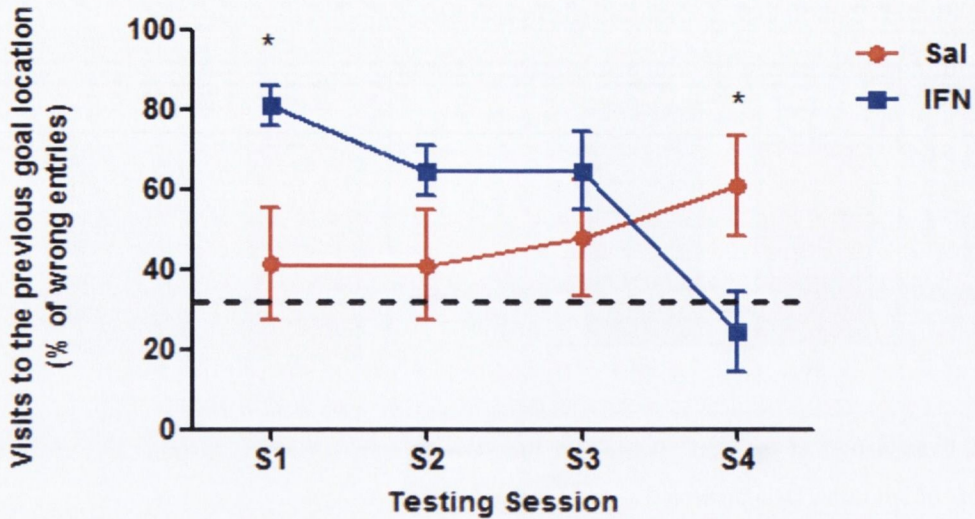


Fig.9. Percentage of incorrect trials spent visiting the previous goal location. Dashed line indicates chance level (33%). Data presented as mean + SEM, IFN- α (n=8) and Saline (n=6), * $p < 0.05$. Abbreviations: S1; Switch 1, S2; Switch 2, S3; Switch 3 and S4; Switch 4.

IFN- α animals make more perseverative errors to the previous arm in the first 10 trials of the first switch

Figure 10 below depicts the number of visits to the previous goal location in the first 10 trials of the new goal switch session in both saline and IFN- α animals. A two-way repeated-measures ANOVA revealed a significant effect of testing session ($F_{(3, 48)} = 5.199, p < 0.01$), a significant interaction between the testing session and group ($F_{(3, 48)} = 4.770, p < 0.01$), however, no significant effect of group was determined ($F_{(1, 12)} = 3.089, p = 0.104$). Subsequent one-way ANOVA's performed for each testing session revealed that there was a significant difference in the number of visits to the previous goal location in the first 10 trials of switch 1 that the IFN- α and saline treated animals made, with the IFN- α animals making more perseverative visits to the old goal location ($F_{(1, 12)} = 8.661, p < 0.05$). However, significant differences were not apparent in the second and third goal switch phase. Nevertheless, there is a significant difference in the errors made in the final goal switch (when the goal returns back to the first goal) with the saline controls performing at chance levels and the IFN- α animals performing below chance ($F_{(1, 12)} = 6.645, p < 0.05$).

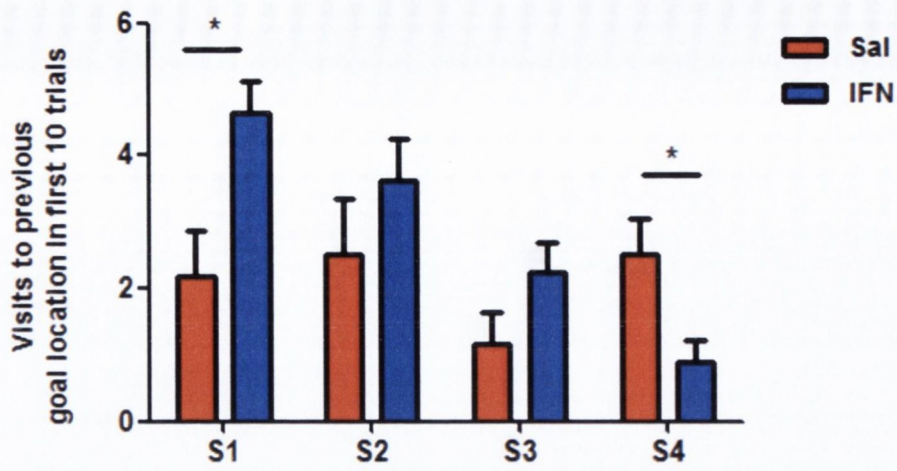


Fig.10. Examination of the number of visits to the previous goal location in the first 10 trials. Data presented as mean + SEM, IFN- α (n=8) and Saline (n=6), * $p < 0.05$. Abbreviations: S1; Switch 1, S2; Switch 2, S3; Switch 3 and S4; Switch 4.

4. Discussion

This series of experiments examines the behavioural flexibility of IFN- α -treated animals. It is determined that there is a subtle impairment in animals treated with IFN- α .

The elevated plus-maze (EPM) was conducted as a probe to verify that IFN- α treatment was effective in inducing an anxiogenic phenotype in the animals. Consistent with previous data presented in our Lab (Fahey et al., 2007) the IFN- α -treated animals presented with an anxiogenic behavioural phenotype in the EPM.

IFN- α -treated animals performed comparably to saline controls on the fixed start phase. This task is a directional learning task (egocentric) that introduces the animal to the purpose of the paradigm (to escape onto a hidden platform). This simply requires the rat to learn to turn left to find the escape location. Both groups perform well on this task; almost all achieve 80% criterion level within 30 trials. This task may be compared to stimulus response paradigms (associative) considering the animal is learning to pair the motor movement with escape from an aversive situation.

A question that emerges from this data is the underlying reason for differences in the acquisition of the variable start phase for the saline and IFN- α animals. IFN- α -treated animals require more trials to reach 80 % criterion on this task. The underlying errors were assessed based on egocentric movements and figure 6b demonstrates that there are no proportional differences in motor direction exploration, thereby ruling out purely egocentric based heading strategy impairments. This could have resulted from a habit based behaviour remaining from the previous (fixed) trial phase. From the variable point of departure motor sequences directed to the previous goal arm were not evident. No clear pattern could be deciphered from the sequences of entries into arms; the most plausible reason resides in an inability to rapidly learn to navigate towards a goal using an external cue. The IFN- α -animals remain topographically disorientated and require many more trials in order to complete this phase of the task. This spatial memory impairment has been supported by previous experiments in our lab group on the Morris Water Maze (Fahey, 2005); IFN- α -treated animals demonstrated a slightly delayed acquisition indicating a spatial deficit.

It is clear from the fixed and variable start location that there are no impairments in motor movements. The egocentric component of the task is thought to reside behind a

dorsomedial striatum; damage to this region would result in impairments in acquiring the response (Ragozzino, Detrick, & Kesner, 2002). In the fixed location the animals are learning a route, which requires less representation than a spatial map (Byrne & Salter, 1983). The basis behind such learning is egocentric (body-centred), the animal will use his egocentric position in relation to the goal and turn in that direction in order to reach the target (Aguirre & D'Esposito, 1997).

It is important to point out that the impaired performance of IFN- α animals on the variable start phase resulted in these animals experiencing this goal location more than the saline-treated animals. This is illustrated in figure 7a, IFN- α animals experience this arm twice as much as the saline counterparts. This may have inferred some overtraining on this task.

On first glance at figure 5, there appears to be no difference on performance of the two groups in the switch (reversal) paradigm. Although acquisition remains unaffected it is apparent that the behavioural performance underlying the initial performance of the task (specifically switch 1) differs between the two groups. IFN- α -treated animals make more perseverative errors to the old goal location (figure 9). The percentage of visits to the wrong arm is twice that of the saline-controls. When deconstructing the behaviour underlying the perseverative behaviour it may be interpreted that the anticipated reward of escape from aversive environment is no longer present in the arm that previously held refuge. Although attempts to escape remain futile when entering this arm the animal repeats the same entry into the arm that has lost reward incentive (valence) (Bechara, 2001). The animal is unable to suppress the inappropriate response even in light of the negative consequences. One explanation may originate from the overtraining in the variable goal location. Overtraining behaviour can lead to the formation of habits. Kilcross and Coutureau (2003) examined this phenomena and assessed specific lesions of the dorsal (prelimbic) and ventral (infralimbic) PFC in order to dissociate subregional contribution to this behaviour (Killcross & Coutureau, 2003). They found that a loss of sensitivity to a goal value (devaluation) underlies a prelimbic contribution. However, animals with infralimbic lesions are able to demonstrate this sensitivity to the goal value. Therefore we speculate that the underlying disruption may occur in the prelimbic region of the PFC.

The central aim of this experiment was to determine the extent of damage to the PFC and decipher specific subregions that may be impaired. As a whole it is well established that the PFC is required for the inhibition of a learning strategy in order to shift to a new task contingency. There are differential contributions that PFC subregions make for the performance in certain tasks, the deconstruction of these subregional contributions to the WPM task is provided here. Interpretations made here suggest that there is focal damage to the PFC in the region of the prelimbic cortex however, widespread damage in PFC cannot be ruled out. Additionally, the orbitofrontal cortex (OFC) may contribute to some of the impairments, considering this is a subregion of the PFC specifically required for devaluation and changes in rewards. However, this region is also particularly susceptible to reversal learning and in this case reversal impairments are not evident. Histological analysis may provide further insight into the extent of the damage to the PFC.

Interestingly, there is a heightened performance of the IFN- α -treated animals on the last switch, figure 9 and 10. The saline-treated animals perform uniformly on the goal switch tasks whereas the IFN- α -treated demonstrate a strong learning curve for the task. A simple explanation for the heightened performance on the 4th switch may arise from the overtraining the animals experienced in the training session. It would therefore be interesting to determine if extinction occurs to the same extent with IFN- α and saline-treated animals in a probe trial. One would predict IFN- α -animals would perseverate, visiting the 4th arm more than the other three arms; however, further studies are required. Some forms of habit forming behaviour may therefore stem from compromise to the PFC; this is demonstrated in ageing, where habits may predominate goal directed behaviour.

5. Conclusion

The results included here demonstrate IFN- α -treated animals display selective impairments in variable goal location tasks. IFN- α -treated animals demonstrate subtle perseverative tendencies, which indicate a prelimbic dysfunction. Accumulating evidence suggests that the HC-PFC pathway may be dysregulated in affective disorders and from these two chapters may be an underlying cause for IFN- α -treated adverse effects. Further indication is provided here that there may be a dysregulation in this pathway resulting from deficits in the prelimbic cortex, which is the main recipient of the fibres from the hippocampus.

Chapter IV

IFN- α -Treatment produces significant behavioural deficits and a decrease in Cell Proliferation suggestive of a decrease in neurogenesis

Restoration of Neurogenesis occurs with Fluoxetine treatment

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Summary

There is a wealth of literature establishing a link between IFN- α -treatment and coinciding neuropsychiatric deficits. These manifest in many forms, with anxiety and depression as the leading culprits. However, there remains a poor understanding of the molecular basis underlying these adverse events.

Consistent with previous research from our lab (Fahey et al., 2007) the data presented here demonstrate anxiogenic and depressive adverse events in IFN- α -treated animals. A rescue of these behavioural responses is found with the co-administration of the commonly used SSRI Fluoxetine. Additionally, a decrease in cell proliferation is also found, indicative of suppression of neurogenesis in rats treated with IFN- α . This decrease is successfully recovered with the administration of Fluoxetine.

The functional significance of a suppression of neurogenesis may provide further clues to the mechanisms underlying IFN- α -induced mnemonic deficits and neuropsychiatric effects.

1. Introduction

1.1. Neuropsychiatric Adverse Events in IFN- α -Treatment

Depression is one of the most detrimental neuropsychiatric side effects in patients receiving IFN- α -treatment (Capuron & Miller, 2004; Loftis & Hauser, 2004). In that clinical population a wealth of knowledge demonstrates a high incidence of depression cases, however, the underlying mechanisms for these adverse events remain unknown. This chapter aims to investigate how immune modulators, specifically IFN- α can have a detrimental effect on mood. The link between the immune system and the incidence of depression is well established (Dantzer et al., 2008; Maier & Watkins, 1998). Immune system activation may be considered an endophenotype of depression. In other words, depression can occur as a non-specific symptom of disease, secondary to immune activation (Dantzer et al., 2008).

1.2. Depression

"I think this man might be useful to me - if my black dog returns. He seems quite away from me now - it is such a relief. All the colours come back into the picture." - Winston Churchill

This quote poignantly illustrates how we can fall hostage to our emotions and the all-consuming nature that can result from depression. But what if those emotions appeared uncontrollable? Depression and anxiety disorders are becoming an increasing problem in the western world, an estimated incidence of 10-20% of people experiencing depression at some time in their lifetimes (Wong & Licinio, 2001). IFN- α -treated patients often experience a reactive depression to the pharmacological treatment; this is unrelated to the disease progression. The incidence of depression may be as high as 40% in patients treated with IFN- α (Bonaccorso, Marino, Puzella et al., 2002), however there is susceptibility involved.

1.3. Modelling Depression and Anxiety in animals

Anxiety and depression can be adequately modelled in rodents with the use of several behavioural paradigms. The mainstay paradigms are the Elevated-Plus Maze (EPM) and the Forced Swim Test (FST)/Porsolt test. The elevated-plus maze is a reliable screen for anxiolytic drugs (Carobrez & Bertoglio, 2005) and the forced swim test

reliably screens depression (Cryan, Valentino, & Lucki, 2005). Fahey *et al* (2007), successfully modelled anxiety and depression in IFN- α -treatment. IFN- α -treated animals spent less time in the open arms of the EPM, indicating anxiogenic tendencies and more time immobile in the forced swim test, demonstrating despair behaviour (Fahey *et al.*, 2007). The development of this animal model was paramount in assisting to unravel the complexities of IFN- α -induced adverse events.

1.4. The Neurogenic Hypothesis

Adult neurogenesis is the generation of newborn neurons from progenitor cells in the adult nervous system. The first report of neurogenesis in the adult brain was revealed by Altman in 1962; however, it was not until the early 1990s that the field regained revival. Until this renewed interest, the hard-wired view of ‘no new neurons’ in the adult brain prevailed, this dogma has now been quashed (Kempermann, Jessberger, Steiner, & Kronenberg, 2004). The areas where new born cells arise are limited in the adult brain, these comprise of the subventricular zone (SVZ), the olfactory epithelium and the subgranular zone (SGZ) of the dentate gyrus.

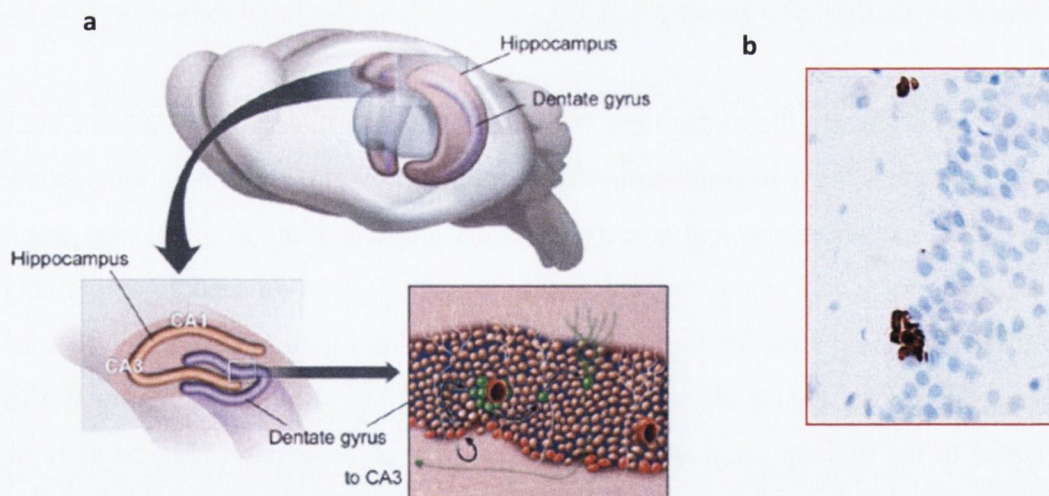


Fig.1. a) Picture depicting the hippocampus in rodents and a coronal section revealing the dentate gyrus. The inset demonstrates the proliferating neurons in the subgranular zone and the migration of neurons through the granule cell layer (green). Picture taken from Morse and Barlow 2006 **b)** Picture demonstrating BrdU staining of a cluster of cells in the subgranular zone of the dentate gyrus. Staining picture from the present study.

In the SGZ the neural precursor cells migrate to the granule cell layer and integrate into the neural network making efferent connections in the molecular layer with the entorhinal cortex and afferent connections in the CA3 region (Fig 1). The hippocampus is a region of great interest and fascination for investigating memory and mood disorders. The revelation that neurogenesis occurs in this region has spurred many theories suggesting that the beneficial functional significance of newborn cells to memory and mood.

The neurogenic hypothesis is one theory stemming from this great interest, proposing that depression may occur as a result of an arrest/suppression in neurogenesis (Duman, 2002). Neurogenesis in the subgranular zone may therefore have great clinical relevance. Support for this notion is plentiful with many studies demonstrating that neuropsychiatric factors such as stress (Fuchs, Landas, & Johnson, 1997; Gould & Tanapat, 1999), anxiety (Gordon & Hen, 2004; Revest et al., 2009; Warner-Schmidt & Duman, 2006) and depression (D'Sa & Duman, 2002; Malberg, 2004) can affect neurogenesis. In addition, there is much research demonstrating the rescue of neurogenesis with antidepressant treatment (Chen, Madsen, Wegener, & Nyengaard, 2008; Perera, Park, & Nemirovskaya, 2008) indicating the route of the functional therapeutic effect of antidepressant.

The increase in neurogenesis by antidepressant action is highly reproducible (Marcussen, Flagstad, Kristjansen, Johansen, & Englund, 2008; Santarelli et al., 2003). The link between antidepressant action and neurogenesis was verified by an elegant study of Santarelli et al (2003). Using x-irradiation to kill dividing cells they determined that the loss of neurogenesis co-occurred with the loss of behavioural responsiveness to antidepressants (Santarelli et al., 2003). This was the first direct causal link relating a role of neurogenesis in the action of antidepressants. The time required for neurogenic integration (2-3 weeks) also conveniently correlates with the delayed onset of antidepressant action.

1.5. Neurogenesis and IFN- α

By virtue of the antiproliferative properties of IFN- α the contributing underlying cause of affective adverse events may be via neurogenic arrest. However, there is only one single report that has examined the direct effects of IFN- α on cell proliferation (Kaneko et al., 2006). However, shortcomings are apparent in this report, as the administration

of IFN- α does not reliably mimic therapeutic treatment. Most evidently the route of administration was i.c.v and of an acute nature (7days), whereas patient treatment is administered s.c and chronically, from 12 months to 48 months. This report found a decrease in neurogenesis in the subgranular zone of the hippocampus with IFN- α -treatment attributing it to the action of IL-1 β .

1.6. Rationale for Experiments

The rationale for investigating neurogenesis in IFN- α -treated rats stems from previous experiments conducted in our lab indicating hippocampal impairments in spatial memory tasks and an inhibition of LTP induction in the perforant path (Fahey, 2004). The aim was to determine molecular mechanisms that may explain an underlying reason for hippocampal impairment. Additionally, Fahey et al (2007) demonstrated IFN α -induced affective impairments in the EPM and FST (Fahey et al., 2007), the intention was to build on these observations and determine if the adverse events could be reliably reversed with SSRI treatment. The aim was to determine if treatment with IFN- α and Fluoxetine co-treatment was accompanied by behavioural changes and hippocampal cell proliferation and neurogenesis. This combined behavioural and molecular approach would be beneficial in unravelling the underlying mechanisms of IFN- α induced adverse events.

2. Materials and Methods

2.1. Animals

32 Adult Male Wistar rats (BioResources Unit, Trinity College Dublin) were used for this experiment. Upon arrival animals were housed in groups of two/three in standard Plexiglas cages, (dimensions 44 x 28 x 18cm) in a Scantainer (Scanbur©) with food and water available *ad libitum*. Rats were 3 months old at the start of the experiment, and weighed between 315g-370g (mean 345g). The Scantainer was maintained at (20-22°C) on a 12:12 light/dark cycle (lights on at 08.00h). Animals were randomly allocated to one of four treatment groups; Saline and placebo (Sal), Saline and Fluoxetine (SalFlx), Interferon and placebo (IFN) or Interferon and Fluoxetine (IFNFlx), n = 8 for each group. Animals were monitored and handled regularly by the experimenter, and all procedures were carried out with carried out in accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experimentation.

2.2. IFN- α Treatment

IFN- α -treatment was administered as described in chapter II (section 2.2). The dose was adjusted once a week to compensate for any increase in weight gain.

2.3. Fluoxetine Treatment

Alongside the administration of IFN- α treatment rats in the SalFlx and IFNFlx group were co-treated with Fluoxetine hydrochloride (LKT Laboratories., Minnesota, USA; 7.5mg/kg) daily. Fluoxetine (Flx) was delivered orally to prevent stress and skin irritation experienced as a consequence of i.p injection. Administration route also reflected the clinical administration of the SSRI. Two days prior to administration of Flx, rats were placed on a restricted food diet of 10g/100g BW/day. During the introduction to the food restriction period rats were trained to approach the bars of the cage to consume small amounts of honey. The consumption of honey was readily adopted and subsequent pieces of chow coated in honey were also well received. Chow pellets calculated for the mean weight of the SalFlx and IFNFlx were soaked in fluoxetine hydrochloride (Flx, 7.5mg/kg/1.5ml). Pellets were counted out for a week supply and coated with the Flx solution and left to dry prior to administration. The drug was administered one hour before the end of the light period throughout the entire

course of IFN- α -treatment. Animals in the Sal and IFN groups received placebo pellets dipped in honey. Dose of Flx was calculated once a week to account for increase in body weight.

2.4. Experimental Procedure

A behavioural battery was performed on the rats prior to molecular analysis to determine the affective state of the animals. The timeline can be seen in figure 2 below

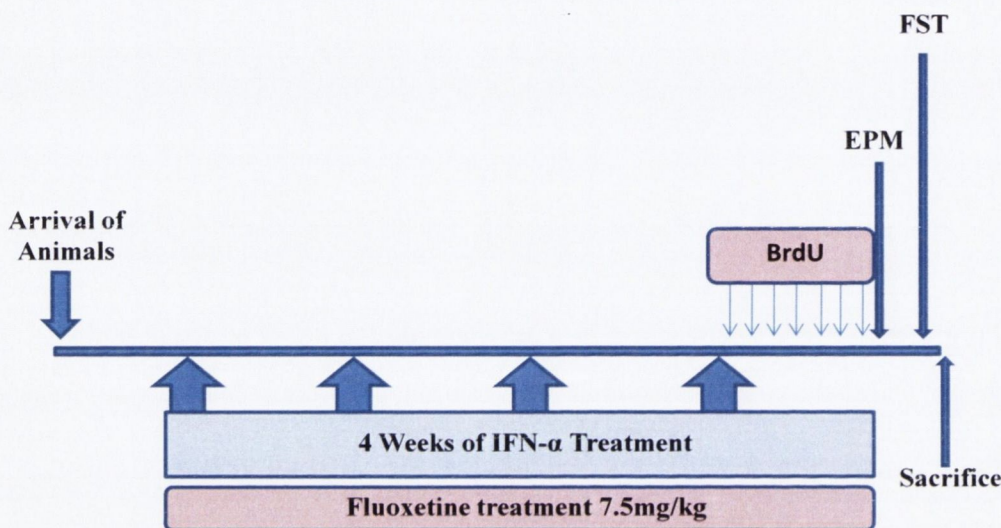


Fig.2. Schematic of the behavioural experiments and treatment regime in the affective battery. Abbreviations: EPM, Elevated plus-maze; FST, Forced Swim Test; BrdU, BrdU administration

2.4.1. Elevated Plus Maze (EPM)

The apparatus and procedure was the same as that described previously in Chapter III (section 2.3.1)

2.4.2. Forced Swim Test (FST)

Apparatus

A custom made clear Perspex forced-swim chamber was used in this test. The dimensions of the chamber were 50cm x 20cm (height x diameter). The cylinder was filled with tepid tap water to a 30cm depth and maintained at a temperature of 20°C. Water was changed after every rat's swim trial. The chamber was placed on a table in a dimly lit room with black curtains around to avoid any distraction from the task.

Procedure

In the original Porsolt test, a two day protocol was used with a pre-exposure to the swim chamber before the testing session (Porsolt, Bertin, & Jalfre, 1977). A modified Porsolt was adopted in this series of experiments, using a one-day protocol. This protocol was chosen to reduce stress exposure and testing time due to the extensive behavioural battery employed in the experiment and has been found to be reliable in measuring depression in rodents (Warner-Schmidt & Duman, 2007) Rats were transferred to the testing room and placed in the chamber for 15 minutes and monitored for behavioural changes. Behaviour was recorded using the Mediacruise® software.

Immobility, a measure of despair, was measured. The operational definition of despair was defined by the animal making little or no attempt to escape, and movement only directed towards keeping the head above water.

Following the testing session, rats were removed from the chamber and dried with a towel. A final injection of BrdU was given and the animal was allowed to dry in an empty cage in front of a fan heater.

2.5. Bromo-Deoxyuridine (BrdU) Labelling

The nucleotide analogue BrdU is incorporated into the newly dividing DNA in the S phase and therefore can be used as a marker of proliferating cells and their progeny. Three weeks after beginning Interferon- α drug treatment rats were injected with BrdU (100mg/kg, i.p; Sigma, UK) once daily for a week (at 18.00hr). A stock solution of 20mg/ml was made by dissolving BrdU in 0.9% saline at 40-50°C and vortexing until dissolved. Individual doses were calculated for each rat each day and adjustments made according to weight fluctuations. Rats were perfused 2 hours after the final BrdU injection.

2.6. Tissue Preparation

Rats were deeply anaesthetised with Urethane (1.3g/kg), and transcardially perfused with chilled 0.9% saline until the animal was exsanguinated and then further fixation perfused with chilled 4% Paraformaldehyde (PFA) in 0.1M Phosphate buffer pH 7.4. Brains were rapidly removed and postfixed for 24 hours in PFA at 4°C and transferred to 30% sucrose in PBS at 4°C for 2 days or until the brains sank. The dehydrated

brains were sectioned into selective parts, loosely wrapped in aluminium foil and rapidly frozen using Isopentane and liquid nitrogen. Tissue was stored at -80°C until further processing for immunohistochemistry. Samples were sent to the IMS Histology & EM Facility, University of Aberdeen for sectioning and slide preparation. Samples were wax-embedded and coronal sections (6µm) cut using a wax sliding microtome. Samples were floated onto Superfrost Plus® Slides. Sections were taken through the entire extent of the dorsal hippocampus (Posterior to bregma -1.8mm to -5.40mm (Paxinos & Watson, 1986). A stringent stereological approach was adopted when deciding the sampling of the sections for immunohistochemistry. Coronal sections were cut every 6µm, in a 5 serial section protocol with 60µm of tissue (10 sections) discarded between levels and 3 slices per slide. This spacing arrangement dictated that every fifth section, (discounting the inter-level interval) was processed for immunohistochemistry. This ensured that the same neuron would not be counted in each section.

2.7. Immunohistochemistry

2.7.1. Antibodies

Table.1. Antibodies used in Light Microscopy

Antibody	Company	Concentration Used
Primary Antibodies		
Bromo-Deoxyuridine	Abcam	1:500
Secondary Antibodies		
Rabbit anti-rat	Dako	1:500
Serums		
Normal Rabbit Serum	Sigma	1:5

2.7.2. Protocol for BrdU staining.

Five slides with 15 sections in total were examined for each animal. Immunohistochemistry was conducted as follows. Slides were cleared from paraffin 2 x 3 min in Xylene and rehydrated for 2 min with 90% and 70% Ethanol. Slides were washed 2 x 3 min in PBS, and 1 x 2 min in ddH₂O. Antigen retrieval was achieved

using a citrate buffer protocol. Slides were microwave heated in a plastic coplin container for 2.5 min at 100%, 2.5 min at 80% and 3 min at 60% power level. At each stage in the retrieval it was ensured that adequate citrate buffer was maintained. Samples were left to cool down for at least 30 min before proceeding. Samples were washed 1 x 2 min in PBS before exposure to 0.25% triton X-100 for 5 min. A wash step with tap H₂O for 3 min preceded the endogen peroxidase blocking; slides were placed in 2% H₂O₂ for 20 min. Samples were washed 2 x 2 min in tap H₂O, 1 x 1 min in ddH₂O and 1 x 5 min in PBS. Slides were incubated with Normal Rabbit Serum (1:5) for 30 min, then the primary antibody BrdU (1:500) overnight at 4°C in a moistened staining chamber. The following day, slides were washed 3 x 3 min PBS, and incubated with the secondary Ab rabbit anti-rat (Dako: 1:500) for 30 min at room temperature. Next, samples were washed 3 x 3 min in PBS and incubated with ABC^{HRP} (Vectastain, Vector Labs) complex for 30 min (ABC^{HRP} complex had been prepared 30 min prior to incubation and stored at 4°C). Samples were washed 3 x 3 min in PBS and incubated with DAB (Sigma, 1:50) for 12-14 min (colour development was monitored closely at 20 x on the light microscope). The reaction was halted in PBS, next, slides were washed 1 x 5 min in tap H₂O, 1 x 2 min in dd H₂O and counterstained for 100 seconds in Meyer's Haematoxylin (Sigma). Samples were cleared of stain in tapH₂O and the samples left to develop for 30 min. Slides were washed 1 x 2 min in dd H₂O, and dehydrated 1 x 2 min in 50%, 70%, 90% and 100% EtOH. Finally, slides were cleared 2 x 2 min in xylene and coverslipped using DPX mountant (Sigma)

2.7.3. Microscopic Data Analysis

After staining the slides were coded with tape placed over the identification labels to ensure the analysis was blind to experimental condition. Slides were analysed using a Zeiss microscope at 20x BrdU positive cells were counted in the dorsal granule cell layer (GCL) subgranular zone (SGZ) and the Hilar region. Cells were included in the SGZ counts if the cell was touching the SGZ (the 3 cell layer thick border of the hilus and GCL). If the cell was more than 2 cell diameters from the GCL it was counted as a hilar cell.

2.7.4. Quantification

Stereological estimates of the total number of proliferating cells in dentate gyrus were calculated using series and inter series interval as a multiplier. A one in 5 series protocol was adopted for staining with a 60µm interval between levels. This ensured that one slice was stained and counted every 90 µm. The following calculation was used to determine the estimated total number of BrdU positive cells in the hippocampus.

$$\textit{Estimated BrdU cells} = \textit{Absolute BrdU positive cells} \times 15$$

2.8. Statistics

All data was analysed using SPSS version 14. Normality was assessed prior to analyses using the Shapiro-Wilk's test. If the data was not normally distributed non-parametric versions of the following statistical tests were chosen. Independent t-tests, repeated measures ANOVA or one-way ANOVA were used where appropriate, *p<0.05, ** p<0.01, ***p<0.001. One-tailed t-tests were used for EPM analysis as previous results suggest typical anxiogenic responses. All data presented are Bonferroni corrected where appropriate.

3. Results

One animal in the IFN- α group was removed from all behavioural analyses due to abnormal home cage behaviour. This animal repeatedly escaped from the home cage therefore was exposed to a more enriched environment.

3.1. Physiological measurements

Fluoxetine results in weight loss in saline controls

Figure 3 displays the weight gain of the animals in the four experimental groups throughout the treatment period. A two-way repeated measures ANOVA revealed a significant effect of timepoint ($F_{(4,140)} = 44.2, p < 0.0001$), a significant interaction between the week and group ($F_{(12,140)} = 3.8, p < 0.0001$), and a significant effect of group ($F_{(3,140)} = 5.9, p < 0.01$). Subsequent one-way ANOVA's conclude differences lie at week 1; ($F_{(3,28)} = 8.8, p < 0.0001$), week 2; ($F_{(3,28)} = 4.8, p < 0.01$), and week 3; ($F_{(3,28)} = 10.7, p < 0.0001$). Tukey HSD *post hoc*s expose differences emerging between the SalFlx and all other three groups, IFN ($p < 0.0001$), IFNFlx ($p < 0.01$) and Sal at week 1 ($p < 0.01$).

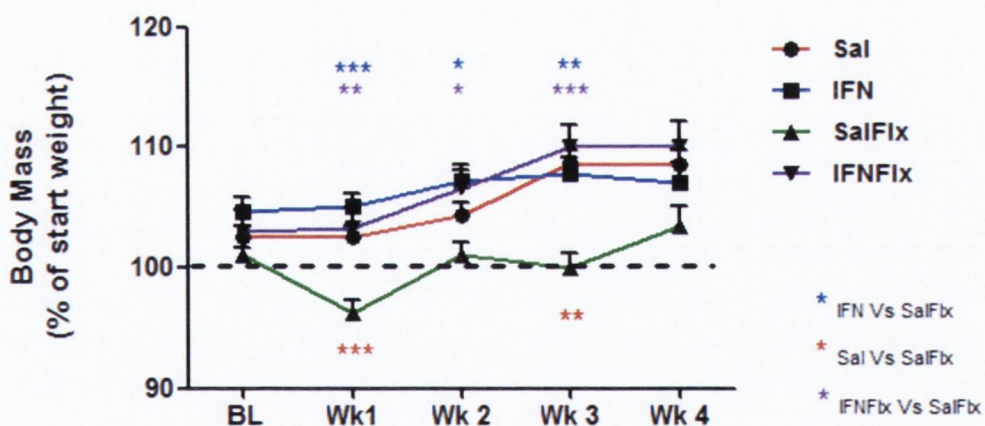


Fig.3. Percentage increase in weight from baseline over the course of the experimental testing session. Data presented as mean percent + SEM, BL, Baseline, $n = 6$ for each group, ($n = 7$ in IFNFlx) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

SalFlx animals display a significantly lower body mass than the other three groups, this pattern persists, with weight differences apparent in week 2; IFN and IFNFlx groups

have a greater body mass than SalFlx ($p < 0.05$) at this time point. In week 3, SalFlx differs from all treatment groups: Sal ($p < 0.001$); IFNFlx ($p < 0.001$) and IFN ($p < 0.01$). However, in week 4 weights were comparable although the SalFlx animals still demonstrate a trend towards lower body masses.

There is an effect of the Fluoxetine on the saline-treated animals throughout the course of the experiment; animals only marginally increase in weight from the baseline body mass measurements. The other three experimental groups (SalFlx, IFN and IFNFlx) exhibit a steady increase in weight over the course of the experiment with a final gain of ~10% in body mass. Anorectic effects of Fluoxetine were only evident in the Saline control groups and not in the IFN- α treated animals.

3.2. Elevated Plus Maze

3.2.1. Time spent in arms

Chronic antidepressant treatment increases exploration in the open arms of the elevated-plus maze

Figure 4 shows the time spent in open (a) and closed (b) arms for the four treatment groups in the elevated plus maze. IFN- α animals spent the least time in the open arms (fig 1), however treatment with Flx increases open arm exploration. A Kruskal-Wallis test was used considering parametric assumptions were not met, the test demonstrated a significant effect of group ($\chi^2 = 11.4$, $df = 3$, $p = 0.010$). Pair-wise comparisons using Mann-Whitney U-tests reveal that the IFN- α animals spent less time in the open arms than saline control animals ($U = 4$, $p = 0.026$, one-tailed). Additionally, a significant difference was revealed between the IFN and the IFNFlx group ($U = 4$, $p = 0.0178$, one-tailed)

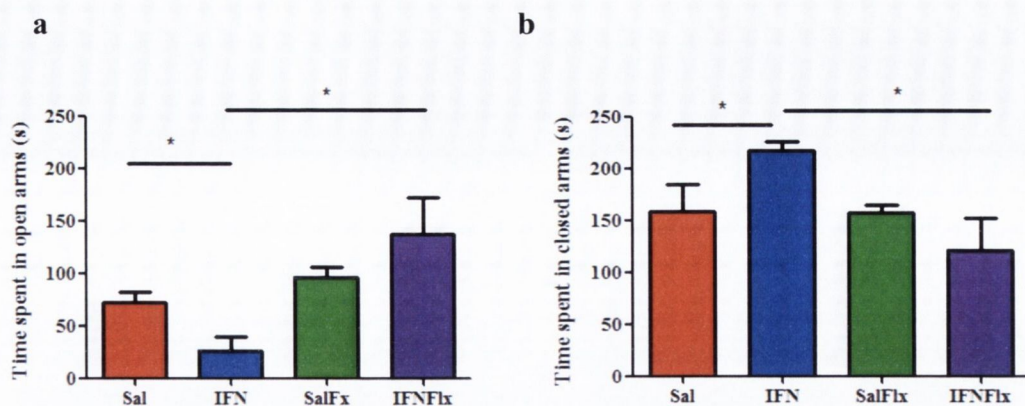


Fig.4. Time spent in the open **a**) and closed **b**) arms of the elevated plus maze of the four experimental treatment groups. Data presented as mean + SEM, n= 6 for each group apart from IFNFlx n=7 *p<0.05

Analysis of time spent in the closed arm also reveals a significant effect of group ($\chi^2 = 10.3$, $df = 3$, $p = 0.016$). Subsequent Mann-Whitney U-tests showed a significant difference in exploration time of the closed arm in the Sal and IFN groups ($U = 4$, $p = 0.026$, one tailed), however significant differences were apparent between the IFN and IFNFlx groups ($U = 5$, $p = 0.022$, one tailed), IFN- α animals spent significantly longer in the closed arms.

3.2.2. Frequency

IFN- α -treated animals make less visits to the open arm, Fluoxetine treatment reinstates visits

The frequency of open and closed arm visits display a similar pattern amongst the experimental groups as the time spent in the arms (figure 5). A Kruskal-Wallis test revealed a significant effect of group ($\chi^2 = 13.0$, $df = 3$, $p = 0.005$). Subsequent pairwise comparisons using Mann-Whitney U-tests expose differing patterns of entries to the open arms, IFN- α -treated entering the open arm less than saline animals ($U = 3.0$, $p = 0.016$), however, IFNFlx animals make more visits to the open arms than IFN- α ($U = 1.5$, $p = 0.0056$).

On examination of the time spent in the closed arms, no significant effect of group was determined ($\chi^2 = 2.6$, $df = 3$, $p = 0.450$). All groups entered the closed arm a comparable number of times indicating equivalent locomotor activity.

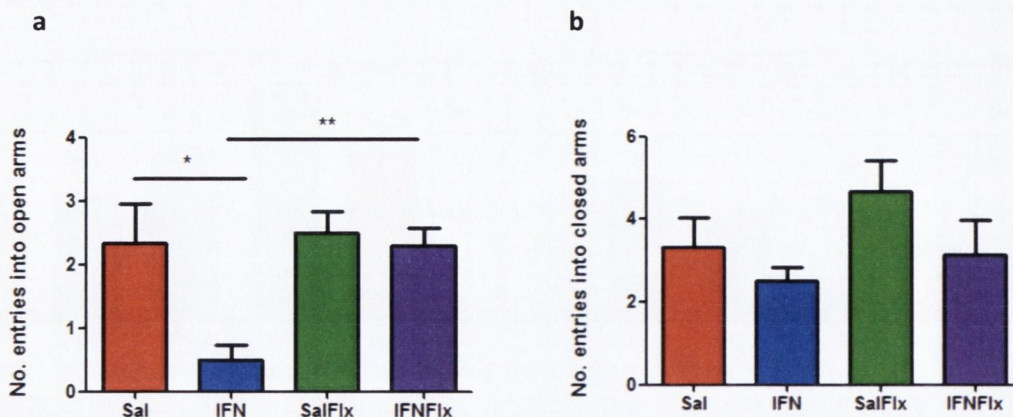


Fig.5. Frequency of entries into open and closed arms in the elevated plus-maze across the 4 treatment groups **a)** Entries into open arms **b)** Entries into closed arms. Data presented as mean + SEM, n= 6 for each group (IFNFlx, n= 7), *p<0.05 **p<0.01

Time Bin Analysis of EPM

Further in depth analysis of the EPM behaviour via minute-by-minute examination in the open and closed arms reveals a significant difference in the pattern of exploration over the course of the task (fig 6). Both IFN- α and SalFlx animals spend a minimal percentage of time in open arms in the first minute of exploration and consequently a reflective near maximal time in the close arm (% time, IFN: 7.9 ± 6.9 ; SalFlx: 23.8 ± 9.3). In contrast, the Sal and IFNFlx group perform comparably in the first minute of exploration (Sal: 55.6 ± 13.7 ; IFNFlx: 78.7 ± 9.6) spending 50-85% of time in the open arm and spend less time in the closed arms. A Kruskal-Wallis test demonstrate a significant effect of group in the first minute of the trial ($\chi^2 = 14.5$, df = 3, p=0.002 (p< 0.01). However, group exploratory behaviour did not differ after the first minute of test, 2 minutes: ($\chi^2 = 3.1$, df = 3, p=0.376); 3 minutes: ($\chi^2 = 3.8$, df = 3, p= 0.284); 4 minutes: ($\chi^2 = 3.1$, df = 3, p= 0.369); and 5 minutes: ($\chi^2 = 0.706$, df = 3, p= 0.872).

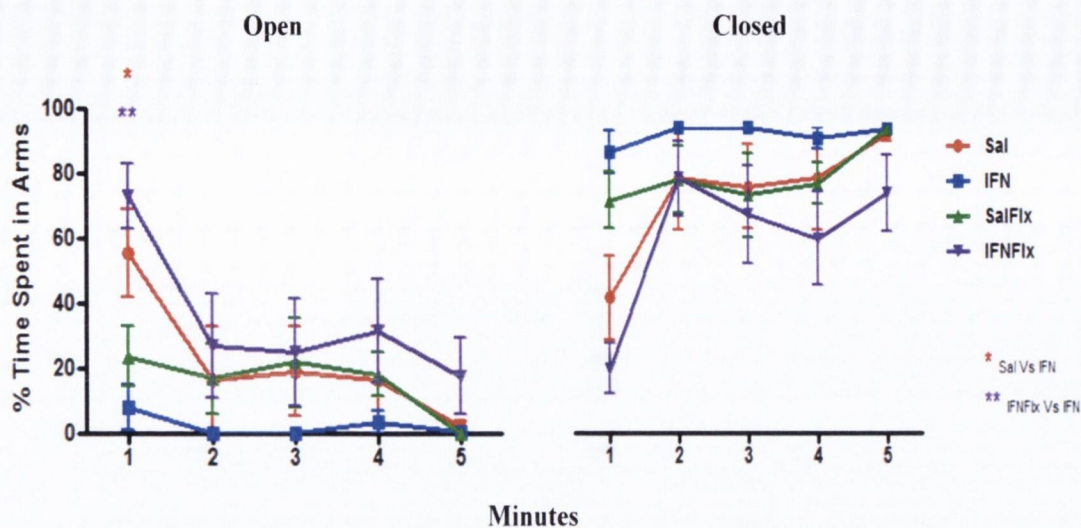


Fig.6. Minute time bin analysis of the elevated plus-maze showing the percentage time spent in the open and closed arms. Data presented as mean percent \pm SEM, $n=6$ for each group apart from IFNFlx ($n=7$), * $p<0.05$ ** $p<0.01$

Subsequent pair-wise comparisons were used to assess where the differences occurred in the first minute of exploration. Mann-Whitney U test reveals IFN- α animals spent less time in the open arm than saline controls in the first minute ($U=4.0$, $p=0.0250$, $p<0.05$). Additionally, IFNFlx spent more time than IFN- α animals ($U=1.0$, $p=0.0047$, $p<0.01$).

3.3. Forced Swim Test

3.3.1. Immobility

IFN- α animals spend more time immobile in the forced swim test, Fluoxetine attenuates immobility

Figure 7 below displays the immobility behaviour of the four treatment groups in the Forced Swim Test. IFN- α spend almost half ($\sim 45-50\%$) the time in the test session immobile, in contrast, the other three groups spent approximately a third of the time immobile ($\sim 30-35\%$). A one-way ANOVA demonstrates there is a significant effect of group ($F_{(3, 29)} = 5.258$, $p<0.01$), *Post Hoc* Newman Keul's analysis determined that IFN- α -treated animals spend a significantly greater percent of time immobile than Sal ($p<0.05$), IFNFlx ($p<0.05$) and SalFlx ($p<0.01$) treated animals. Indeed, co-treatment

with Flx reduced immobility behaviour down to control levels, however, Flx did not further decrease the time spent immobile in saline controls.

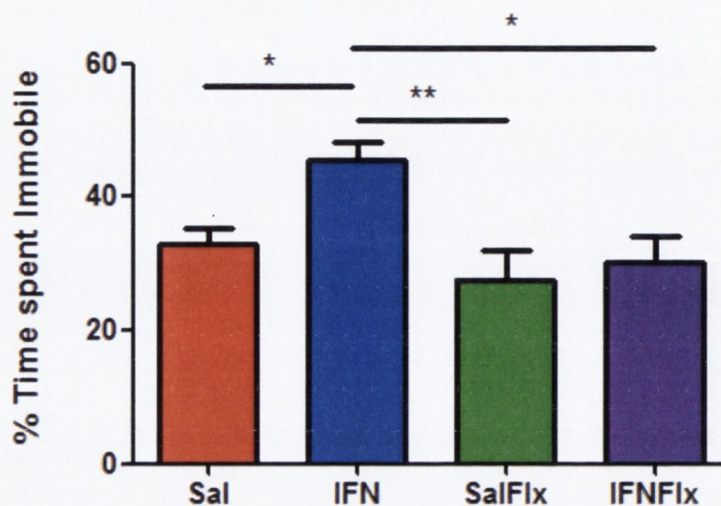


Fig.7. Time spent immobile in the Forced Swim Test of the four experimental treatment groups, Sal and SalFlx $n=8$ and IFN and IFNFlx $n=7$. Data presented as mean percent + SEM, * $p<0.05$, ** $p<0.01$

Correlations exist between affective behaviours

Correlations of the two affective tests used in this series of experiments demonstrate a moderate correlation between the Elevated-Plus Maze (EPM) and the Forced Swim Test (FST). A negative correlation exists (figure 8a, correlation coefficient = 0.3328) demonstrating the more time spent immobile the less time animals spend in the open arms. Analyses of clustering in the groups demonstrate that IFN- α rats primarily cluster at extreme levels demonstrating poor performance on both of the behavioural affect tasks. In contrast saline-treated animals demonstrate average performance on the two tasks and cluster together as a fairly homogeneously distributed group. Fluoxetine treated animals demonstrate a distributed pattern with several animals presenting atypical behaviour; exhibiting anxiogenic and depressive phenotypes. However, several of the antidepressant treated animals perform to a superior extent on the tasks. An inverse measure is also demonstrated using time spent in the closed arm (figure 8b, correlation coefficient = 0.305) revealing a moderate positive correlation between time in the closed arm and the time spent immobile.

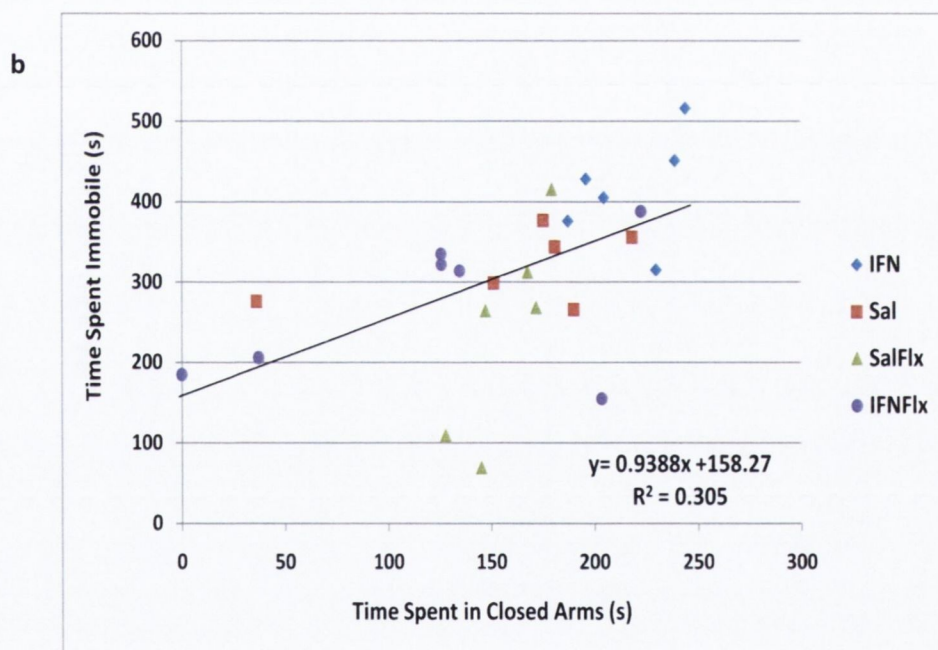
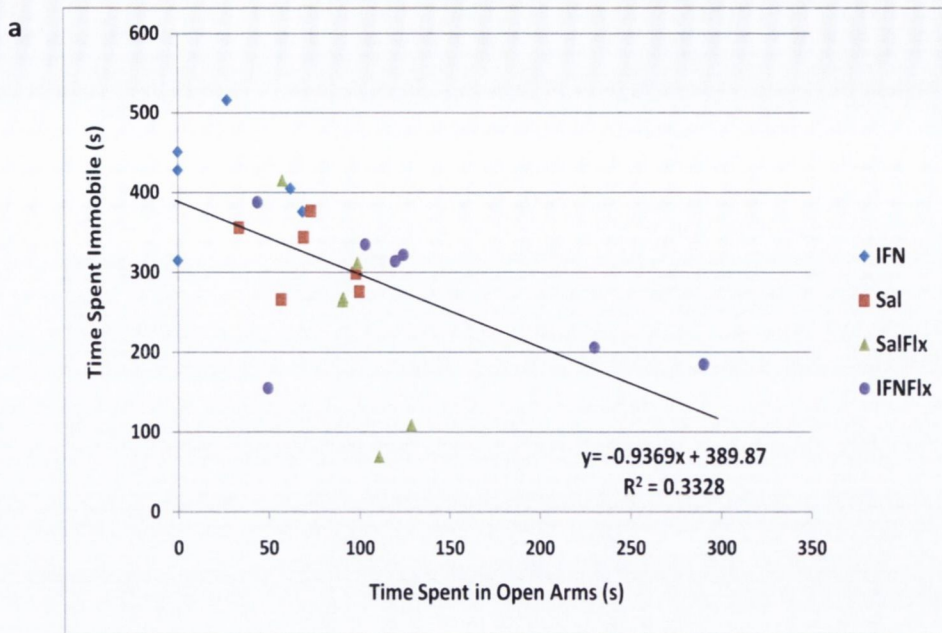


Fig.8. Correlation between the elevated plus-maze and forced swim test **a)** time spent in the open arms *versus* time spent immobile **b)** time spent in the closed arms *versus* time spent immobile n=6

3.4. Neurogenesis – BrdU staining

Figure 9 below displays neurogenesis staining pictures from the four experimental groups.

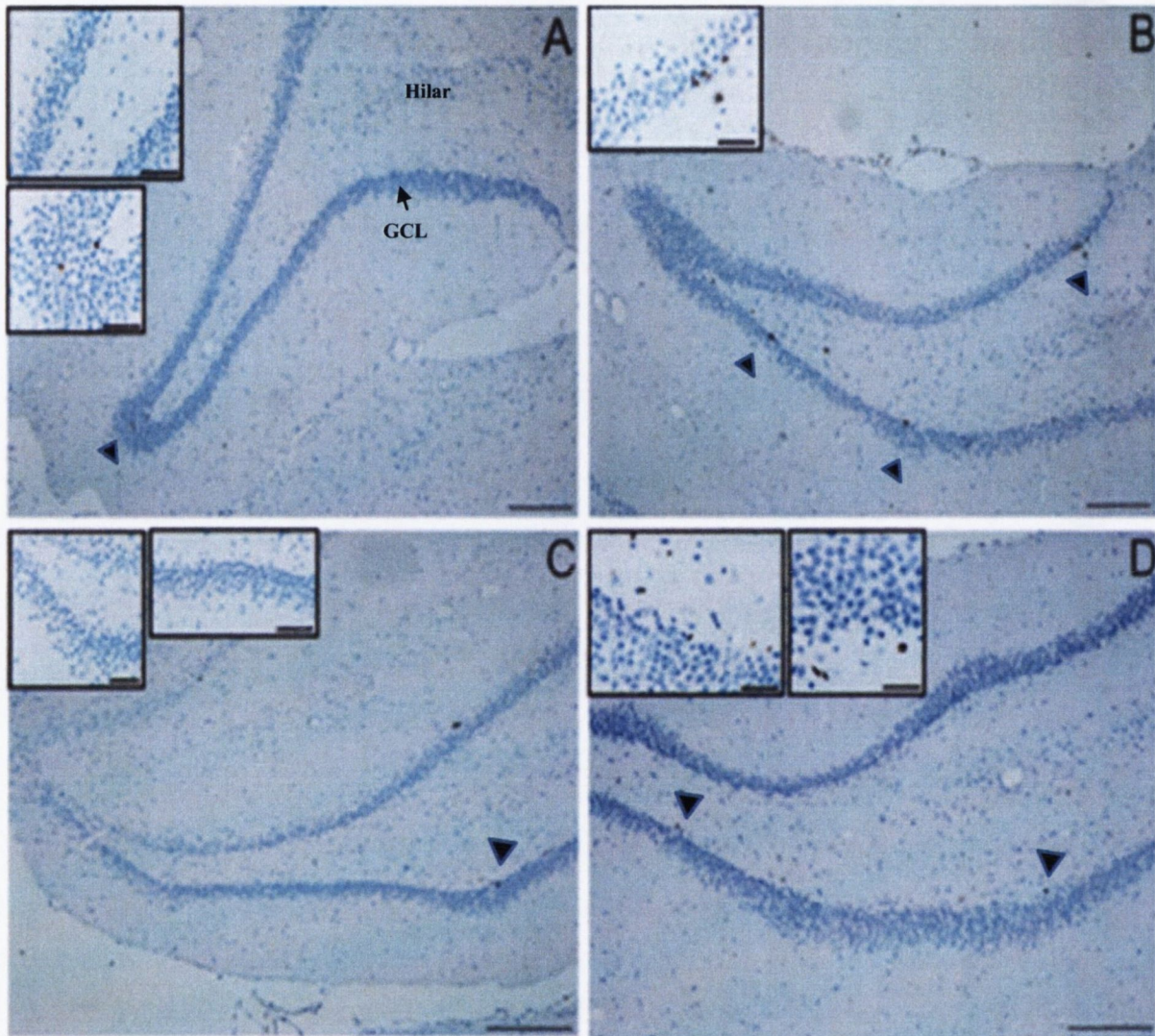


Fig. 9. BrdU staining for neurogenesis A) Saline-treated animals, B) Saline and Fluoxetine treated animals, C) IFN- α -treated animals and D) IFN- α and Fluoxetine treated animals. On larger panels, scale bar marks 200 μ m on smaller panels scale bar marks 100 μ m. GCL: Granule Cell Layer

3.4.1. Subgranular Layer of the Dentate Gyrus

IFN- α -treatment induces a significant decrease in cell proliferation, Fluoxetine restores cell proliferation

Examination of new born proliferating cells in the subgranular zone of the dentate gyrus reveals that cell proliferation in the IFN- α -treated animals is reduced when compared with saline controls (fig 10a)(IFN- α : 1314 ± 127.9 ; Sal: 1851 ± 64.5 , respectively). Antidepressant treatment with Fluoxetine recovers the suppression in cell proliferation (IFNFlx: 2328 ± 238.0). A Kruskal-Wallis one-way ANOVA demonstrates a significant effect of group ($\chi^2 = 9.7$, $df = 3$, $p = 0.022$, $p < 0.05$). Subsequent pair-wise Mann-Whitney tests revealed a significant decrease in the number of new born cells in the subgranular zone of the dentate gyrus in IFN- α -treated rats compared to saline-treated counterparts ($U = 4$, $p = 0.019$) ($p < 0.05$). Additionally, there is an increase in the number of cells in the animals co-treated with IFN- α and Fluoxetine ($U = 1.5$, 0.004) ($p < 0.01$).

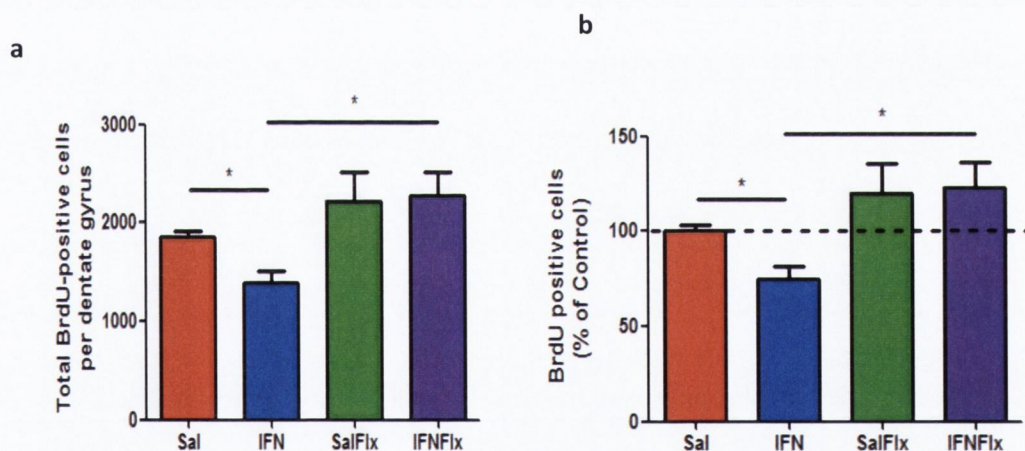


Fig.10. a) Number of BrdU positive cells in the subgranular cell layer of the dentate gyrus of the hippocampus in all four treatment groups. b) BrdU positive cells in the subgranular cell layer as a percentage of the saline control group. Dashed line represents 100%. Data presented as mean percent \pm SEM, $n = 7$ IFN, IFNFlx and SalFlx, Sal: $n = 5$. * $p < 0.05$

Figure 10b above demonstrates that there is a significant decrease in the BrdU positive cells in the subgranular cell layer in the IFN- α rats when compared to saline controls. There is a 20-30% decrease in cell number in IFN- α treated rats. In contrast, there is a

25-40% increase in the number of proliferating cells in the subgranular zone in the two antidepressant treatment groups (SalFlx and IFNFlx).

3.4.2. Granule Cell Layer of the Dentate Gyrus

The migration of new born cells through the granule cell layer of the dentate gyrus indicates the migration and integration of the cells into the network. IFN- α -treatment results in a decrease in proliferating cells found in the granule cell layer (Figure 11a, Saline: 117 ± 11.0 ; IFN- α : 72.5 ± 6.0). In contrast, an increase in the number of proliferating cells in the granule cell layer is apparent with co-treatment with antidepressants (IFNFlx: 235.7 ± 39.5). IFN- α treated animals exhibit a ~30-40% decrease in number of proliferating cells in the granule cell layer. Antidepressant treatment increases the number of cell in this layer (Figure 8b, IFNFlx: ~60-130%; SalFlx: ~0-60%), with the potential to double the number of new born cells migrating in this layer. A Kruskal-Wallis one-way ANOVA demonstrates a significant effect of group ($\chi^2 = 12.5$, $df=3$, $p = 0.006$, $p<0.01$). Subsequent pair-wise Mann-Whitney tests showed that there was a significant difference in the number of new born cells in the molecular layer of the dentate gyrus in IFN- α -treated rats compared to saline-treated counterparts ($U = 2.5$, $p=0.0274$, $p<0.05$). Additionally, there is an increase in the number of cells in the animals co-treated with IFN- α and Fluoxetine ($U = 1$, $p= 0.003$) ($p<0.01$) compared to the IFN- α treated counterparts.

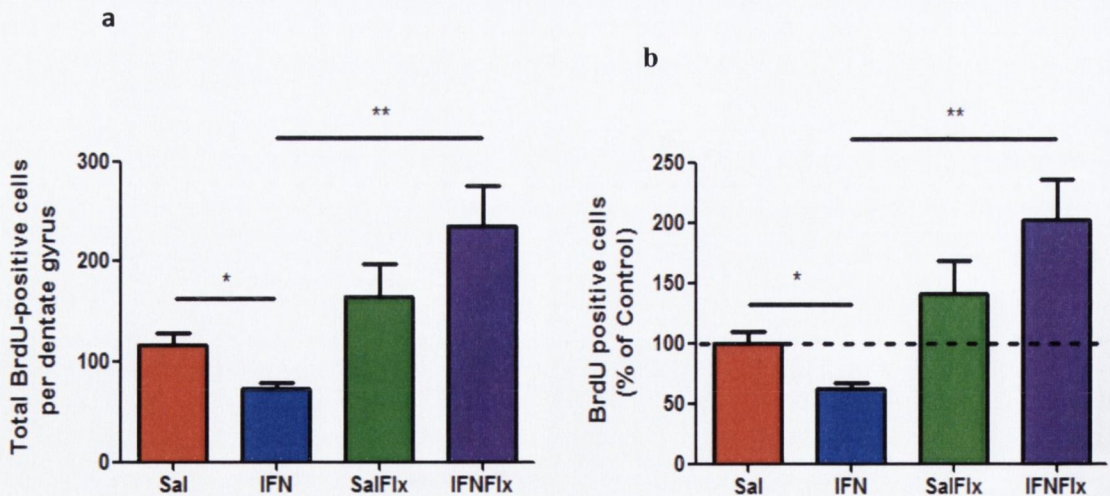


Fig.11. a) Number of BrdU positive cells in the granule cell layer of the dentate gyrus of the hippocampus in all four treatment groups. **b)** BrdU positive cells in the granule cell layer as a percentage of the saline control group. Dashed line represents 100%. Data presented as mean percent \pm SEM, $n= 7$ IFN, IFNFlx and SalFlx, Sal: $n= 5$ * $p<0.05$ ** $p< 0.01$

3.4.3. Hilar region of the Dentate Gyrus

A Kruskal-Wallis one-way ANOVA demonstrates a marginally significant effect of group ($\chi^2 = 7.1$, $df = 3$, $p = 0.069$). Subsequent pair-wise Mann-Whitney tests showed that there is no significant difference in the number of new born cells in the hilar region of the dentate gyrus in IFN- α -treated rats compared to saline-treated counterparts (fig 12 a)($U = 10$, $p=0.138$) ($p>0.05$). However, there is an increase in the number of cells in the animals co-treated IFNFlx group ($U = 5$, $p= 0.007$, $p<0.01$) compared to the IFN treated counterparts. There is a significant rescue of the number of newborn cells in the hilar region with the addition of the antidepressant exceeding control cell number (fig 12b).

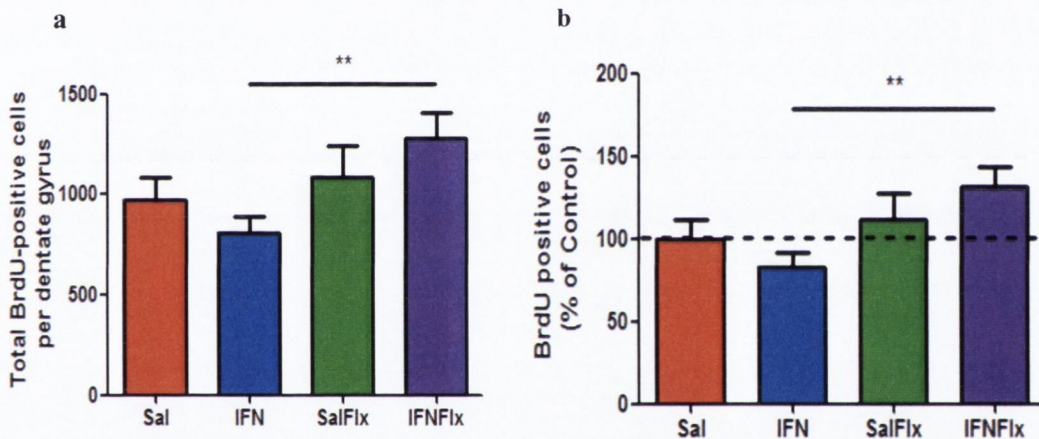


Fig.12. a) Number of BrdU positive cells in the hilar region of the dentate gyrus of the hippocampus in all four treatment groups. **b)** BrdU positive cells in the hilar region as a percentage of the saline control group. Dashed line represents 100%. Data presented as mean percent \pm SEM, $n = 7$ IFN, IFNFlx and SalFlx, Sal: $n = 5$. ** $p < 0.01$

Correlations exist between new proliferating cells and affective behaviour

Correlations of the number of BrdU positive cells in the dentate gyrus and the affective tasks demonstrate a moderate correlation between both the elevated plus-maze (EPM) and the forced swim test (FST) and cell number. A negative correlation exists between cell number and immobility (figure 13a, correlation coefficient = 0.2997) demonstrating the more time spent immobile the less cells there are in the dentate

gyrus. Analyses of clustering in the groups demonstrate that IFN- α rats primarily cluster at extreme levels demonstrating poor performance on the behavioural task and decreased cell number. In contrast saline-treated animals demonstrate average performance on the two tasks demonstrate average cell number, centred on the middle of the correlation line. Fluoxetine treated animals demonstrate a distributed pattern with several animals presenting atypical behaviour; exhibiting anxiogenic and depressive phenotypes. However, several of the antidepressant treated animals perform to a superior extent on the tasks. A similar inverse correlation is evident in the time spent in the open arm and the number of BrdU positive cells.

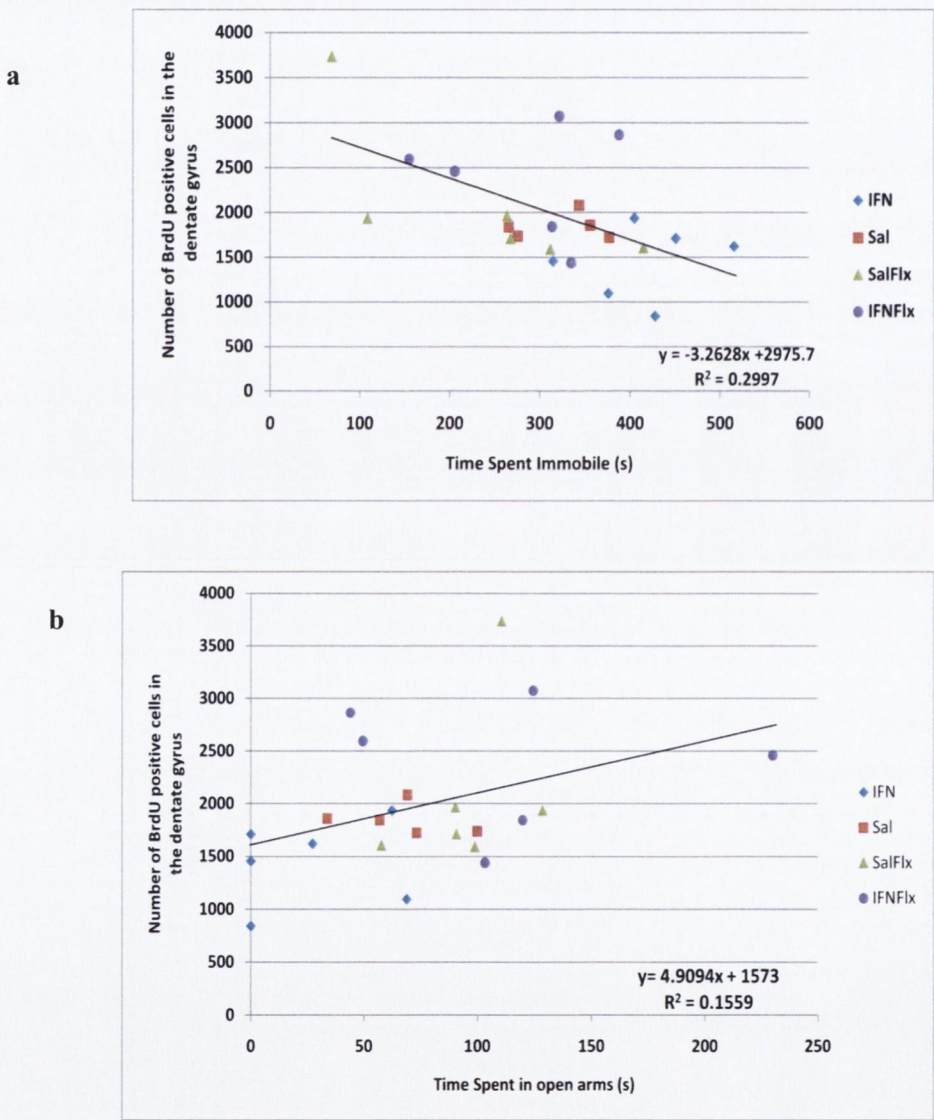


Fig.13. Correlation between the behavioural tasks and BrdU positive stained cells in the subgranular zone **a)** Time spent Immobile and the number of proliferating cells **b)** time spent in the open arms and number of proliferating cells

4. Discussion

The data presented here demonstrate, first, altered behavioural responses in IFN- α -treated animals in affective tasks; second, a decrease in cell proliferation with this treatment; and third, the proneurogenic effect of Flx results in an increased cell proliferation.

Physiological changes with Flx treatment

Figure 3 displays an anorexic side effect of Fluoxetine (Flx) treatment in saline-treated rats, SalFlx rats only marginally increase in weight over the testing period. The anorectic effects of Flx have commonly been documented in the literature (Currie, Coscina, & Fletcher, 1998; Fuller et al., 1994) however; it is interesting that there are no anorexic side effects associated with rats co-treated with IFN- α . This is surprising as IFN- α -treated rats typically show signs of sickness behaviour, including appetite loss (Konsman, Parnet, & Dantzer, 2002), this suggests that the co-administration of Flx would result in a more pronounced decrease in body mass. Reasons for the maintenance in body mass in the IFNFlx group are unknown, however, we may speculate that Flx may be aiding in the alleviation of sickness behaviour which may result in a sudden revival of appetite ensuring the maintenance of body weight.

Affective Behavioural Performance

The performance of the IFN- α -treated and saline-treated animals in the elevated-plus maze demonstrated here are consistent with results demonstrated previously in our lab (Fahey, 2004; Fahey et al., 2007). Additionally the results presented here include a restoration of the affective behaviour (amelioration of anxiety and depressive responses) with the co-administration of an SSRI. Exploration in the open arms when co-treated with Flx is restored to control levels (Fig 4a) ; also IFNFlx-treated animals spend less time in the closed arms than IFN α -treated animals further illustrating this recovery (Fig 4b). Confirmation of these data is also demonstrated in the frequency of entries into arms. IFN- α -treated animals visit the open arms the least number of times, visits to open arms are recovered with Flx treatment (Fig 5a). All animals enter the closed arms in a comparable way (fig 5b), if controlled for locomotor changes. The decreased performance in the open arms was examined further using a time-bin breakdown (fig 6); which revealed that the exploration of the open arms of IFN- α -animals was uniformly poor throughout the entire test session. This illustrates the

pronounced angiogenic response and aids to separate the baseline angiogenic response experienced by control animals.

In addition, IFN- α -treated animals demonstrate increase immobility (despair) in the forced swim test; immobility is successfully alleviated with SSRI treatment. IFN/Flx-treated animals spent less time immobile suggesting a decrease in behavioural despair. The behavioural benefit of Flx (recovery of affective behaviours) is in line with previous data in clinical data supporting the prophylactic potential and positive regulation of Flx for IFN- α adverse events (P. Hauser et al., 2000; Levenson & Fallon, 1993). Furthermore, scatter graphs (fig 13) demonstrate a moderate correlation between the two behaviour tasks indicating the co-morbid nature of anxiety and depression in individuals treated with IFN- α .

Cell Proliferation

Figure 9c and 10a demonstrates a significant decrease in cell proliferation in IFN- α -treated animals in the subgranular zone (SGZ) of the dentate gyrus. Comparisons to baseline controls (fig 10b) demonstrate a substantial ~25% decrease in neurogenesis. A decrease in neurogenesis has only been investigated once previously (Kaneko et al., 2006), however the treatment was not clinically relevant, the mode of administration was via i.c.v and the time span of treatment very short (7days) compared to treatment in the clinic. Given the conflicting evidence surrounding peripheral administration of IFN- α and access to the brain, it is of great interest to determine if a clinically relevant route of administration (systemic s.c.) would also result in the suppression of neurogenesis. Here we provide indication that neurogenesis is substantially suppressed with clinically relevant chronic IFN- α -treatment. A limitation in this study is the absence of supporting phenotypic data to determine the fate of the proliferating cells. We tentatively suggest that the cell proliferation decrease demonstrated here may reflect a decrease in neurogenesis. However, glial and endothelial fate is also of great interest. Previous studies suggest that approximately half of the cells phenotyped are neuronal and 10-15% glial based (Elder, De Gasperi, & Gama Sosa, 2006). However, it is unknown if chemical or environmental manipulations will specifically target neurons or glia. The supportive role of glial cells to neuronal survival, both for metabolic support and repair is well characterised (Duman, 2005) suggesting that a consequent decrease in glia may also be disadvantageous. One study found that astrocytes can

actually induce neurogenesis, therefore evidence of astrocytic proliferation may also indicate neurogenesis (H. J. Song, C. F. Stevens, & F. H. Gage, 2002). Additionally, endothelial cell proliferation suppression may also have a detrimental effect on the nervous system, reducing the blood supply thereby impacting on the neurovascular niche (Warner-Schmidt & Duman, 2007). Therefore although further investigation is warranted in order to characterise the fate of the cells it is important to note that any decrease in cell proliferation will have a drastic impact on the neuronal microenvironment that already exists. Admittedly, many of the proliferating cells will not survive past the three week critical window; however, those that do will integrate into the existing circuitry and provide functional significance (Doetsch & Hen, 2005; Snyder, Kee, & Wojtowicz, 2001). It may also be interesting to investigate the terminal fate of the neurons in further experiments to determine the percentage of cells that remain. This may aid in the assessment of the functional significance of the cell proliferation.

It was also found that proliferating cells in the hilar region and the granule cell layer reveal some interesting findings. IFN- α and saline-treated animals produce a similar number of proliferating cells in the hilar regions (fig 12a) whereas the IFNFlx numbers are significantly increased. In addition, saline-treated rats were found to have more cells in the granule cell layer than IFN- α animals (fig 11a), this is suggestive of neuronal migration and potential for integration. There appears to be a delay in the advancement of neurogenic integration in the IFN- α -treated animals. Moreover, IFNFlx animals exhibit significantly enhanced levels of cells in the granule cell layer, the proliferation is astoundingly high, almost twice the number of saline-treated animals (Fig 11b).

Overall, IFN- α -treatment results in a decrease in cell proliferation in the SGZ and GCL. Flx treatment increases the pool of cells in the SGZ, GCL and hilar regions, aiding in rescuing behavioural responses.

What is the significance of this decrease in cell proliferation?

The decrease in cell proliferation may have significant impact on both learning and memory and affective behaviour (as seen here). Shors et al (2001), demonstrated that the inhibition of neurogenesis using a chemical cell blocker could block hippocampal-dependent learning (Shors et al., 2001). The decrease in cell proliferation seen in the

IFN- α animals may explain the spatial memory impairments demonstrated previously in our lab highlighting further hippocampal impairments (Fahey, 2004).

It is demonstrated in the data here that there are mild correlations between the number of BrdU positive cells (cell proliferation) and affective behavioural performance (fig 13). This is suggestive of a functional role of neurogenic suppression (IFN- α) in disrupting affective behavioural responses. The role of neurogenesis in affect was first investigated by Gould et al (1992), who demonstrated that cell proliferation in the SGZ was suppressed by corticosteroids (Gould, Cameron, Daniels, Woolley, & McEwen, 1992). The HPA axis is regulated by the hippocampus, therefore hippocampal impairment may reduce the feedback in this region resulting in an increasing glucocorticoid attack on neurogenesis. The implication of Flx treatment recovering both affective behavioural response and neurogenesis suggests a role for increased neurogenesis and rescue of affect.

Future work is required to verify the phenotype of the cell proliferation included here, this work is ongoing. Additionally, it would be interesting to examine neurogenesis along the septo-temporal (dorso-ventral) axis, and determine if there are differences in the dorsal and ventral neurogenesis that may explain any preferential effects for memory and affective behaviour respectively. This may provide more insight into the extent of the hippocampal impairment in IFN- α . Another potential avenue to follow would be to investigate neurogenesis in the neocortex; Controversy still remains over the presence of neurogenesis in these so-called non-neurogenic regions. From early conceptions of Gould et al 1999, conflicting results have suggested that these progenitors are glial based (Kornack & Rakic, 2001). The implication of the HC-PFC in the pathophysiology of neuropsychiatric disorders suggests that impairments in the hippocampus may result in consequent impairments in this pathway and the PFC (Jay et al., 2004). Examination of cell proliferation in the PFC would be beneficial to indicate the molecular status of the PFC.

5. Conclusion

The data presented here demonstrate a rescue of affective disorder associated with IFN- α with the co-treatment of an SSRI. Cell proliferation is decreased in IFN- α -treated animals; however, this is restored with Fluoxetine treatment. The restoration of behavioural responses and correlation with increased neurogenesis suggests a mechanism for affective deficits in IFN- α animals.

Chapter V

Magnetic Resonance Imaging in IFN- α -treated rats

**Volumetric analysis demonstrates a substantial decrease in hippocampal volume
in rats treated with IFN- α**

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Summary

Magnetic Resonance Imaging is a favourable technique for investigations into structural and functional changes in the nervous system. It can be used reliably to assess changes with pharmacological treatment over time and is becoming an increasingly popular method to use in the field of neuroscience.

T1 and T2 relaxation times are sensitive measures thought to signify injury in the brain. There were no apparent differences found in these data in the T1 and T2 relaxation times in animals treated with IFN- α . A second explorative analysis, volumetric analysis was used to elucidate a remarkable decrease in hippocampal volume in IFN- α -treated animals. This decrease is profound, 7-8% in the IFN- α -treated animals; comparable intracranial volumes indicate that this is a specific decrease in mass in this specific anatomical region. These data are in line with decreases in hippocampal volumes resulting from stress and depression (McEwen, 1999). It remains unknown whether this decrease in hippocampal volume is a precipitating factor in the induction of depression in IFN- α -treated animals or arises as a result of circulating cytokines and corticosteroids.

This decrease in hippocampal volume may have a detrimental impact on other connected regions such as the prefrontal cortex. This hippocampal decrease in volume demonstrated in IFN- α -treated animals may be suggestive of a more extended limbic-cortical dysregulation. Future experiments are required to clarify this proposed dysfunction, it is suggested the use of voxel-based morphometric analysis or diffusion tensor imaging would clarify this proposal.

1. Introduction

1.1. Applications of small animal MRI in Neuroscience

Magnetic Resonance Imaging utilises proton resonance to generate anatomical images for medical and scientific practice. It is a powerful tool to provide useful insights into the underlying structural and functional changes of disease states. The implementation of MRI in the neuroscience field to investigate neuropsychiatric dysfunction is gaining in momentum (Benveniste & Blackband, 2002; Warach, Gaa, Siewert, Wielopolski, & Edelman, 1995). There are several advantages to this technique, it is non-invasive and can be repeated for longitudinal drug studies, which enables within sample comparisons of anatomical and cerebral blood flow information. Additionally, MRI can yield a high volume of information from a few pre-defined scans.

Developments in small animal imaging have been delayed in comparison to the advancements of human MRI experiments due to technical considerations. There are caveats in small animal imaging, one major limitation is the requirement for anaesthetic, not only concerning the unconscious status of the animal but also the effect anaesthetic can have on cerebral blood flow. Some potential avenues that are being exploited in order to assess pharmacological MRI activity using the rat somatosensory cortex and whisker barrel cortex as markers (Gyngell, Bock, Schmitz, Hoehn-Berlage, & Hossmann, 1996; Yang, Hyder, & Shulman, 1996).

1.2. T1 and T2 weighted scans

Proton resonance is the principle basis underlying MRI image acquisition. Excitation of protons by the application of a radiofrequency pulse will instil a high energy state in that proton. The return of the excited protons from a high energy state to a low energy state is associated with a loss of energy which produces a contrast. Spin Relaxation is the name given to the time it takes for an MR signal created by a specific RF to decay.

It is possible to exploit static contrasts of the tissue of interest to assess brain tissue injury. Parameters in the magnet field of the tissue are altered in order to determine differences between the signal of the tissue. Normal and abnormal tissue respond differently to the alteration and produce different signals. There are two measures that can be manipulated to alter static contrast, one in the longitudinal plane (T1) and one transversal (T2) these will be discussed in turn.

The longitudinal contrast is dictated by T1 or spin-lattice relaxation time measurements. T1 is the time it takes to reach magnetisation following a radiofrequency pulse. T1 relaxation time is the time it takes for relaxation of the protons to return to 63% of its original length. With increasing T1 times the number of protons that have returned to their original state increase exponentially (after 2 T1 times 86%, 3 T1 times 95% and so on), an asymptotic level is reached after 3-5 T1 times.

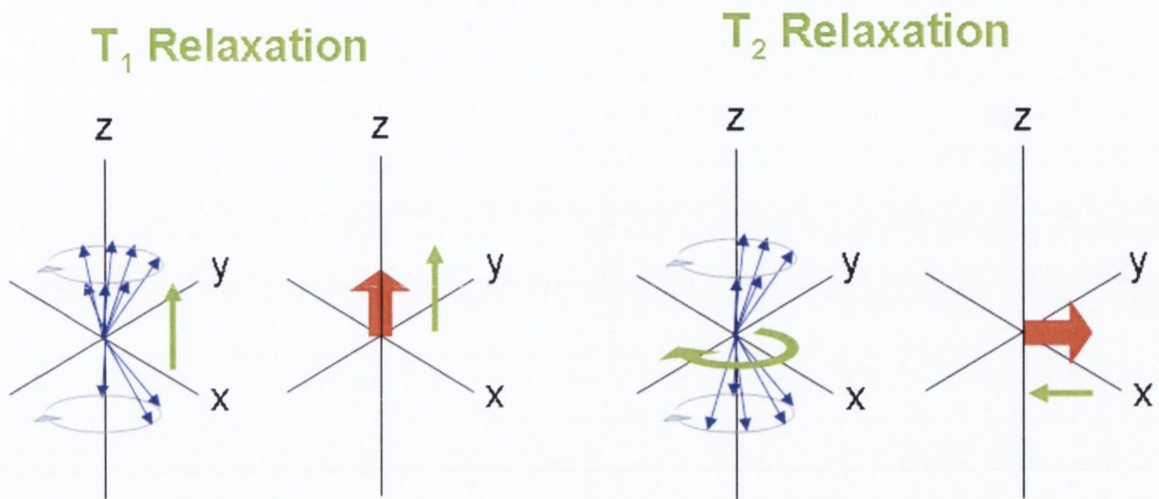


Fig.1. Annotation of T1 and T2 relaxation. T1 relaxation involves the return of nuclei to equilibrium of spin up and down states, this restores magnetisation in the Z plane. T2 is a more rapid dephasing reducing the overall magnetisation vector in the XY plane. Cardiff University, www.Cardiff.ac.uk

Conversely, T2, transverse relaxation or spin-spin relaxation is produced by an exchange of energy between spins in the high and low energy state. This results in a loss of transverse magnetization; once again T2 occurs exponentially similar to T1, although measurements result from a 63% loss in magnetization. It is important to note, T1 time is always considerably slower than T2 time.

But what do they measure?

The neural correlates of T1 and T2 remain ill-defined; however, some indications of the basis of histological changes in these time constants exist. It is suggested that T2 changes may occur when the perivascular space enlarges, with gliosis and demyelination (Braffman et al., 1988). It is also suggested that T1 and T2 relaxation times increase with free water content (Calderan et al., 2005), this interpretation results from the basis that water molecules are small in size therefore their motion frequency is faster. Dense tissue i.e. grey matter can indirectly be distinguished from tissue with

more water content such as CSF. This can result in a very sensitive measure of tissue injury, than conventional T2-weighted scans. Broom et al (2005) suggest regions of T1 and T2 hypointensity may appear to reflect hypercellularity (Broom et al., 2005)

The employment of T2 relaxation analysis in neuroscience research has been successful in determining subtle changes in tissue integrity of the brain in epilepsy patients (Bengner, Siemonsen, Stodieck, & Fiehler, 2008); hippocampal sclerosis (Duncan, Bartlett, & Barker, 1996; Woermann, Barker, Birnie, Meencke, & Duncan, 1998) and age related changes in Alzheimer's disease (El Tannir El Tayara et al., 2006; Falangola et al., 2007).

1.3. High resolution scanning for Volumetric analysis

A second exploratory focus in small animal imaging utilises high resolution scanning to determine volume changes of specific brain regions (Cerqueira et al., 2005). The utilisation of high resolution volumetry in preclinical models aims to bridge the gap between animal MRI scanning and human MRI scanning, providing translational measurements. There is a substantial literature base reporting volumetric changes occurring alongside memory deficits and mood disorders (Sheline, Wang, Gado, Csernansky, & Vannier, 1996). In particular, there is specific evidence indicating hippocampal atrophy may be attributable to affective disorders. The volume of the hippocampus may be decreased by factors such as prolonged stress and depression (Bremner et al., 2000; Czeh et al., 2001). The implementation of volumetric MRI analysis was instrumental in the diagnosis of a 8-19% decrease in hippocampal volume in patients with depression (Bremner JD et al 2000; Sheline, Mittler, & Mintun, 2002). The significance and molecular origins of this decrease in hippocampal volume remain elusive. Stressful events or drugs that act as systemic stressors can impact heavily on the morphology of hippocampal neurons (McEwen, 1999). This action may be via a suppression of neurogenesis or a decrease in dendritic arborisation (complexity) of the CA3 pyramidal neurons.

1.4. Imaging Studies related to IFN- α -deficits

To date there are only a few studies that have examined the effects of IFN- α using imaging, most have examined metabolic changes in the brain. A SPECT (single photon emission computed tomography) study conducted by Tanaka and colleagues (2006) examined cerebral blood flow in patients taking IFN- α compared with those not on

treatments. Two brain regions the left-angular and the left temporal region showed reduced cerebral blood flow (CBF). These regions have been associated with language (angular) and memory (temporal region). The decrease in cerebral blood flow may be indicative of impairments in brain functioning in these regions (Tanaka et al., 2006). The power of this study is very small with only 8 patients examined however, observed results are in line with IFN- α -induced memory related adverse events. Hypometabolism has also been observed in the PFC in another study (Juengling et al., 2000a).

Two elegant studies are of note that utilise MRI in patients receiving IFN- α (Capuron et al., 2005; Capuron et al., 2007). An fMRI study using a visuospatial attention task in patients treated with low-dose IFN- α demonstrated activation in the anterior cingulate cortex. This activation correlated highly with task-related errors. This increase in activity was rationalised to result from an increased effort required for IFN- α -patients to complete the task. It was also suggested that there was an increased vulnerability to conflicting efforts. Another study completed by this group found increases in basal ganglia metabolism indicative of dopaminergic fibre degeneration (Capuron et al. 2007)

1.5. Rationale for Experiments

The rationale for conducting this MRI study was to assess underlying tissue changes that result from IFN- α -treatment. The intention was to assess volume changes and more subtle contrast changes. It is well-established that many mood disorders and mnemonic deficits often demonstrate underlying changes in anatomical structure. To date hippocampal integrity and volumetrics have not been examined in preclinical or clinical investigations of IFN- α -treatment. The experimental design laid out here will examine the volume of the hippocampus in IFN- α -treated animals compared with saline controls. We hypothesise that there will be structural changes in the hippocampus of animals treated with IFN- α which may explain hippocampal dependent deficits demonstrated previously.

2. Materials and Methods

2.1. Animals

18 Male Wistar rats (BioResources Unit, Trinity College Dublin) were used for this experiment. Commencing arrival, rats were randomly allocated to home cage groups of two or three in standard Plexiglas cages, (cage dimensions 44 x 28 x 18cm) in a Scantainer (Scanbur®) with free access to food and water. The Scantainer was maintained at (20-22°C) on a 12:12 light/dark cycle (lights on at 0800h). Rats were 3 months old and weighed between 280-375g (mean 339g) at the beginning of the experiment. Animals were monitored and handled regularly by the experimenter, and all procedures were carried out with accordance with institutional guidelines and approval from the ethical committee.

2.2. IFN- α -treatment

Roferon-A (human recombinant interferon-alpha 2a, Roche Pharmaceuticals, USA) (~170,000 IU/kg, 0.2mL, diluted in saline, s.c.) , or vehicle (0.2mL 0.9% NaCl, s.c.) was administered three times a week for four weeks prior to MRI scanning as described in the previous chapters.

2.3. Experimental Procedures

Prior to the start of the experiment the animals were given a 1-week period of acclimatisation and handling. During this period rats were randomly allocated to the experimental groups (n=8), Interferon (IFN α), Saline (Sal). Rats were handled on a regular basis in order to familiarise the rats with behavioural handling by the experimenter prior to the start of the treatment regime.

2.3.1. Behavioural Phenotyping

Behavioural testing was conducted to act as a probe to determine that there is indeed a pharmacological effect of the drug on behaviour.

Elevated Plus Maze

The Procedure was carried out as described in previous chapters, Chapter III (section 2.3.1).

2.4. *In-Vivo* MRI Scanning

Unfortunately, one animal from each group died prior to scanning due to anaesthetic effects.

2.4.1. Preparation of animals prior to Scan Acquisition

Animals were anaesthetised with Urethane (1.3g/kg) i.p and checked for pedal reflex before placement in a custom-built Perspex cradle with ear and tooth bars to support the head. Respiration rate and core body temperature were monitored using purpose-built MRI-compatible monitoring equipment (S.A. Instruments, Stony Brook, NY). Temperature was maintained at 37.0°- 38.0°C using a feedback-controlled water-circulating heating system.

Following the secured fixture in the cradle the tail was heated to ~40°C using a heat lamp and warm water to dilate the vein and cannulated using a 22 gauge paediatric intravenous cannula (Introcan-22G, Abbott, Ireland). Towards the end of the MRI experiment the patency of the cannula was re-checked, before it was loaded with MRI contrast agent, meglumine gadopentate (Magnevist, Bayer). During acquisition of the penultimate scan, a bolus of 0.2 mmol/kg of contrast agent was injected into the tail vein by means of a custom-built pump (Gadopentate administration was required for investigation of diffusion scans which will not be included in this chapter).

2.4.2. MRI data acquisition

A 7 Tesla (7T) Avance BioSpec animal scanner 70/30 magnet (Bruker BioSpin, Ettlingen, Germany) equipped with a 20cm actively-shielded gradient system and actively decoupled transmit (12 cm Helmholtz) and receive (3cm surface quadrature) coils was used for data acquisition. Data were acquired using ParaVision software (Bruker, Germany) and analysed using Bruker manufacturer supplied software and MIPAV (Medical Image Processing, Analysis and Visualisation, www.mipav.cit.nih.gov/)

2.4.3. Scan Parameters

A series of 10 scans were conducted, four are outlined below and were used for subsequent data analysis, and 6 other scans were conducted for future analysis.

1. **Tripilot** – A tripilot scan was used to form a reproducible position of the animal in the scanner. The tripilot ensures that the positioning of the animal is correct and targets the region of interest in three orthogonal planes (transversal, horizontal, sagittal), ensuring that the hippocampus was typically centred on a point 3.60mm posterior to Bregma and was in the isocentre. Placement in the isocentre of the magnet aids to minimise imaging artefacts. An echo time (TE) of 5s was used to acquire images of 15 slices (1mm thickness) with the central slice positioned over the hippocampus.

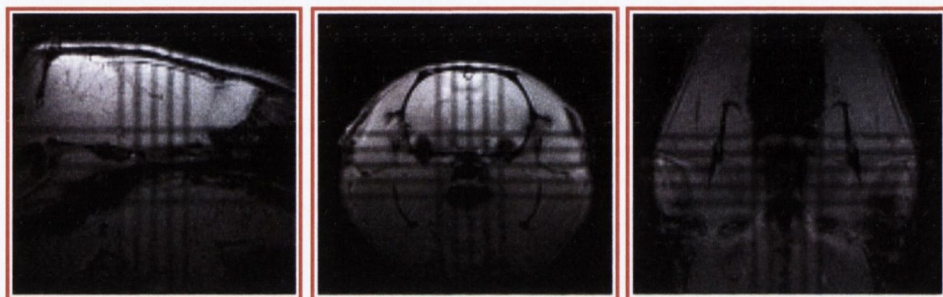


Fig.2. Orthogonal views of the positioning tripilot scan demonstrating the localisation of the scanned region over the hippocampus.

2. High Resolution Average scan – Volumetry

A Rapid-Acquisition Relaxation-Enhancement (RARE) sequence was used for structural imaging (TR = 6267ms, TE = 36 ms, echo train length: 8, number of averages: 4, number of slices: 54, slice orientation: axial, slice thickness: 0.5mm, inter-slice gap: 0.5mm, field of view: 6 x 3cm², matrix: 512 x 128).

3. T1 Weighted Spin-spin scan

Imaging parameter: 7 time series of 11 signals (slices) were acquired using increasing inversion times (TR = 608.4, 788.2, 1007.7, 1289.2, 1682.5, 2340.1, 5000.0ms) with constant echo time (TE = 25ms)

4. T2 Multi-Spin Multi-Echo (MSME) scan

Imaging parameters: 5 time series of 12 signals were acquired using increasing echo times (TE = 8.06, 16.12, 42.18, 32.24, 40.3, 48.36, 56.42, 64.48, 72.54, 80.6, 88.66, 96.72, ms; TR = 8.059 s)

2.4.4. T1 and T2 analysis

Measures of the T1 and T2 time constants were performed using Paravision 2.1.1. software (Bruker, Germany). A slice with an easily definable dorsal hippocampal region was selected from the T1 and T2 scan sequences. Regions of interest (Mizoi et al.) were drawn on the hippocampus and cortex and the ROIs were analysed using the Image Sequence Analysis (Isaacs & Lindenmann) programme. A non-linear exponential line of fit was calculated from the signal intensity and the T1 and T2 equations.

2.4.5. High Resolution – Volumetric Analysis

Measuring hippocampal volume

Volumetric analysis utilised high resolution scans of the hippocampus, analysis was performed using the MIPAV software package. Samples were processed prior to ROI segregation using the algorithm for midsagittal alignment. The hippocampus is a well defined region; the hippocampus was shaded from Bregma -2.16 to -6.36 throughout the dorsoventral extent (Paxinos & Watson, 2005). Volume measurements for each shaded area of the brain slice were calculated automatically in MIPAV. Subsequent hippocampal volume was calculated from the brain slices. Samples were coded by a colleague to ensure they were analysed blind to treatment group and analysed twice with a week interval in between, to allow for inter-rater reliability calculations.

Intracranial volume measurements

Measurement of total brain volume was conducted using the MIPAV software package. The intracranial vault volume was determined by completing whole brain extraction, using the Brain Extraction Tool (BET) algorithm in MIPAV. This separated the skull from the brain tissue. Samples were manually corrected where missing sections were evident. The olfactory bulbs and cerebellum were removed from all samples for this analysis due to difficulties with brain extraction on these areas.

2.5. Statistics

All data was analysed using SPSS version 14. Normality was assessed prior to analyses using the Shapiro-Wilk's test. Independent t-tests were used where

appropriate, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. An inter-rater reliability analysis was completed using a bi-variate correlation analysis.

3. Results

3.1. Behavioural Phenotyping

3.1.2. Elevated plus maze

Figure 3 below shows the performance of saline and IFN- α -treated animals on the elevated plus-maze. Independent t-tests reveal that saline-treated animals spend more time in the open arms (M=16.9, SEM 4.6) than IFN- α -treated animals (M= 4.0, SEM 6.6) ($t = 2.599$, $df = 13$, $p = 0.022$, two-tailed). IFN- α - treated animals spend more time in the closed arms (M= 490.9, SEM 16.6) than saline animals (M = 424.3, SEM = 24.1) ($t = -3.385$, $df = 13$, $p = 0.005$, two-tailed).

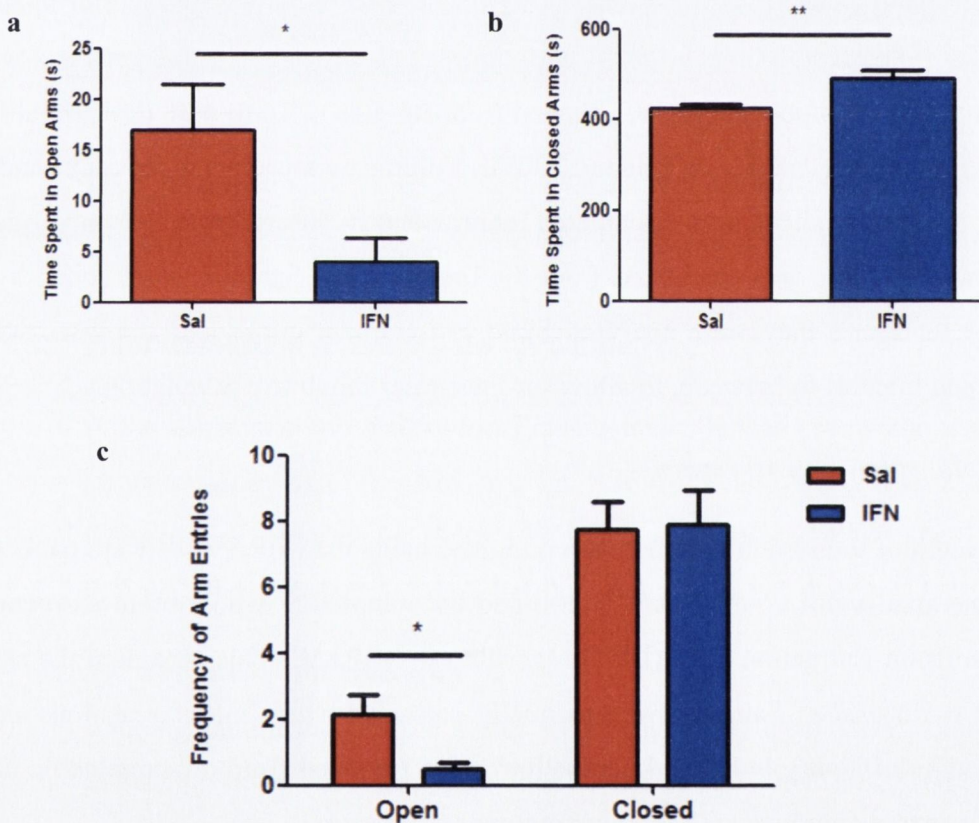


Fig.3. Performance of saline and IFN- α -treated animals on the elevated plus-maze, a) IFN- α -treated animals spent less time in open arms, b) IFN- α -treated animals spent more time in closed arms c) Visits to the open arms was less frequent in IFN- α -treated animals. Data presented as mean + SEM, $n = 8$ for each group, * $p < 0.05$, ** $p < 0.01$

Furthermore, the saline-treated animals ($M=2.1$, SEM 0.6) visited the open arms more than the IFN- α -treated animals ($M = 0.5$, SEM 0.5). Levene's test for equality of variance determined that equal variances could not be assumed, therefore $t= 2.633$, $df = 7.210$, $p =0.033$, two tailed). Conversely, Saline- ($M= 7.7$, SEM 7.9) and IFN α -treated animals ($M= 7.9$, SEM 3.0) did not differ in visits to the closed arm ($t= -1.114$, $df = 13$, $p=0.911$, two-tailed).

3.2. MRI Analysis

3.2.1. T1 Analysis

IFN- α -treatment does not modify T1 recovery time

Figure 4 is a representative T1 saturation curve, demonstrating that log intensity values increase with increased repetition time. The hippocampus and cortex were examined.

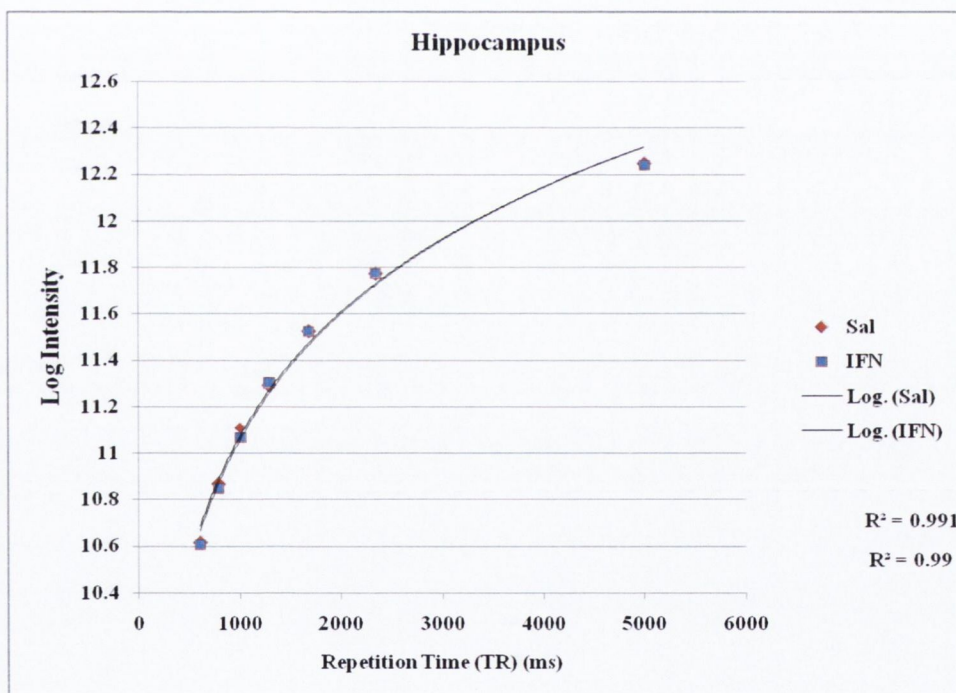


Fig. 4. A representative mono-exponential Longitudinal (T1) saturation curve demonstrating the recovery of T1 with repetition time. There are no differences in saturation between saline and IFN- α -treated animals. Data presented as mean \pm SEM, $n=8$ for each group

Examination of the mono-exponential graph above demonstrates no differences in T1 recovery in saline-treated and IFN- α -treated animals. The hippocampus and cortex did not exhibit differing T1 recovery rates between groups.

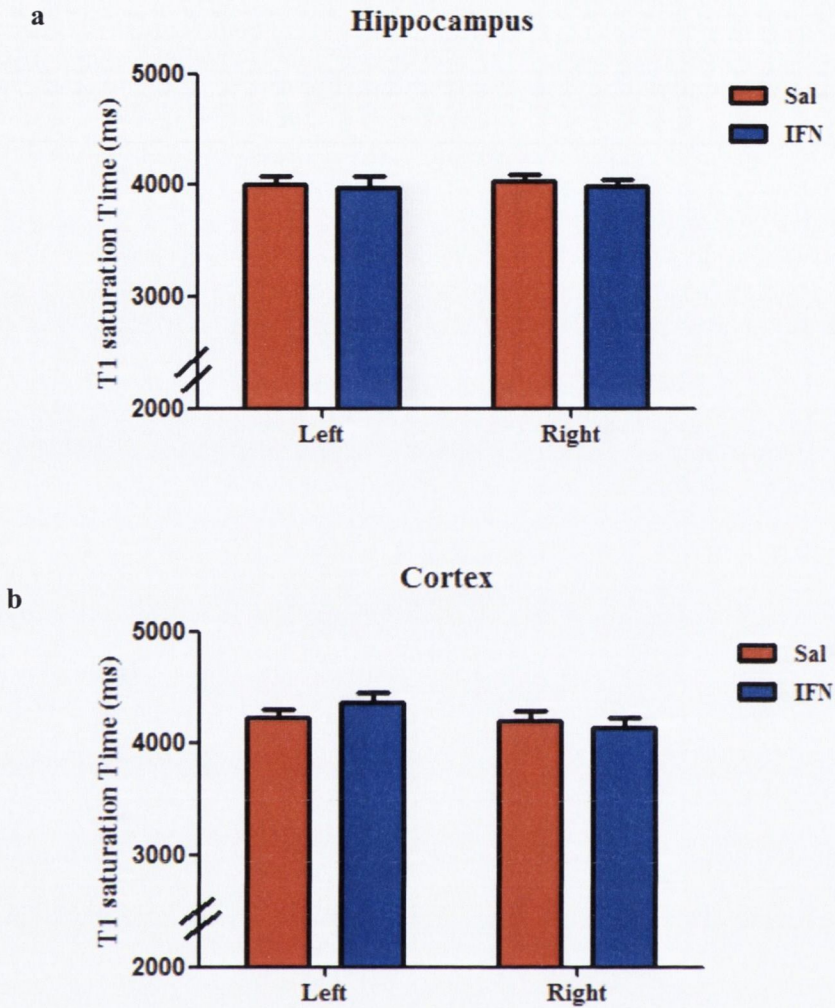


Fig.5. T1 saturation times for a) hippocampus and b) cortex of the saline-treated and IFN- α -treated animals, Data presented as mean + SEM, n = 8 per group

Independent samples t-tests demonstrate that there are no differences in the T1 relaxation times in the hippocampus of IFN- α and saline-treated animals of the: left hippocampus ($t = 0.1359$, $df = 14$, $p = 0.8939$) and right hippocampus ($t = 0.5594$, $df = 14$, $p = 0.5848$) (Fig 5a). There were also no differences determined in the cortex of IFN- α and saline-treated animals: left cortex ($t = 1.104$, $df = 14$, $p = 0.2882$) and right cortex ($t = 0.4711$, $df = 14$, $p = 0.6449$) (Fig 5b).

3.2.2. T2 Analysis

IFN- α -treatment has no effect on T2 relaxation time

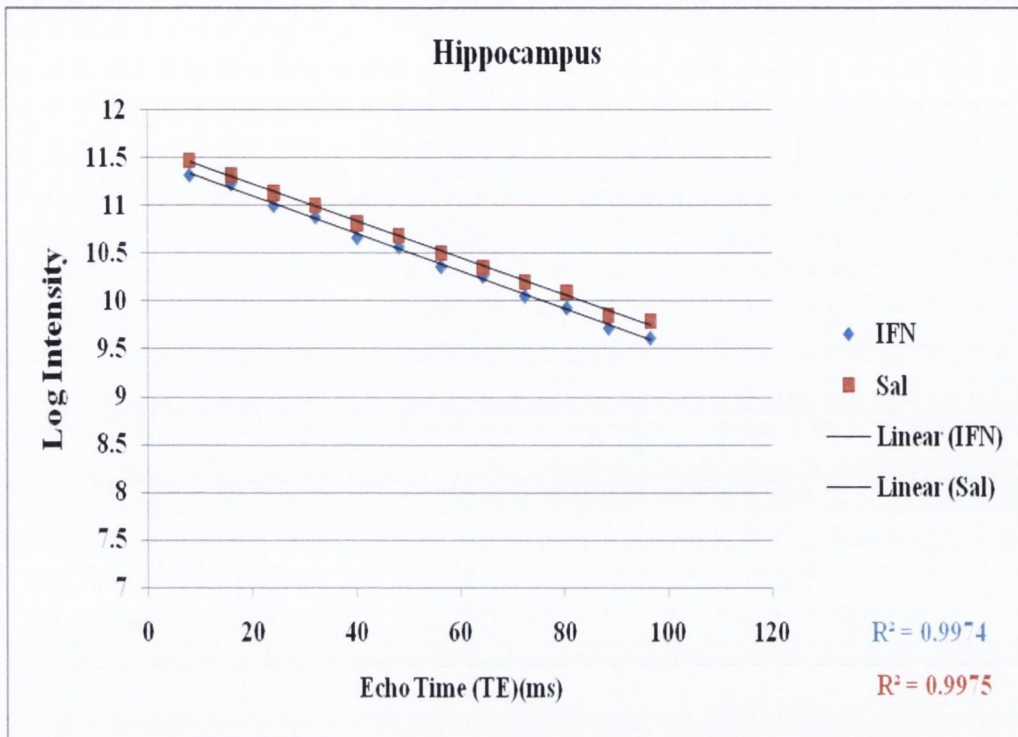


Fig. 6. A representative Transversal (T2) relaxation curve demonstrating the recovery of T2 with echo time. Data presented as mean \pm SEM, n=8 for each group

Figure 6 above demonstrates a representative T2 relaxation curve from the hippocampus, demonstrating there are no clear differences between relaxation times of the two groups. The lines run almost parallel and in extreme close proximity suggesting there are no differences, this is verified with the T2 times demonstrated below (fig 7 a and b)

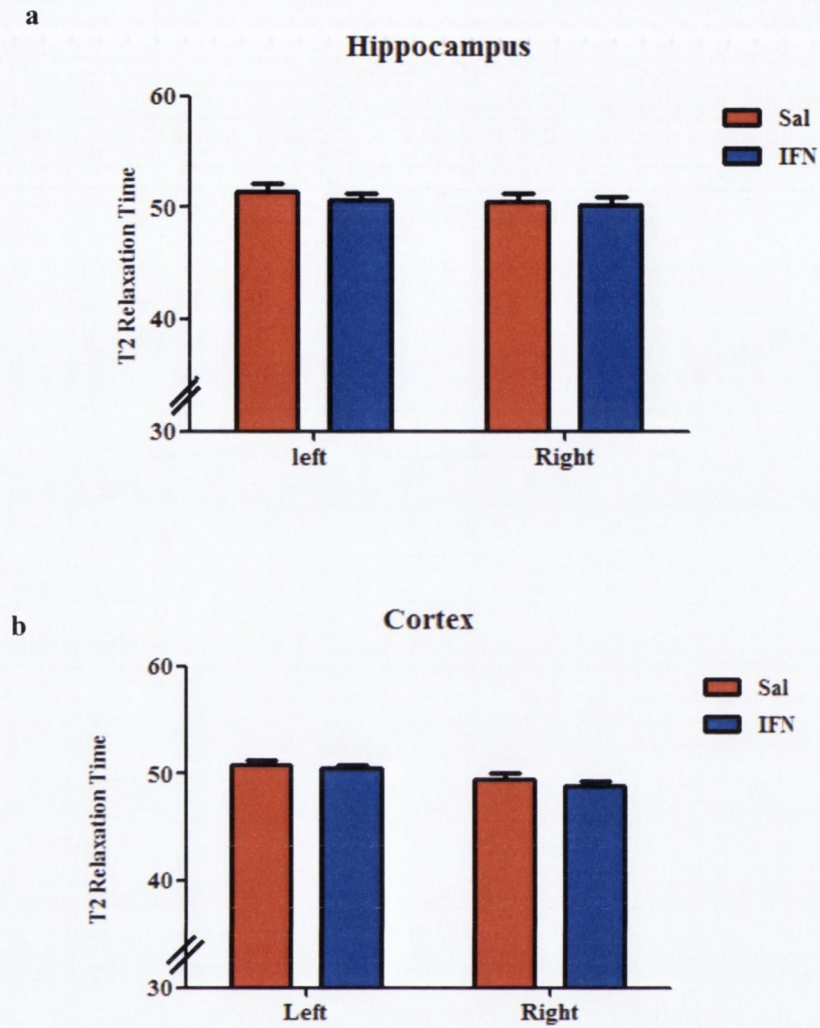


Fig .7. T2 relaxation times in IFN- α -treated and Sal-treated animals of the right and left **a)** hippocampus and **b)** cortex. Data presented as mean + SEM, n= 8 per group.

Independent samples t-tests demonstrate that there are no differences in the T2 relaxation times in the hippocampus of IFN- α and saline-treated animals of the: left hippocampus ($t=0.7940$, $df=14$, $p=0.4404$) and right hippocampus ($t=0.3069$, $df=14$, $p=0.7634$) (Fig 7a). There were also no differences determined in the cortex of IFN- α and saline-treated animals: left cortex ($t=0.6885$, $df=14$, $p=0.5024$) and right cortex ($t=0.8753$, $df=14$, $p=0.3962$) (Fig 7b).

MR Volumetry

Intracranial volumes do not differ between IFN- α -treated and saline-treated animals

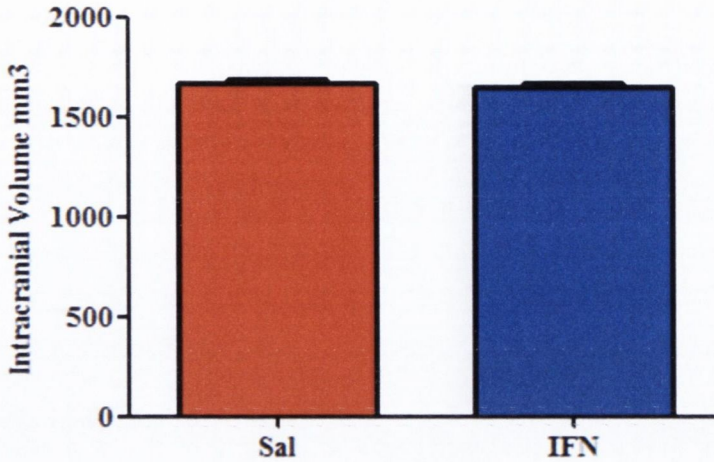


Fig. 8. Intracranial volumes of saline and IFN- α -treated animals. Data presented as mean + SEM, Sal n = 7, IFN, n=8

Figure 8 above displays the intracranial volumes of saline and IFN- α -treated animals. There were no differences found between the volumes of these two groups, Sal:1665.2 \pm 20.6 and IFN: 1648.9 \pm 20.6, (t=0.5518, df = 13, p =0.5905).

3.2.3. Volumetric Analysis of the Hippocampus

Volumetric analysis reveals a significant decrease in hippocampal volume in IFN- α -treated animals

Figure 9 below depicts the decrease in hippocampal volume that occurs as a consequence of chronic IFN- α -treatment. The respective absolute hippocampal volumes are Sal: 112.8 \pm 1.92 and IFN: 102.8 \pm 1.23 and a relative volume decrease in volume compared to saline controls (Sal: 100% \pm 1.09 IFN: 91.2% \pm 1.09). The decrease in hippocampal volume in IFN- α -treated animals is ~7-8%. This is also demonstrated qualitatively in the MRI pictures and 3D rendered reconstructions in figure 11c below.

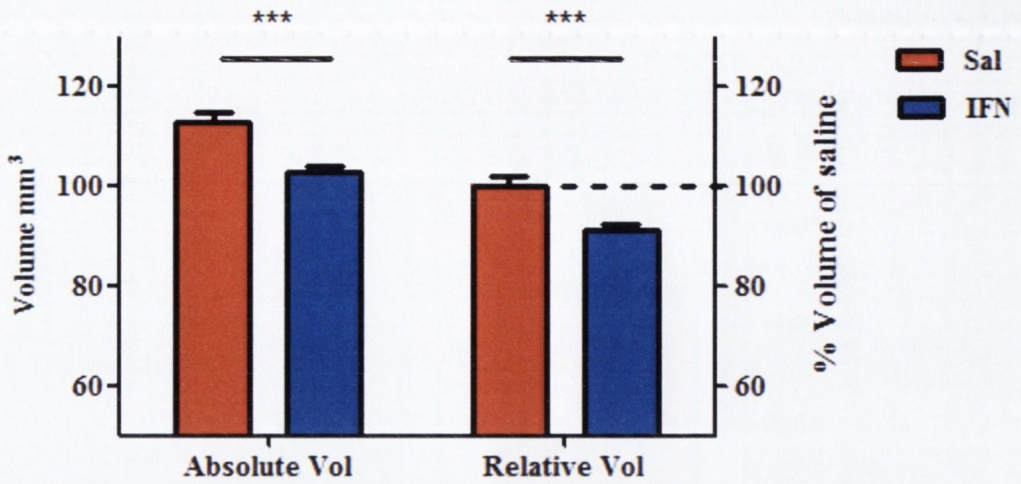


Fig.9. Absolute and relative volume changes demonstrated in saline and IFN- α -treated animals. Data presented as mean + SEM, n= 8 per group

An independent t-test demonstrates a significant difference between the absolute volume and the relative volumes of the hippocampus in the two treatment groups ($t=4.370$, $df = 14$, $p = 0.0006$).

Ratio of hippocampus to total brain volume

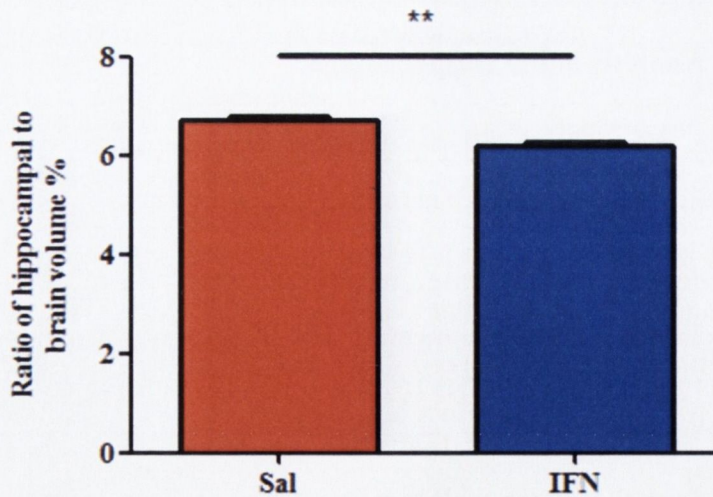


Fig. 10. Ratio of hippocampal to total brain volume of Saline and IFN- α -treated animals. Data presented as mean + SEM, Sal n = 7, IFN, n=8

Fig 10 above demonstrates a difference between the ratio of the hippocampus to brain volume, further demonstrating a decrease in hippocampal volume in the IFN- α -treated animals (Sal: 6.69 ± 0.1 , IFN: 6.18 ± 0.1), ($t= 3.448$, $df=13$, $p= 0.0043$).

Inter-rater reliability

A Pearson's correlation, determined that there was a strong correlation between the two different analysis sessions ($r = 0.917$ (16), $p = 0.000$).

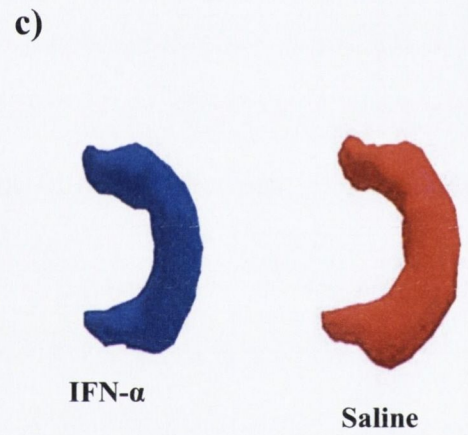
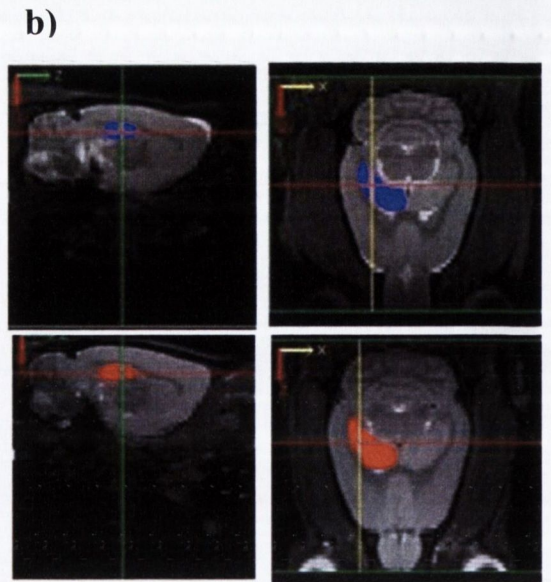
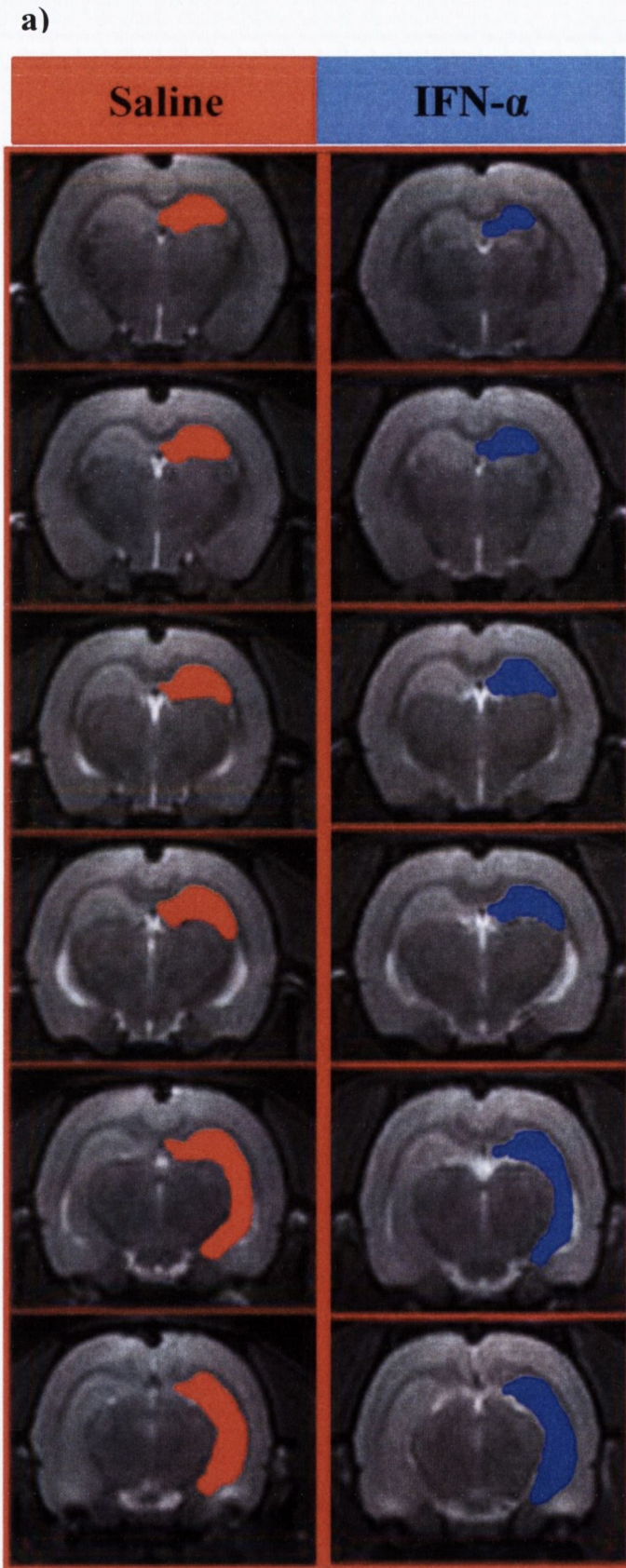


Fig.11. a) Representative coronal High resolution structural MRI Pictures, indicating the highlighted Hippocampal region of interest, b) High resolution horizontal pictures c) 3D rendered images of the hippocampi in the previous pane (a) using a trial version of the imaging software 3D doctor ®.

Table.1. Volumetric measures seen in IFN- α -treated rats and saline controls.

Measure	Saline (n=8)	IFN- α (n=8)	Saline Vs IFN α (p)
Physiological Measures			
BW (g)	437.5 13.19	450.0 11.18	p=0.4817 NS
Volumetric Measures			
BV (mm ³)	1665.22 \pm 21.1	1648.89 \pm 20.60	0.5905
RHV (mm ³)	55.14 \pm 1.07	50.38 \pm 0.71	0.0023 **
LHV (mm ³)	57.65 \pm 0.92	52.44 \pm 0.69	0.0005 ***
THV (mm ³)	112.79 \pm 1.92	102.81 \pm 1.23	0.0006***
RHV/BV (%)	3.33 \pm 0.05	3.06 \pm 0.05	0.0016**
LHV/BV (%)	3.38 \pm 0.08	3.13 \pm 0.05	0.0237 *
THV/BV (%)	6.69 \pm 0.11	6.18 \pm 0.10	0.0043 **
ASY	8.77 \pm 0.94	3.44 \pm 0.87	0.001***

Abbreviations: BW; Body weight, BV; Brain Volume, RHV; Right Hippocampal Volume, LHV; Left Hippocampal Volume, THV; Total Hippocampal Volume, ASY; Asymmetry, Left-hippocampal Dominance

Statistics are provided here for the results included in the summary table above.

An independent t-test revealed no differences in body weights of the saline- and IFN- α -treated animals ($t=0.7228$, $df = 14$, $p=0.481$). The intracranial volume did not differ between groups, ($t= 0.5518$, $df = 13$, $p=0.5905$). Saline-treated animals had larger right hippocampal volumes ($t= 3.717$, $df = 14$, $p =0.0023$) and left hippocampal volumes ($t= 4.521$, $df = 14$, $p= 0.0005$) than IFN- α -treated animals. Saline-treated animals also exhibited larger proportional size of hippocampus in the right ($t =3.984$, $df = 13$, $p = 0.0016$) and left ($t= 2.560$, $df = 13$, $p=0.0237$) hemisphere than IFN- α -treated animals. Asymmetry was measured as left-hippocampal dominance ($(LHV-RHV)/(LHV+RHV)100$). Saline-treated animals demonstrate more left-sided dominance than IFN- α -treated animals ($t= 4.154$, $df = 13$, $p =0.0010$).

4. Discussion

The data presented here demonstrates two separate exploratory analyses of MRI parameters to assess tissue integrity and volume with IFN- α -treatment.

Time Constant Measurements

Estimation of T1 saturation and T2 relaxation were performed in the hippocampus and cortex. The saturation and relaxation times were in line with the values reported for gray matter in the literature (Cudalbu, Cavassila, Rabeson, van Ormondt, & Graveron-Demilly, 2008). However, the estimations demonstrate no differences between the treatment groups (figure 5 and 7), both IFN- α - and saline-treated animals demonstrate comparable relaxation times in the two regions assessed. This indirect measure of tissue kinetics suggests that there are no differences in the stability of the tissue that has been assessed. However, differences cannot be ruled out without further analysis via histological methods it is therefore suggested further experiments are warranted. Previous studies have demonstrated high correlations between volumetry in small animal imaging and histological volumetry (Redwine et al., 2003)

MRI Volumetry of the Hippocampus

A second exploratory analysis utilised MRI volumetry, representative high resolution images are shown in figure 11a and b. Volumetric analysis of the hippocampus highlights a most interesting effect; IFN- α -treated animals demonstrate a remarkably significant reduction in volume of the hippocampus (fig 9). The effect is pronounced, with a 7-8% decrease in volume relative to saline control levels. This confers with previous studies using chronic mild stress (CMS) for 4 weeks conducted also demonstrating a decrease in hippocampal volume in the range of 7-8% (McEwen, 1999). This treatment is often used to induce depressive side effects and the behavioural effects reflect those of IFN- α -treated animals, indicating stress precipitates depression. Sheline (1996) was the first to make the correlation between the time spent depressed and the severity of depression and the amount of hippocampus volume loss, indicating that the volume loss was a consequence of the depression and not a secondary cause (Sheline, 1996).

There were no differences determined in the IFN- α -treated animals and saline controls between the overall brain volumes (fig 8). The volume reduction was substantiated by

assessing the ratio measurements of hippocampal volume to intracranial volume, the ratios were significantly different (fig 10). This indicates that it is not a global reduction in volume of the brain but a more specific reduction in this one well-defined anatomical region.

It is surprising that volumetric analysis has not been studied in patients receiving IFN- α -treatment previously. Consistent with the notion that IFN- α produces adverse events which in some patients reflect those of major depressive disorder (MDD) it is probable that there is a structural effect of IFN- α on the hippocampus.

In the previous chapter we established a decrease in cell proliferation in the subgranular zone of the dentate gyrus. However, the functional significance of the newborn cells remains unknown, is there the possibility that neurogenic suppression may contribute to the decrease in volume reported in this expression? To answer this question it is important to evaluate the quantity of neurogenesis that can arise in the dentate gyrus. It has been reported that approximately 9000 new cells can be generated in the dentate gyrus per day (Cameron & McKay, 2001), although some calculations determine that at peak proliferation rate this could be as high as 50,000 cells per day (Schlessinger, Cowan, & Gottlieb, 1975). West et al (1990) suggest that if all the young cells were to survive for 4 weeks, the number of young neurons in the dentate gyrus could be as much as 138,000, which calculates as 6% of the total granule cell population (West & Gundersen, 1990). However, it is suggested that two thirds of newborn cells at the proliferative stage die by programmed cell death and only one third survive to maturity (Elder et al., 2006). Although this may be a contributing factor to the decrease in volume loss it is unlikely to explain the morphological changes in their entirety. Additionally, the hippocampus possesses a high level of receptors for glucocorticoids, providing the link between the HPA axis and the production of depressive symptomology (Holsboer & Barden, 1996). Elevated glucocorticoids can lead to atrophy of the CA3 pyramidal neurons could lead to loss of feedback inhibition to the HPA axis (McEwen, 1999).

Of further note, this reduction in volume may be an underlying reason for the impairments found in IFN- α -treated animals in hippocampal dependent tasks (Fahey, 2004). The reduction in volume stated here is a global reduction in hippocampal volume. Further avenues to investigate would be the cingulate cortex. Cerqueira et al

(2005) determined volume changes in the anterior cingulate cortex with hypercortisolaemia using small animal imaging.

Traditional morphometric analysis was used in this experiment, involving drawing manual regions of interest in order to calculate the volume enclosed. The hippocampus in rats is a well defined unambiguous structure; however, other regions of interest are difficult to establish using fixed boundaries. Alternative methods of volumetric analysis are developing in the small animal imaging field. A more pragmatic approach uses Voxel-Based Morphometry (VBM) a technique that compares brain regions on a voxel-to-voxel basis after the images have been normalised using deformation parameters (Mechelli, Friston, Frackowiak, & Price, 2005). This enables the entire brain to be analysed rather than a pre-defined structure and provides an unbiased objective analysis of the regions that are differentially altered to the template. Although this is an attractive technique, yielding results fast and en masse, the gold standard for morphometric analysis still favours manual analysis.

Interestingly, one study has suggested that a decrease in hippocampal volume may be a result of an increase in cell packing density (Stockmeier et al., 2004). However others have reported no changes in cell packing in this region (M. B. Muller et al., 2001). Consistent with the latter findings, the T1 and T2 results contained herein suggest that there are no changes in the density of packing in the hippocampus. However, this would warrant further examination with histological staining.

5. Conclusion

These data, show for the first time a decrease in hippocampal volume in animals treated with IFN- α . The functional significance of this decrease may pertain to the presentation of mnemonic and affective adverse event in patients. The volume decrease may contribute to a dysregulation of the limbic-cortical pathway via the HC-PFC connections.

Chapter VI

Microarray analysis of IFN- α -treated rats

A preliminary investigation of gene targets involved in IFN- α -induced deficits and recovery with combined Fluoxetine administration

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Summary

Here we present a preliminary microarray study. Microarray technology is an attractive approach to assess large quantities of genes in a high-throughput manner. Benefits lie in the yield of data and hypothesis free screening of multiple gene candidates.

This pilot study aimed to deconstruct mechanisms underlying IFN- α -induced deficits. The results presented here indicate several potential pathways that may be of interest. Of specific interest one of the enzymes involved in the kynurenine pathway is up-regulated in IFN- α -treated animals which may indicate an increase in oxidative stress alongside a decrease of available tryptophan for the metabolism of 5-HT. Additionally the down-regulation of STAT-3 has been associated with memory deficits and a down-regulation of genes associated with tight junctions may indicate an altered integrity of the BBB. Additionally of note, IFN- α up-regulates calcium signalling and this is reflexively down-regulated with SSRI treatment, this may indicate another potential role for toxicity through glutamate excitotoxicity. There are several other lines of interest that may be followed.

Further analysis and validation is required to unravel the expression of these genes further and to reveal their functional significance in the pathophysiology of IFN- α -induced mnemonic and affective disorders.

1. Introduction

1.1. Microarray technology

Microarray analysis is a method that enables the whole genome to be assessed for alterations in expression of a large quantity of genes. Gene expression changes may indicate cellular and metabolic changes. Prior to the advent of microarray technology (P. O. Brown & Botstein, 1999) conventional assessments of gene expression involved a considerably more labour intensive approach. Although, initially they appear to be an expensive technique on informed second glance the high throughput nature and wealth of information make this an attractive option.

1.2. Application of Microarray to Neuroscience

A caveat arises in the use of microarray in the field of neuroscience, considering the heterogeneous nature of brain cells and the small quantity of tissue usually available. The expected fold change in brain tissue is ~ 2 whereas in other organs where the tissue is more homogeneous the expression is greater. Mirnics et al (2001) state 'True gene expression changes are small in psychiatric diseases' (Mirnics, 2001). Additionally the dynamic nature of the ever-changing plastic brain can result in complications with interpretations of the data. Of particular benefit is the hypothesis free nature of this application which may in turn lead to hypothesis generation. Microarray facilitates the screening of diseases to provide insight into the nature of molecular underpinnings of the brain's 'function' or 'dysfunction' in a global fashion and assesses gene expression in parallel (Reimers, Heilig, & Sommer, 2005).

Microarray analysis is not a final definitive analytical tool; it requires further verification with in-situ hybridisation and real time polymerase chain reaction (rtPCR). Although the large yield of results is informative the principles behind analysis are labour intensive unless strict bioinformatic tools are implemented. There are however user-friendly software tools that can perform pathway analysis to indicate specific gene pathways that may be differentially expressed.

1.3. Microarray and Interferon-Alpha

Microarrays that have been performed using IFN- α centre on peripheral measurements in plasmacytoid dendritic cells (pDC) and peripheral blood mononuclear cells (PBMCs) (Ji et al. 2003) or cell lines (Cai et al., 2005; Radaeva et al., 2002). To our knowledge, a study of this kind in the context of IFN- α -treatment has not been completed previously. Although, this is a pilot study with only a small sample size the results gained from this study may be pivotal for future experimental designs.

1.4. Rationale for Microarray Investigation of genes

This experiment was intended to provide more insight into the molecular mechanisms that may underlie neuropsychiatric and mnemonic adverse events that occur as a consequence of IFN- α -therapy. Four treatment groups have been investigated to ascertain differential gene expression with IFN- α given alone and when the system is rescued by antidepressant co-administration. It still remains unclear how fluoxetine may rescue the adverse events that present alongside this treatment when given prophylactically, therefore further investigations are required. Due to the small sample size included in this pilot study the results will be descriptive, inferential statistics will not be applied considering statistical restraints.

2. Materials and Methods

2.1. Animals

24 Male Wistar rats (BioResources Unit, Trinity College Dublin) weighing 275-320g were housed two/three to a cage in a controlled environment (Laminar airflow unit, 12h light/dark schedule with lights on at 08:00-20.00). Rats received food and water *ad libitum*. After a weeklong acclimatisation period rats were randomly assigned to one of four treatment groups (n=6 per group), Sal (Saline and placebo), Salflx (Saline and fluoxetine), IFN (Interferon-alpha and placebo) and IFNflx (Interferon-alpha and fluoxetine). All behavioural testing was conducted during the light phase of the schedule. Prior to behavioural testing all rats were handled to acclimatise them to the experimenter. All experiments were carried out in accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experiments.

2.2. IFN- α -Treatment

Administration of IFN- α -treatment was the same as described in Chapter 2, section 4.2.1.

2.3. Fluoxetine Treatment

Fluoxetine hydrochloride (LKT laboratories), (7.5mg/kg) treatment was given once a day in the evening time (18.00hr) throughout the course of a 4 week IFN- α drug regime. Fluoxetine (flx) was administered orally in a pellet form as described in experimental chapter 3. Animals were lightly food deprived for one hour prior to separation to administer the flx. If the rat did not eat the pellet, they were left overnight with the flx and a small amount of food. Adaptation to the feeding routine was quickly adopted.

2.4. Behavioural testing

Temporal Order Memory task

This task was performed as previously described in chapter 2 (section 2.2.1) with the exception that general laboratory objects were used which had been adapted. In line with former experiments a six hour delay was imposed between sample 2 and the test phase.

Elevated Plus Maze Task

This task was performed as previously described in chapter 3 (section 2.3.1), however, a 10 minute trial length was adopted.

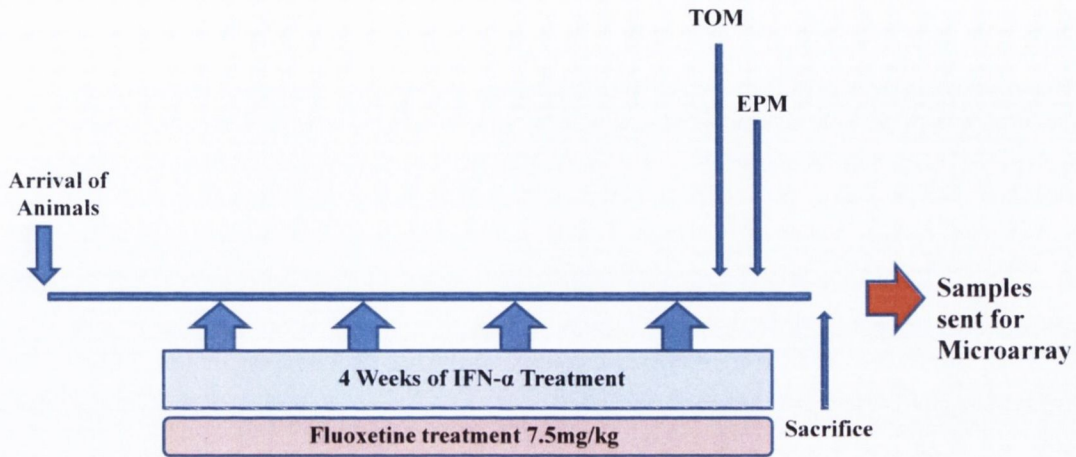


Fig. 1. Schematic of the behavioural and drug administration schedule. Abbreviations: TOM, Temporal Order Task; EPM, Elevated-Plus Maze.

2.5. Tissue Sampling

Tissue was sampled after 4 weeks of treatment and 24 hours after the last behavioural testing session. Animals were killed by rapid decapitation and the brain was swiftly removed from the skull and dissected. Dorsal and ventral hippocampi were sampled separately. The hippocampus was dissected according to established anatomical landmarks (Paxinos & Watson, 2005), tissue was transected into three pieces, the middle section was discarded as intermediary hippocampus. The right dorsal hippocampus was placed in cryotubes and rapidly frozen in liquid nitrogen. All samples were stored at -80°C prior to further analysis. Prefrontal cortex, cerebellum, and ventral and dorsal hippocampus of the left hemisphere were preserved in Krebs Calcium solution with protease inhibitor for future analysis.

Samples for microarray analysis were chosen on the basis of the animal's performance in the aforementioned behavioural tests. Three samples for each group were chosen for analysis resulting in the requirement for 12 microarray chips. The author decided to

keep the samples as individual tissue samples and not pool the samples in order to provide more accurate information from the microarray (Reimers et al., 2005). Dorsal hippocampal samples were sent off to Almac Diagnostics (www.almacgroup.com) for RNA extraction and subsequent microarray analysis. The microarray array platform used was the commercially available Affymetrix Rat Genome 230 2.0 arrays. The quantity of RNA sent was $\sim 5\mu\text{g}$. A summary outline of the microarray process performed by Almac diagnostics may be seen in the figure 2 below.

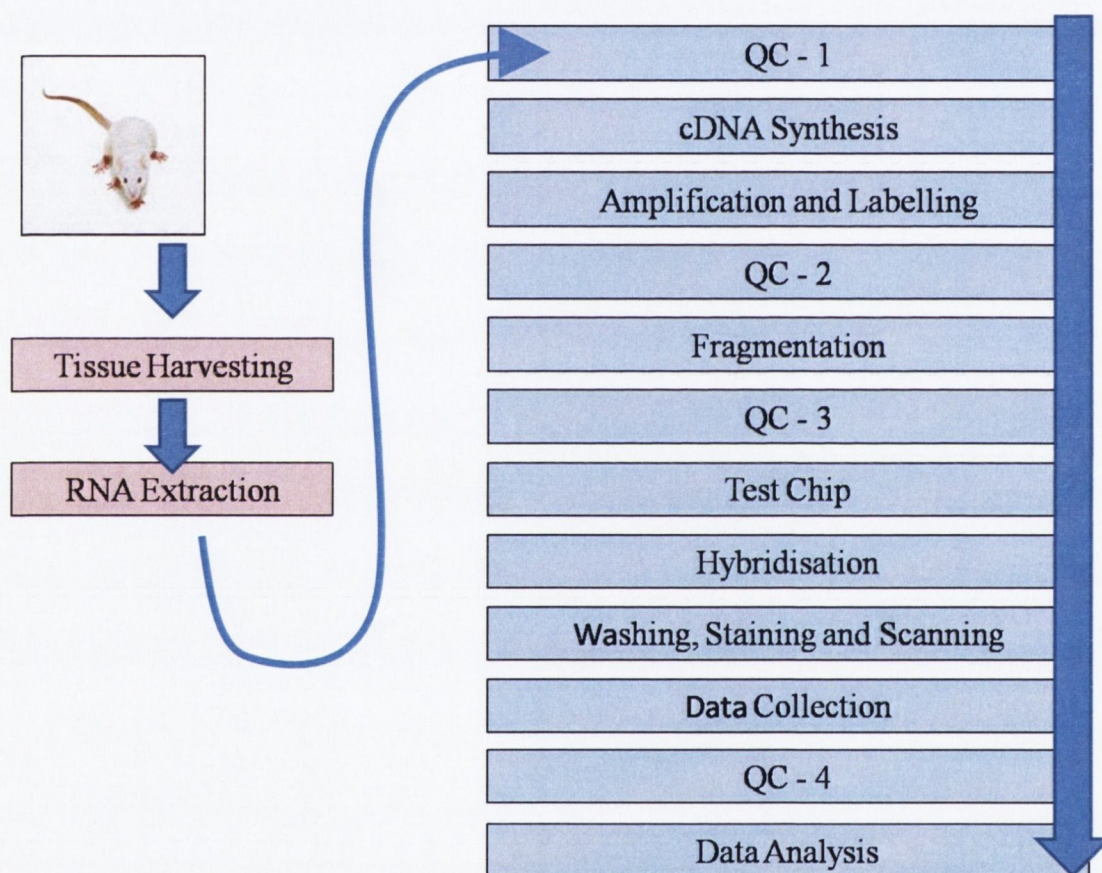


Fig. 2. Schematic of the processes required for microarray gene expression analysis.

Almac provided the RNA extraction service this procedure is outlined in appendix 2.

2.6. Exploratory Data Analysis - Cluster Analysis

Almac provided a comprehensive analysis of the clustering of individual profiles of expression within the treatment groups. Cluster analysis was performed to determine if the gene expression in the treatment groups demonstrated similar patterns of expression, therefore exhibiting clustering. This was performed using two measurements, distance metrics (Euclidean distance and Pearson's Correlation) and a clustering algorithm (Principal Component Analysis (PCA)).

Distance Metrics

Distance measurements are based on the similarities and differences in intensity measures and measures the distance between two data points in a cluster. These may be measured using the Euclidean distance and Pearson's correlation.

The Euclidean Distance

The Euclidean distance is defined by Pythagoras theorem as the distance between two points in space. To derive this distance between two data points, the square root of the sum of differences between the corresponding values is calculated.

Pearson's Correlation

Utilises the application of a correlation matrix to the data and measures the similarity between the shapes of the profiles, the degree to which the samples are related.

Hierarchical Clustering

Hierarchical clustering was applied to both of these distance metric analyses to produce dendrograms representing the relationship of the samples to each other. Strong relationships appear on one branch, increasing branches increases the relationship to each other.

Principal Components Analysis (PCA)

Principal components analysis takes into account the 31,100 genes that are analysed on the rat genome chip alongside the 12 animals in 4 treatment groups. The analysis aids in the elimination of background noise by verifying trends (clusters) within the data groups and determining outliers. The samples are clustered in a 3D space (x, y, and z

axis) where obvious outliers are easily distinguishable from the other experimental samples.

Data Analysis

Almac incorporate the use of a number of stringency parameters on the data, details of these are provided in the appendix 3.

Pathway analysis

Two software packages were used to analyse the data provided by Almac; the open source software Bio Resource for Array Genes, (Biorag) (Arizona Cancer Center and Southwest environmental Health Science Bioinformatics Core, www.Biorag.org) and a trial version of Biosphere.

Author's notes

This experiment was designed as a pilot study and utilises only a small number of animals due to financial limitations, therefore data is presented descriptively for the purpose of indications into future experimental targets. In addition, the Affymetrix Rat Genome 2.0 microarray assesses 31,000 genes, many of the genes expressed have unknown function and are characterised by accession number alone. For the purpose of investigating candidate genes for IFN- α -induced neuropsychiatric and mnemonic deficits, only genes with known function that are differentially expressed will be discussed.

3. Results

3.1. Behavioural Results

3.1.1. Temporal Order Memory

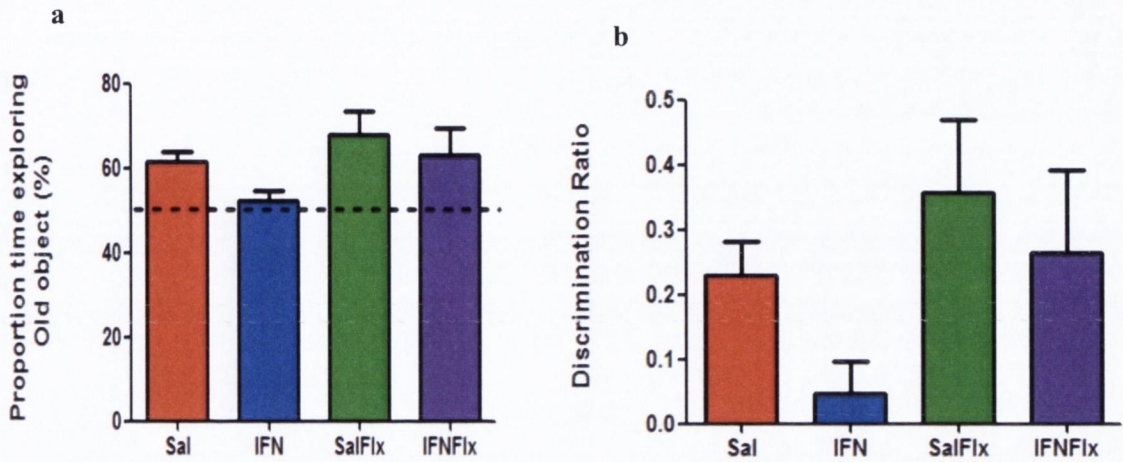


Fig. 3. Performance of the animals on the temporal order memory task that were used in the microarray experiment **a)** proportion of time **b)** discrimination ratio. Data presented as mean + SEM, n=3 of the four experimental groups

Figure 3 above demonstrates the mean performance of the animals that were chosen for microarray analysis. It is evident that all experimental (Sal: 61.5 ± 2.5 , SalFlx: 67.4 ± 4.0 and IFNFlx: 56.8 ± 6.0) groups apart from IFN (52.0 ± 1.3) can discriminate temporal order. IFNFlx treated animals discriminate temporal order comparably to saline controls.

The table below demonstrates the time spent exploring the sample phases, all animals explored sample phase one and two equally. There does appear to be a lower exploration of objects in the test phase for IFN- α -treated animals.

Table.1. Time spent exploring the sample phases and test phase

Group	Expl. Sample 1 (s)	Expl. Sample 2 (s)	Expl. Test Phase (s)
Sal	93.3 ± 15.53	83.1 ± 9.01	61.1 ± 3.0
IFN	102.9 ± 8.01	124.2 ± 11.74	49.7 ± 6.9
SalFlx	102.4 ± 19.55	98.8 ± 10.80	67.8 ± 5.6
IFNFlx	99.8 ± 5.87	95.1 ± 8.92	63.2 ± 6.4

Expl. Exploration, n=3

3.1.2. Elevated Plus-Maze

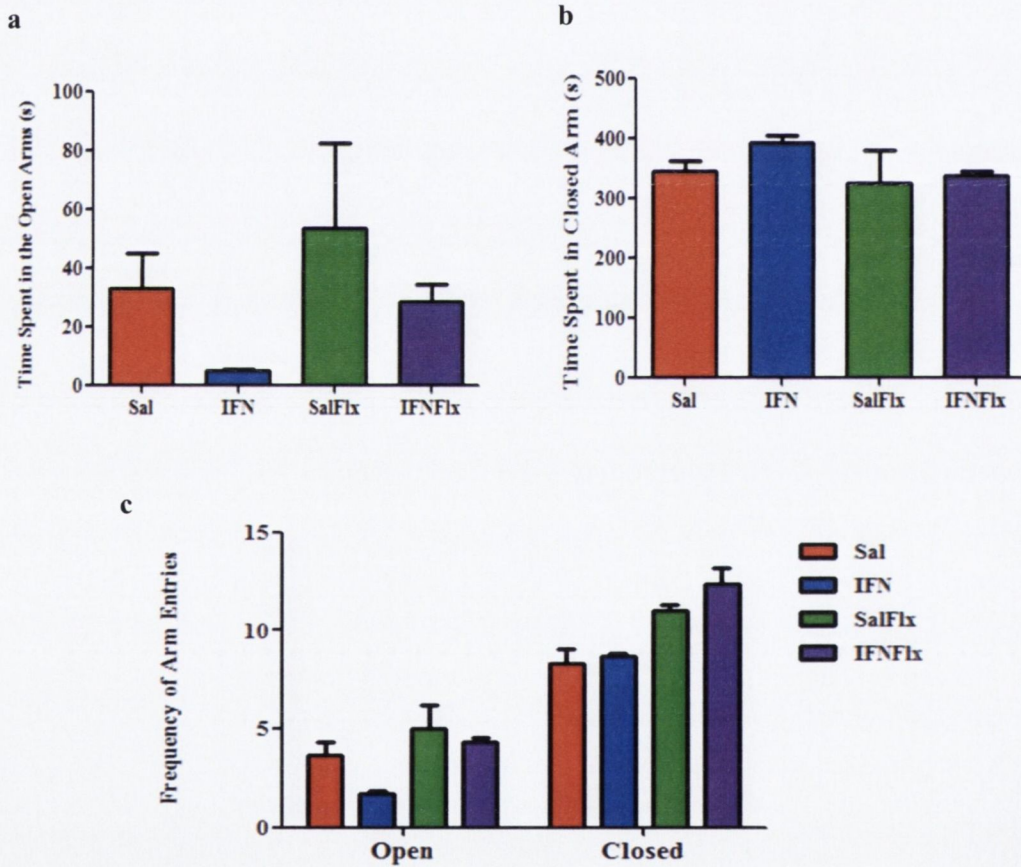


Fig.4. Performance of the animals from the four experimental groups in the elevated plus-maze, a) time in open arms, b) time in closed arms and c) frequency into arms. Data is presented as mean + SEM. N= 3 for all groups.

Performance of the animals on the elevated plus-maze demonstrates that the IFN- α -treated animals explore open arm to a lower extent (4.7 ± 0.6) than the other three groups (Sal: 33.0 ± 11.9 , SalFlx: 53.3 ± 29.1 and IFNFlx: 28.3 ± 6.1). It appears that IFNFlx animals explore the open arm to similar levels as the saline-treated group; however the SalFlx group perform quite variably (fig 4a). Fig 4b reveals IFN- α -treated animals explore the closed arm more than the other three groups.

In addition, the frequency of entries into the open and closed arms is displayed in figure 4c, IFN- α made the least number of visits to the open arms, Sal, SalFlx, and IFNFlx made comparable number of visits. IFN and Sal groups visited the closed arms comparably demonstrating similar motivational and locomotor behaviour, however, IFNFlx and SalFlx appear to visit the closed arms more frequently, demonstrating slightly heightened mobility.

3.1.3. Correlation of behaviour on the two Tasks

There is a moderate- strong correlation evident between the two behavioural tasks ($R^2 = 0.5633$). The rats that spend more time on the open arms also spend more time exploring the old object thereby indicating good discriminative ability. The behaviour of the IFN treated animals is very poor with animals clustering around the extreme level, performing both poorly on the temporal order task and the elevated plus-maze.

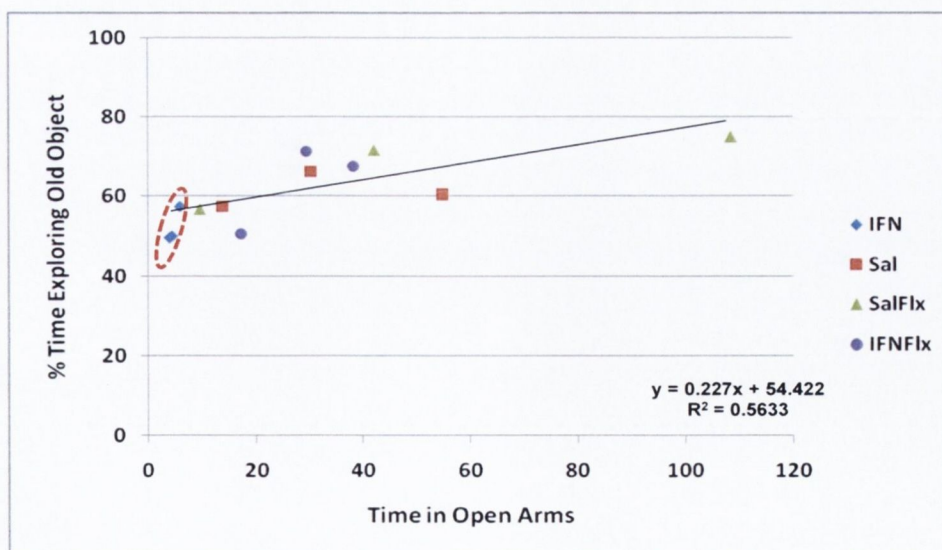


Fig.5. Correlation of performance on the elevated-plus maze with performance on the temporal order memory task.

3.2. Microarray Analysis

3.2.1 Exploratory Data Analysis - Cluster Analysis

Exploratory data analysis was performed by Almac; the results obtained from the analysis will be highlighted here.

First Pass Analysis

Initial cluster analysis was performed on all the data from the 12 chips to determine the profile relationships.

Hierarchical Clusters

Analysis of the Euclidean distance and the formation of a dendrogram from the distance metric data demonstrates that two of the samples (S0397F011_1 (IFN) and S0397F010_IP (IFNFlx) highlighted in figure 6 are separated from the group and do not share similar expression profiles. The other 10 samples cluster weakly together. Some of the treatment characteristics of the group appear to be fairly closely related. Four samples on the left of the dendrogram all share the common saline-treatment and samples in the middle are treated with IFN. However some branches demonstrate a common pathway between IFN and saline.



Fig.6. Hierarchical dendrogram created using the Euclidean distance metric, profiling the relationship between the samples of the four experimental treatment groups. The dashed line indicates samples that are not as related as the other samples.

Figure 7 includes the second distance metric measure using Pearson's correlation to determine profile commonalities. In this dendrogram it is demonstrated that once again sample S0397F011_1 (IFN) and S0397F010_IP (IFNFlx) cluster together away from the core group. Alongside these two strays another sample joins the branch Sample S0397F06_IP (IFNFlx) joins the cluster. Sample S0397F01_IP (Sal), S0397F08_IP (SalFlx) and S0397F02_IP (SalFlx) all cluster together on the left side of the dendrogram, however S0397F01_IP (Sal) is more closely related to S0397F08_IP (SalFlx). Similarly, S0397F04_IP (Sal) appears closely related to S0397F02_IP (Sal), however S0397F02_IP (Sal) is more closely related to S0397F05_IP (IFN).

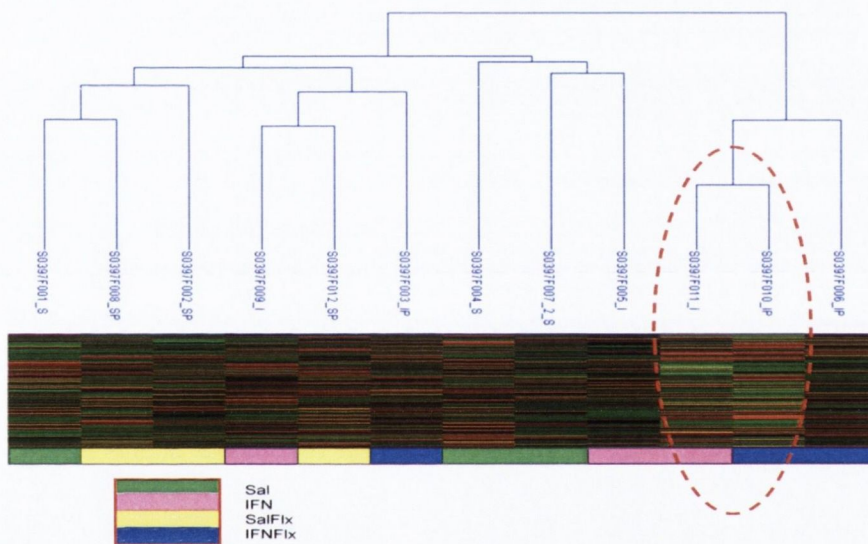


Fig.7. Hierarchical dendrogram created using the Pearson's correlation distance metric, profiling the relationship between the samples of the four experimental treatment groups, n= 3 per group. The dashed line reveals samples that are not closely related to the other samples processed.

Principal Components Analysis

Figure 8 below shows the principal components analysis performed using all of the chip samples. It is evident once again using this exploratory technique that there appear to be some outliers in the clusters, sample S0397F011_1 (IFN) and S0397F010_IP (IFNFlx). The other samples cluster together fairly weakly but there does not appear to be much overlap in the cluster groups. It is interesting to note that the identities of the disparate samples from the core cluster group have a common treatment of IFN α .

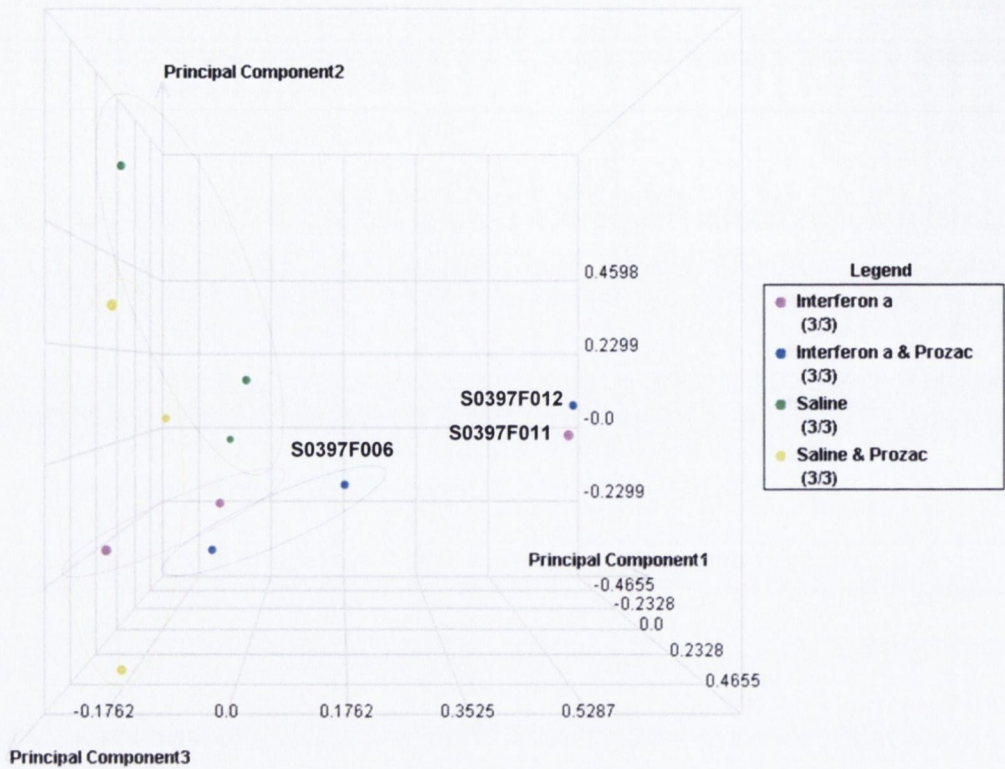


Fig.8. Principle components analysis showing all the samples from the four treatment groups from the 12 chips.

From the above exploratory analysis performed by Almac, it was determined that there were two distinct outliers in the chip dataset, samples S0397F011_1 (IFN) and S0397F010_IP (IFNflx). These samples were removed and exploratory analysis performed again to determine if the cluster pattern altered.

Second Pass analysis

Hierarchical Clusters

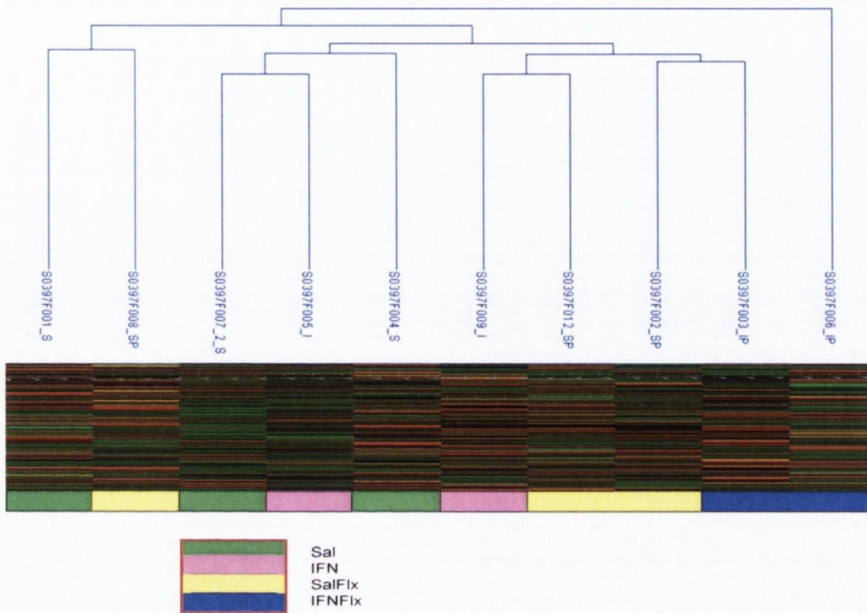


Fig.9. Hierarchical dendrogram created using the Euclidean distance metric, profiling the relationship between the samples of the four experimental treatment groups with outliers S0397F011_1 (IFN) and S0397F010_IP (IFNFlx) removed.

The hierarchical dendrogram above yields some closer related clusters. Samples S0397F06_1 (IFNFlx) and S0397F03_1 (IFNFlx) cluster together, however S0397F03_1 (IFNFlx) is more closely related to S0397F02_1 (SalFlx). These share the same treatment group of Flx. Additionally, S0397F012_1 (SalFlx) also clusters alongside the S0397F02_1 (SalFlx). However sample S0397F02_1 (SalFlx) is more closely related to S0397F09_1 (IFN). The IFN samples are samples that are disrupting the cluster pattern.

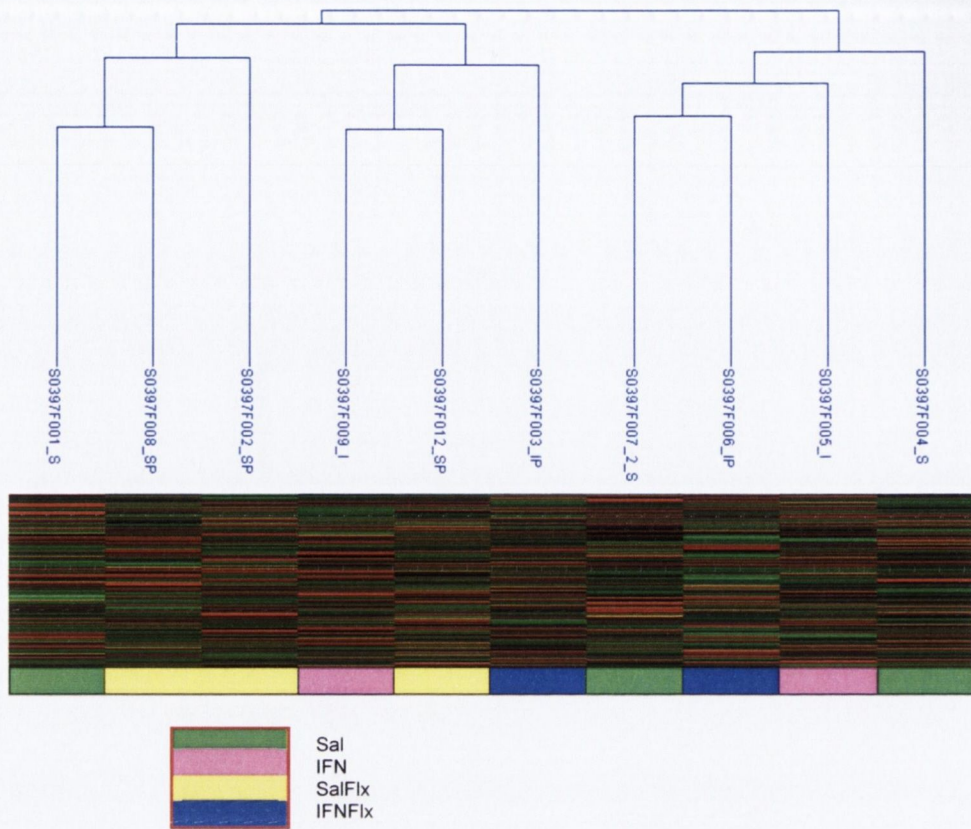


Fig.10. Hierarchical dendrogram created using Pearson's correlation distance metric, profiling the relationship between the samples of the four experimental treatment groups with outliers S0397F011_1 (IFN) and S0 397F010_IP (IFNFlx) removed.

Once again the hierarchical dendrogram was produced for Pearson's correlation (Figure 10). It appears that there is little clustering in the analysis above. However if the IFN- α samples were removed it appears that there would be more clustering across the groups.

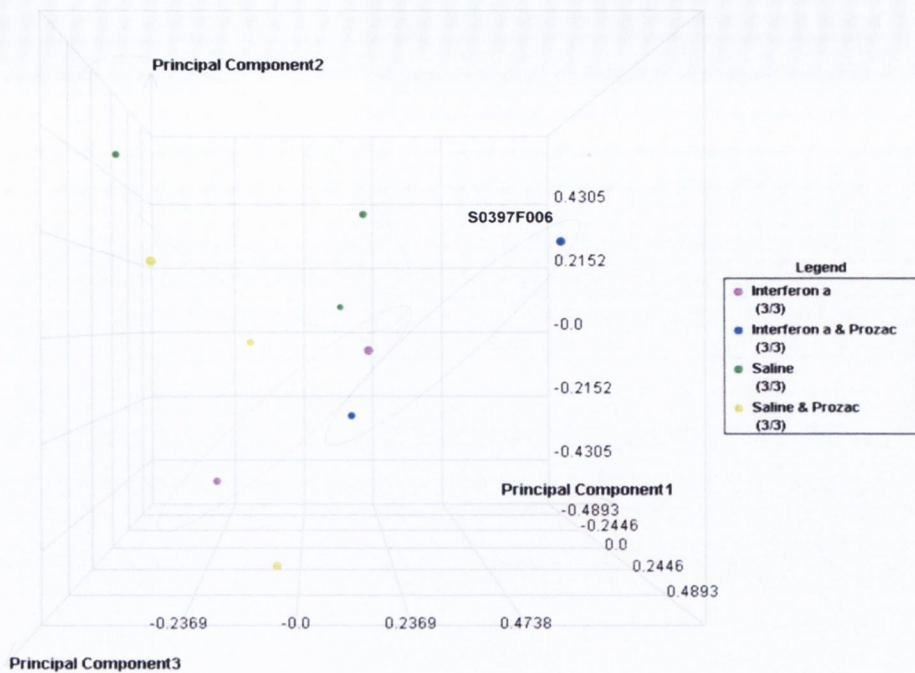


Fig.11. Principle components analysis showing all the samples from the four treatment groups from the 12 chips

The removal of the outliers does not have significant impact on the spread of the data in the principal components analysis, although it does aid to reframe the data.

3.2.2. Differential Gene Expression

Union List

Almac produced a union list of all differentially expressed genes compared to saline-treated animals. The number of genes and overlapping common expression are demonstrated in the Venn diagram in figure 12 below. Only 19 genes are common to all groups; however the group with the most distinctly expressed genes is the IFNFlx group. This would be expected as this experimental group is further removed than the other treatment groups.

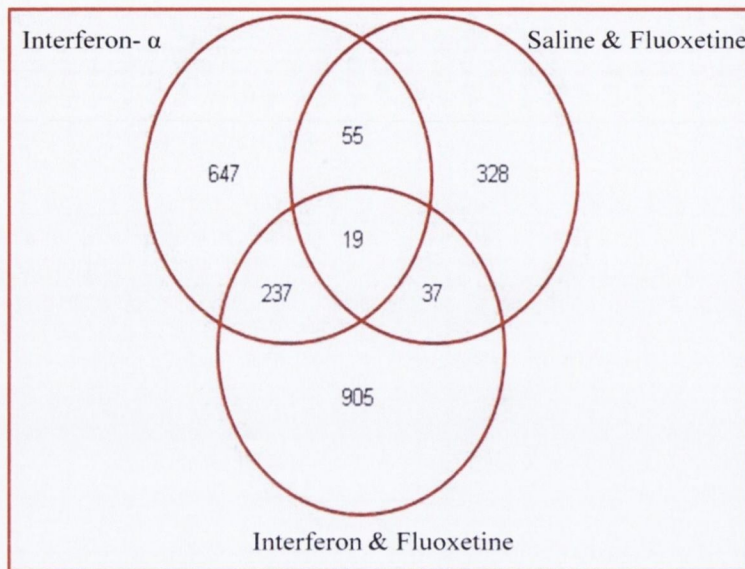


Fig.12. Venn diagram demonstrating the pattern of spread of the differentially expressed genes.

Table 2 below demonstrates the total number of genes expressed segregated into those that have known function and those that remain unknown. Only the known genes were investigated further.

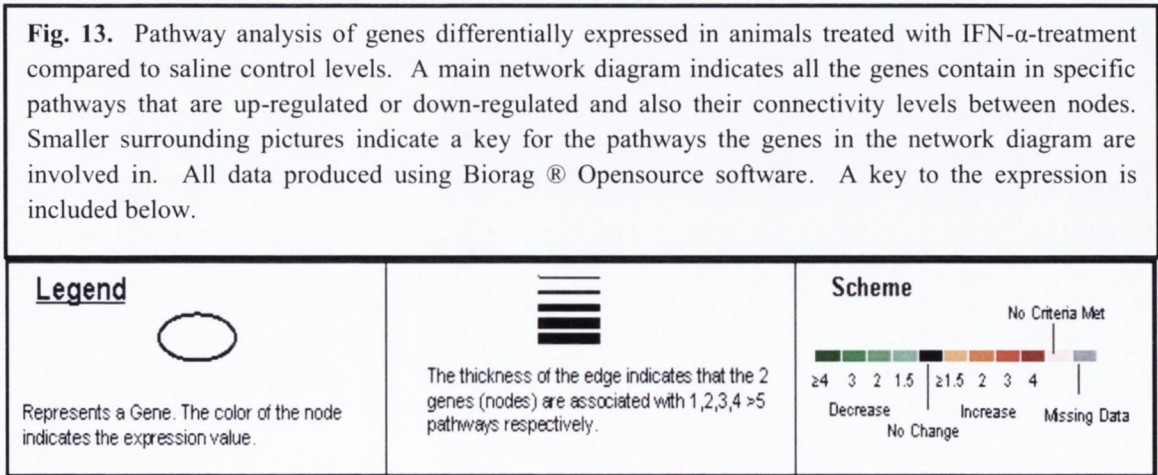
Sal versus IFN

Figure 13 reveals some genes that are involved in pathways that are differentially expressed in IFN- α -treated animals when compared to baseline levels in saline treated animals. Up-regulation (Redwine et al. 2003) and down-regulation (green) of genes are coded with varying gradients of colour to denote the fold change. Individual pathway diagrams have been included as a key to the involvement of genes in specific pathways. It is apparent that many pathways have altered gene expression; some of the genes are expressed in multiple pathways.

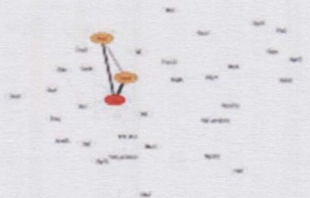
Table.2. Number of genes differentially expressed in different combination of comparisons.

Treatment Comparisons	No. genes expressed	Known Genes	Unknown genes
Sal Vs IFN	647	360	287
Sal Vs SalFlx	328	188	140
Sal Vs IFNFlx	905	530	385
Sal Vs IFN Vs IFNFlx	237	140	97
Sal Vs Salflx and IFNFlx	37	15	22
Common in all groups	19	10	9
IFN Vs IFNFlx	1296	772	524

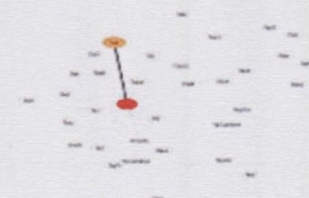
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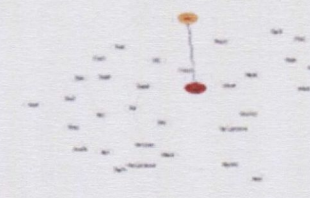
Apoptosis



Toll like Receptor



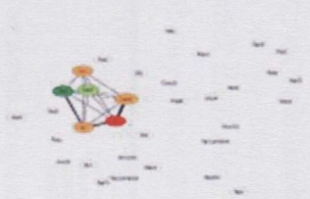
Actin Cytoskeleton



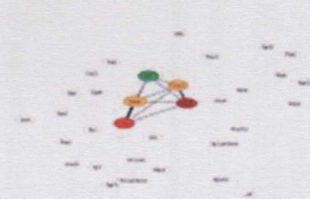
Calcium Signalling



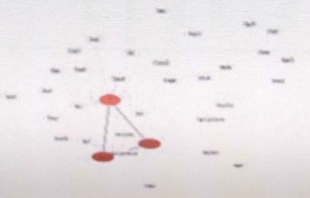
Cytokine-Cytokine Interaction



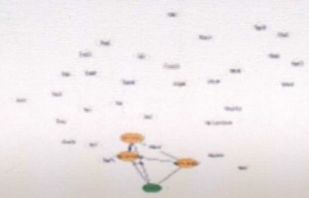
MAPK Pathway



T-Cell Signalling



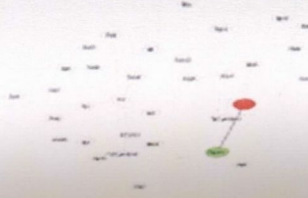
Cell Adhesion Molecules



Wnt Signalling



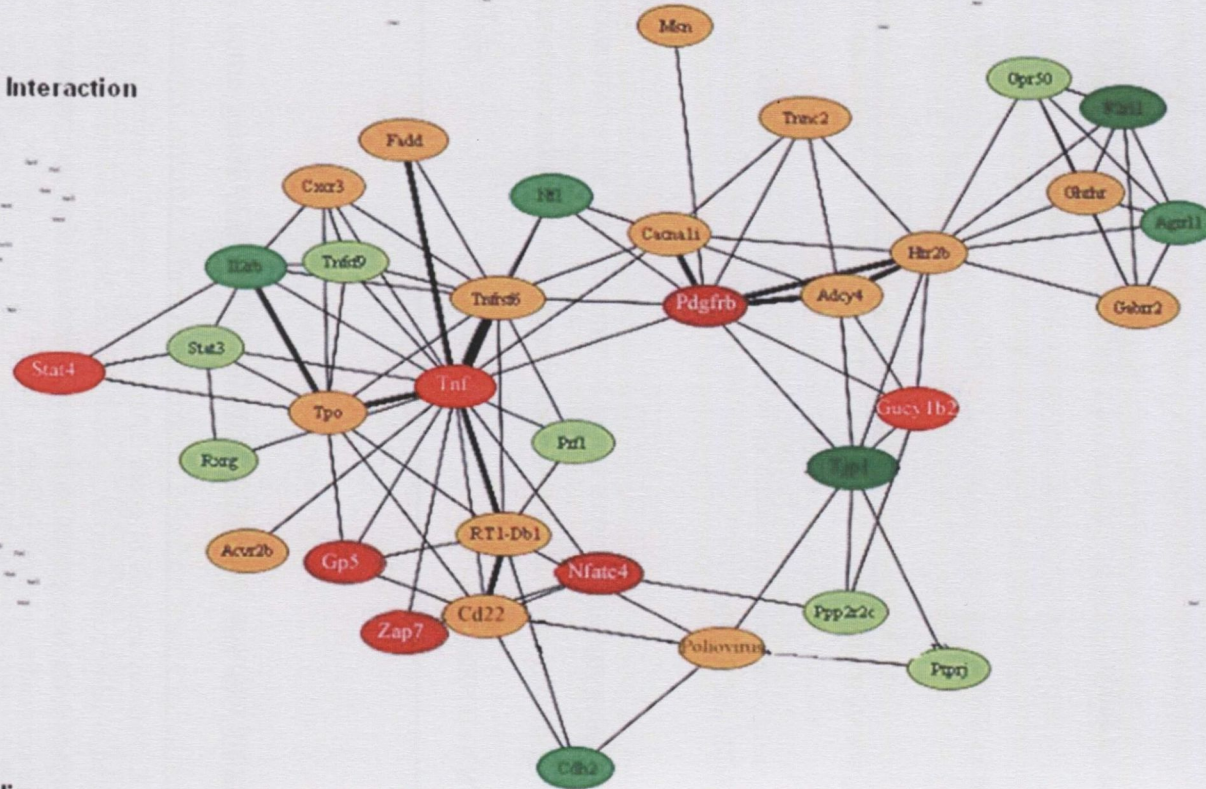
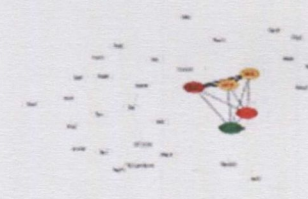
LTD



Neuroactive Ligands



Gap Junction



Pathway analyses were completed for IFNFlx versus IFN, a vast number of genes were found to be differentially expressed in many different pathways. A network diagram of the volume of genes in specific pathways is included in the figure below. Due to the complexity of this network the specific individual pathway diagrams are included in appendix 4.

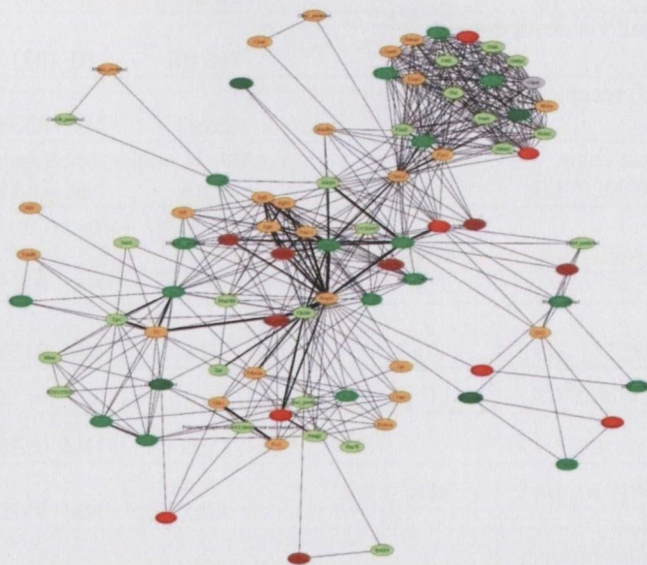


Fig.14. A network diagram demonstrating the volume of genes differentially expressed in the IFNFlx treatment group compared to IFN treated animals. Nodes highlighted in red denote up-regulated genes and those in green are down-regulated genes. All data produced using Biorag ® Open source software.

The network diagram above reveals a large cluster of genes that are altered in close proximity and with rich connectivity between them. This cluster of nodes represents the differential expression of neuroactive ligand-receptor interactions.

The expressions of genes were compared between the genes differentially expressed in both pathway network analyses. Additionally some extra sifting of interesting genes was also completed using prior knowledge of pathways and genes that may be involved in the activity of IFN- α and the recovery of behavioural deficits with SSRI antidepressant treatment. A summary table is included below displaying the most interesting genes (table 3). A more comprehensive table is included in appendix 4.

Table.3. Differential gene expression in the treatment groups. A key is provided with figure 13.

Pathway Name	Gene Name	Accession	IFN Vs Sal	IFN Vs IFNFlx	Sal Vs SalFlx
Cytokine-cytokine receptor interaction					
Thyroid Peroxidase	Tpo	NM_019353	Orange	Green	White
tumor necrosis factor (TNF superfamily, member 2)	Tnf	AA819227	Red	White	White
tumor necrosis factor (ligand) superfamily, member 9	Tnfsf9	BF394106	Green	Orange	White
Fas (TNFRSF6)-associated via death domain	Tnfrsf6	BE108106	Orange	White	White
chemokine (C-X-C motif) receptor 3	Cxcr3	NM_053415	Orange	White	White
interleukin 2 receptor, beta chain	Il2rb	NM_013195	Green	White	White
interleukin 3	Il3	NM_031513	White	Orange	White
interleukin 1 alpha	Il1a	NM_017019	White	Green	White
chemokine (C-C motif) receptor 3	Ccr3	NM_053958	White	Green	White
growth hormone receptor	Ghr	NM_017094	White	White	Green
Neuroactive ligand-receptor interaction					
5-hydroxytryptamine (serotonin) receptor 1A	Htr1a	NM_012585	White	Orange	White
5-hydroxytryptamine (serotonin) receptor 2B	Htr2b	NM_017250	Orange	White	White
5-hydroxytryptamine (serotonin) receptor 2C	Htr2c		White	White	White
growth hormone releasing hormone receptor	Ghrhr	NM_012850	Orange	White	Green
growth hormone releasing hormone	Ghrh	NM_031577	White	Green	White
growth hormone receptor	Ghr	NM_017094	White	White	Green
gamma-aminobutyric acid (GABA-C) receptor, subunit rho 2	Gabbr2	NM_017292	Orange	Green	White
gamma-aminobutyric acid (GABA) B receptor 1	Gabbr1	NM_031028	White	Green	White
gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 3	Gabra3	NM_017069	White	Orange	White
gamma-aminobutyric acid (GABA-A) receptor, subunit delta	Gabrd	AI228492	White	Red	Orange
dopamine receptor D3	Drd3	NM_017140	White	Green	White
Galanin	Gal	NM_033237	White	Green	White

opioid receptor, delta 1	Oprd1	NM_012617		
cholinergic receptor, muscarinic 2, cardiac	Chrm2	NM_031016		
MAPK signaling pathway				
tumor necrosis factor (TNF superfamily, member 2)	Tnf	AA819227		
Fas (TNFRSF6)-associated via death domain	Tnfrsf6	BE108106		
neurofibromatosis 1	Nf1	NM_012609		
calcium channel, voltage-dependent, alpha 1I subunit	Cacna1i	NM_020084		
platelet derived growth factor receptor, beta polypeptide	Pdgfrb	AI071374		
fibroblast growth factor 8	Fgf8	NM_133286		
fibroblast growth factor 12	Fgf12	AW252096		
fibroblast growth factor 18	Fgf18	NM_019199		
fibroblast growth factor 6	Fgf6	NM_131908		
mitogen activated protein kinase kinase kinase kinase 1	Map4k1_predicted	AI237423		
mitogen activated protein kinase 1	Mapk1	NM_053842		
mitogen-activated protein kinase kinase kinase 8	Map3k8	NM_053847		
interleukin 1 alpha	Il1a	NM_017019		
guanine nucleotide binding protein, alpha 12	Gna12	NM_031034		
Calcium signaling pathway				
5-hydroxytryptamine (serotonin) receptor 2B	Htr2b	NM_017250		
calcium channel, voltage-dependent, alpha 1I subunit	Cacna1i	NM_020084		
platelet derived growth factor receptor, beta polypeptide	Pdgfrb	AI071374		
adenylate cyclase 4	Adcy4	NM_019285		

troponin C2, fast	Tnnc2	BG663128	Orange	
phospholipase C, beta 2	Plcb2	NM_053478		Green
	P2rx11	NM_012721		Green
cholinergic receptor, nicotinic, alpha polypeptide 7	Chrna7	NM_012832		Red
purinergic receptor P2X, ligand-gated ion channel, 7	P2rx7	NM_019256	Orange	
prostaglandin E receptor 3 (subtype EP3)	Ptger3	NM_012704		Green
calcium/calmodulin-dependent protein kinase II gamma	Camk2g	BF390092		Red
ATPase, Ca ⁺⁺ transporting, plasma membrane 2	Atp2b2	NM_012508	Orange	
cholinergic receptor, muscarinic 2, cardiac	Chrm2	NM_031016	Orange	
Protein phosphatase 3, catalytic subunit, alpha isoform	Ppp3ca	A1146158		Green
Jak-STAT signaling pathway				
thyroid peroxidase	Tpo	NM_019353	Orange	Green
interleukin 2 receptor, beta chain	Il2rb	NM_013195	Green	
signal transducer and activator of transcription 3	Stat3	BE113920	Green	
signal transducer and activator of transcription 4	Stat4	AA955354	Red	Green
	Il3	NM_031513	Orange	
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053481		Green
growth hormone receptor	Ghr	NM_017094		Green
Apoptosis				
tumor necrosis factor (TNF superfamily, member 2)	Tnf	AA819227	Red	
Fas (TNFRSF6)-associated via death domain	Fadd	BE100715	Orange	
Fas (TNFRSF6)-associated via death domain	Tnfrsf6	BE108106	Orange	
protein kinase, cAMP dependent regulatory, type II alpha	Prkar2a	AW919085		Orange
interleukin 3	Il3	NM_031513	Orange	

phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053481			
interleukin 1 alpha	Il1a	NM_017019			
Protein phosphatase 3, catalytic subunit, alpha isoform	Ppp3ca	AI146158			
T cell receptor signaling pathway					
tumor necrosis factor (TNF superfamily, member 2)	Tnf	AA819227			
zeta-chain (TCR) associated protein kinase	Zap70	BM387946			
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	Nfatc4	AI175320			
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053481			
	Lat	NM_030853			
mitogen-activated protein kinase kinase kinase 8	Map3k8	NM_053847			
Protein phosphatase 3, catalytic subunit, alpha isoform	Ppp3ca	AI146158			
CD3 molecule, gamma polypeptide	Cd3g	AI044631			
Regulation of actin cytoskeleton					
platelet derived growth factor receptor, beta polypeptide	Pdgfrb	AI071374			
Moesin	Msn	NM_030863			
Rho guanine nucleotide exchange factor (GEF) 12	Arhgef12	BI303053			
fibroblast growth factor 8	Fgf8	NM_133286			
fibroblast growth factor 12	Fgf12	AW252096			
fibroblast growth factor 18	Fgf18	NM_019199			
fibroblast growth factor 6	Fgf6	NM_131908			
mitogen activated protein kinase 1	Mapk1	NM_053842			
IQ motif containing GTPase activating protein 3	Iqgap3	AI071398			
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053481			
	LOC316457	BE098860			
cholinergic receptor, muscarinic 2, cardiac	Chrm2	NM_031016			

guanine nucleotide binding protein, alpha 12	Gna12	NM_031034			
Toll-like receptor signaling pathway					
tumor necrosis factor (TNF superfamily, member 2)	Tnf	AA819227			
Fas (TNFRSF6)-associated via death domain	Fadd	BE100715			
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053481			
Long-term potentiation					
phospholipase C, beta 2	Plcb2	NM_053478			
calcium/calmodulin-dependent protein kinase II gamma	Camk2g	BF390092			
mitogen activated protein kinase 1	Mapk1	NM_053842			
Protein phosphatase 3, catalytic subunit, alpha isoform	Ppp3ca	A1146158			
Long-term depression					
guanylate cyclase 1, soluble, beta 2	Gucylb2	NM_012770			
Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), gamma isoform	Ppp2r2c	BF414575			
phospholipase C, beta 2	Plcb2	NM_053478			
mitogen activated protein kinase 1	Mapk1	NM_053842			
guanine nucleotide binding protein, alpha 12	Gna12	NM_031034			
phospholipase A2, group V	Pla2g5	NM_017174			
VEGF signaling pathway					
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	Nfatc4	A1175320			
mitogen activated protein kinase 1	Mapk1	NM_053842			
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053481			
Protein phosphatase 3, catalytic subunit, alpha isoform	Ppp3ca	A1146158			
phospholipase A2, group V	Pla2g5	NM_017174			

4. Discussion

Some of the key interesting gene expression changes will be discussed here. A more in depth analysis may reveal further interesting markers.

Immune Response

Cytokine-cytokine Interactions and Apoptosis

Several Interferon stimulated genes (ISGs) were revealed. Tumour necrosis factor (Tnf) is raised in IFN- α -rats. This would be expected in the immune activation that occurs with IFN- α administration. Tnf has many properties in immune reactions and inflammation and may induce apoptosis (Van Antwerp, Martin, Verma, & Green, 1998).. It also has other properties in the reduction of tumorigenesis and viral replication, which may be where its role lies here. A subtype of Tnf, Tnfrsf6 (Fas) is also up-regulated, this subtype is specifically associated with apoptosis and cell death. Additionally a downstream effector of Fas, Fadd is also up- regulated and also involved in apoptosis. Another member of the family, Tnfsf9 (member 9) is down-regulated in IFN- α however, this is recovered by combined Flx treatment. Additional ISGs such as the chemokine motif receptor 3 (Cxcr3) is upregulated, Apolinario et al (2007), noted increased expression of Cxcr3 in antiviral treatment which induces T-cell signalling in chronic hepatitis C.

T cell signalling

From table 3 it can be seen that several t-cell signalling molecules are up-regulated with IFN α , such as Tnf, Zap70 and Nfatc4. The up-regulation of T-cells is expected given the role IFN α has in immune reactivity. These T-cells may contribute to the initiation of adaptive immunity. Consequently, IFNFlx treatment results in the down regulation of T-cell signalling, suggesting Fluoxetine may aid in fighting or dampening down the immune response.

Interleukin 2 receptor, (beta chain) (Il2rb) is down-regulated in IFN α ; this is again involved in T cell signalling but also indicated in Jak stat pathway. Barnes et al (2009) found there is a decrease in IL2 in patients taking IFN- α that can return after the cessation of treatment (Barnes et al., 2009). IL-2 is sometimes given as a combination therapy for cancer patients to boost the immune response.

Treatment with IFN α alone results in an increase in Thyroid Peroxidase (Tpo). Tpo is key enzyme in the balance of thyroid hormone production. A decrease in Tpo expression would lead to decreased iodination of thyroglobin resulting in a decrease in thyroid hormone production (hypothyroidism). However, alternatively, an increase could lead to hyperthyroidism, both hypo and hyperthyroidism have been demonstrated in patients treated with IFN α (Bini & Mehandru, 2004). A raised Tpo level in this case may stimulate hyperthyroidism symptoms, some of which overlap with IFN α symptoms such as irritability, and memory impairments. It is interesting to note that co-treatment with IFN β in this study brought the Tpo levels back down to Saline baseline levels. The functional significance of the rescue may reside on the alleviation of hyperthyroid-based irritability and fatigue.

Neuroactive ligand-receptor interaction

Serotonin receptors

5-HT_{2c} (Htr2c) is up-regulated in IFN α -animals and down-regulated in IFN β animals to baseline saline levels. A similar blockade/down-regulation of 5-HT_{2c} was found by Ni and Miledi (1997). This receptor is involved in general anxiety disorder antagonism and is the basis of mechanistic action for agomelatine, which aids to normalise sleep patterns. Down-regulation of this key receptor may provide insight into the axiogenic nature of adverse events in IFN- α -treated patients. Interestingly, 5-HT_{1a} (Htr1a) receptor is up-regulated in IFN β animals but remains unchanged in IFN and saline animals. Activation of these receptors inhibits serotonin release on a global scale. Newman et al (2000) found a hypersensitivity of these receptors with antidepressant treatment. Although there is some clarification required as there are contrary findings, another alternative explanation for the increase in Htr1a in IFN β animals may be an internalisation of the receptors. Riad et al (2004) found that with Fluoxetine treatment the autoreceptors internalise, which they suggest may account for their desensitisation with antidepressant treatment. However, it is plausible that the internalisation may result in an increased expression of Htr1a due to a sequestration of the receptors in the cytoplasm.

Additionally, Tyrosine Hydroxylase (Th) is increased in IFN β animals compared to IFN. This may be a mechanism of rescue of decreased dopamine levels in IFN- α -treated animals. In addition, DBH (dopamine- β -hydroxylase) gene expression is also

up-regulated, suggesting an increased conversion of dopamine to noradrenaline. Decreases in plasma DBH are associated with major depression (Mod et al., 1986), therefore this is also one of the rescue avenues for Fluoxetine treatment.

Kynurenine Pathway

An additional interesting gene, Kynureninase (KynU) is down-regulated in IFN- α -treated animals. Also Kynurenine 3-monooxygenase (Kmo) is increased in IFN- α -treated animals. These enzymes are important for the IDO conversion of Tryptophan to Kynurenine (Kyn) instead of 5-HT. Kmo is an enzyme that converts Kyn to 3-hydroxy-kynurenine (3OH-KYN). 3OH-KYN is a metabolite of Kyn, known to result in increased microglia activation and increased oxidative stress and apoptosis. KynU decrease, would result in less of the Kyn metabolising via the other pathways such as the kynurenic acid pathway and the anthranilic acid pathway to quinolinate. Both 3OH-KYN and quinolinate are metabolites that can induce neurodegenerative processes in the brain (Wichers & Maes, 2004). It is apparent here that activation of the Kyn pathway is via the 3OH-KYN track and not the other pathways to cellular destruction. Fluoxetine does not appear to modulate the Kyn pathway, this is consistent with studies demonstrating an alternative SSRI, paroxetine also does not modulate this pathway (Capuron, Hauser et al., 2002).. These results are in line with many studies now suggesting a causal link between decreased 5-HT availability and IDO conversion in IFN- α (Wichers, Koek, Robaey, Praamstra et al., 2005).

GABA

GABA-C (Gabrr2) receptor is up-regulated in IFN and normalised with co treatment with Flx. This gene is involved in conducting chloride ions across the cell membrane contributing to stabilising the resting membrane potential. GABA B receptor 1 (Gabbr1) is decreased with IFNFlx co-treatment is a g-protein coupled receptor linked to K channels, hyperpolarise the neuron and decrease Ca conductance. CGP36742 is an active GABAB and C antagonist that has been shown to enhance cognition (Getova, Bowery, & Spassov, 1997). GABA A receptor (Gabbrd) is increased in both IFNFlx and SalFlx. GABA A agonists such as benzodiazepines and barbiturates work via reducing anxiety and sedating chemical activity. Interestingly, the levels of GABA A were much lower in IFN- α treated animals compared to saline. Moreover, GABA-A (α subunit) is increased in IFNFlx animals compared to IFN.

MAPK

There are several up-regulated genes in this pathway with IFN- α treatment. Tnf and Tnfrsf6 are two of them which work on the negative side of the pathway stimulating apoptotic responses. Calcium channel voltage dependent α 1 subunit (Cacna1i), Platelet derived growth factor receptor (Pdgfrb) which is involved in cell differentiation. However, this is down-regulated with IFNFlx which normalises Pdgfrb to saline control levels. Down-regulation of Nf 1 is evident in IFN- α -treated animals, Nf 1 which is a negative regulator of the Ras signal transduction pathway. It has also been shown that there is an involvement of Nf1 in long term memory and mental retardation (Ho, Hannan, Guo, Hakker, & Zhong, 2007). Neurofibronin the gene product of Nf 1 is required for normal neuronal differentiation (Silva et al., 1997)

Fibroblast growth factors (FGF) have an increased tone in the (Turner, Akil, Watson, & Evans, 2006) and are up-regulated with fluoxetine treatment in IFN- α -treated animals. However they are not up-regulated in SalFlx treated animals. Fgf8, 12, 18 and 6 ligands are all up-regulated in IFNFlx. These genes are also vital for the actin cytoskeleton so they may indicate structural remodelling. MAPK4k1 and Map3k8, mapk15 are down-regulated in IFNFlx however Mapk 1 and Mapk4 are up-regulated. Mapk4 up-regulation brings the inhibitory action of IFN back to baseline levels.

Il-1a is decreased in IFNFlx groups; IL1a is linked to the stress response therefore indicating a mechanism of action of Flx (Goshen & Yirmiya, 2009) and may cross the BBB by induction of Cox 2 (cyclooxygenase)

There is an up-regulation of muscarinic, cholinergic receptor, (Chrm2) in the IFNFlx group, however there is a down-regulation in the Chrm5 in the IFN groups. Alterations in Chrm2 binding has been determined in the frontal lobes in bipolar and MDD depression (Gibbons, Scarr, McLean, Sundram, & Dean, 2008) However Shi J et al. (2007) found no link between 19 cholinergic genes and bipolar depression (Shi et al.).

Jak Stat Pathway

Signal transducer and activator of transcription (Stat3) is down-regulated in IFN- α -treated animals. Stat 3 has been demonstrated to be neuroprotective in nature and a pathway for the source of activity for transcription factors (Dziennis & Alkayed, 2008). Additionally, research suggests that Stat 3 is vital alongside CNTF for neurogenesis

induction (S. Muller, Chakrapani, Schwegler, Hofmann, & Kirsch, 2008). Interestingly, Chiba et al (2009) found that the pharmacological inhibition of the JAK2/STAT3 produced a significant loss in spatial working memory and this occurred by the down-regulation of the acetylcholine-producing enzyme choline acetyltransferase and also desensitized the M(1)-type muscarinic acetylcholine receptor (Chiba et al., 2009). Stat 1 is up-regulated in IFN and down-regulated in IFNFlx returning stat1 regulation to baseline levels. Wang determined the role of Stat 1 in IFN activity; Stat1 knock-out mice do not have the respond to IFN (Wang et al.,2004 ; Wang et al. 2008). It is also found that stat 1 is not present in non responders. This now begs the question, if fluoxetine returns Stat1 levels back down to saline-treated levels will this make IFN α -treatment less therapeutically effective? Further research needs to be conducted to address this issue.

Tight junctions

Interestingly, the expression of two key tight junction genes (Tight junction protein 1 (Tjp-1) and Protein phosphatase 2 (Ppp2r2c)) are down-regulated in IFN- α -treated animals. Tight junction genes are conversely up-regulated with Fluoxetine treatment. This may indicate a compromise of the BBB, tight junctions are vital for the endothelial support in the BBB. Reports have suggested loss of BBB integrity previously (Pavlovsky et al., 2005; Schaefer et al., 2004) however; these have not been consistent (de Boer & Gaillard, 2006).

The gene expression profiles of IFNFlx and SalFlx appear to be distinct and common pathways are minimal. The stressed background of the IFN- α -treated animals and activated immune system dictates that the action of Flx in IFN- α -treated would produce a more pronounced activation of genes.

Of the 19 genes that have shared pathways

From the 19 genes that were differentially regulated in all the groups compared to saline controls only 2 of them demonstrate differential regulation between the groups. All other genes respond to a similar fold change and therefore expression is likely a result of non specific drug effects. It is apparent that there is a large decrease in the expression of Mx2 in the IFNFlx treated animals (-33 fold). Mx2 is an interferon stimulated gene (ISG), it is surprising that in IFN animals there is a fold decrease in gene expression. However, more surprising is the remarkably large decrease in Mx2 in

IFN β , it may be of concern that the Mx2 levels are decreased so much, could this infer some immune activation decrease in Fluoxetine treated individuals? Could fluoxetine make patients more susceptible to infection?

It is evident from these expression changes that have been discussed above, that there are many interesting avenues to approach for future experiments. The large volume of information gained from this experiment raise many more questions that need to be addressed regarding IFN- α treatment and the potential for prophylactic treatment of adverse events.

It is important to note that IFN- α -treated animals and patients are not a homogenous population. There is a susceptibility to adverse events which most likely is due to the underlying genotype; however there are also other risk factors that contribute. The removal of outliers in this study will determine the extreme effect of IFN- α on adverse events. If a larger sample size had been used it may have been interesting to look at subpopulations of responders versus non responders. In clinical studies 40% of IFN- α -treated patients experience severe affective adverse events (Bonaccorso et al., 2001). This appears to be reflected in the animal model with some animals seemingly less cognitively and emotionally challenged by the treatment, resulting in a dissociation/variability in the treatment group. This has often led to a masking of the effects of the treatment in animal models with some groups reporting no adverse effect of IFN- α on cognition and affect (De La Garza et al., 2005). The assessment of specific subpopulations may highlight molecular functions governing the predisposition to adverse effects.

It is not surprising that with the utilisation of more microarrays used the larger the power of the experiment. The limit is usually set at 4/5 but if budget permits 'safety in numbers' 7/8 arrays per experimental group is desired. A limitation in this experiment is the small sample size (3 in each group and 2 in the IFN and IFN β group after the removal of outliers), precluding the possibility of performing inferential statistics on the expression changes and also reducing the clustering in the exploratory data analysis.

It is important to emphasise once again that although some genes of interest have been shown to be differentially expressed this is a basic analysis to probe for potential targets for more future analysis. Subsequent verification experiments and analyses would be required prior to further analysis. A disadvantage of studying microarray in

the neurosciences is the heterogeneous nature of the cell population and the physiological properties of the tissue (Reimers et al., 2005)

5. Conclusion

The genes that were differentially expressed in all the treatment groups were plentiful. A pathway analysis was completed here to determine what genes may be differentially expressed in specific pathways. The restorative benefit of fluoxetine highlights the recovery of function by modulating the MAPK pathway, tight junctions, calcium channel modulation, T-cell inhibition, and neuroactive ligands. Highlighted here are some pathways that may be useful for future investigation, although further work is required to decipher which pathways may be of most interest.

Chapter VII

Conclusions and future directions

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1. Introduction

This chapter will summarise the main findings of this thesis and unify the results to provide insight into the putative underlying mechanisms of IFN- α -induced mood and neurocognitive deficits. In addition, remaining questions will be discussed and future directions highlighted.

2. Chapter II – Prefrontal Deficits in IFN- α -treatment

This set of experiments extends the framework of the IFN- α animal model to include the assessment of prefrontal function. To date in our laboratory research has focussed on hippocampal deficits arising from this treatment (Fahey, 2005), here we explore prefrontal deficits. These data will be discussed in light of previous data acquired by Fahey, 2005. Mild behavioural stress of an acute nature and chronic, systemic, IFN- α -treatment, produce temporal order memory deficits. This is reflective of a dysfunction of the prefrontal cortex. Ameliorative strategies aid in the restoration of temporal order memory function.

In summary:

- The induction of mild behavioural stress of an acute nature results in a decrease in temporal order memory performance. Animals remain able to distinguish the order of object presentation; however performance is significantly modified by the induction of stress in this task.
- IFN- α -treatment results in a complete loss of temporal order memory proficiency, animals perform discriminations at chance level. Nevertheless there is a sparing of novel object recognition memory.
- Temporal order memory deficits in IFN- α -treated animals persist long after the cessation of treatment.
- The implementation of a chronic forced exercise routine restores temporal order memory performance in IFN- α -treated animals to the equivalent levels of saline controls.

These data present evidence of a prefrontal dysfunction in animals exposed to chronic mild stress and IFN- α -treatment. Impairment in prefrontal function in stressed animals

is in line with the stress literature suggesting physiological and morphological changes in the PFC (McEwen, 1998a; Radley et al., 2006).

There is a shortage of research examining prefrontal function in the context of IFN- α -treatment; this is surprising given the prevalence of adverse events. Many of the deficits mimic those of patients with prefrontal damage. Patients treated with IFN- α often exhibit loss of concentration and memory impairments both of which rely on the integrity of this structure (Schaefer et al., 2002). Additionally the PFC is central to the pathological basis of depression and anxiety (Drevets, 2000). These data demonstrate deficits in IFN- α -treated animals in a task that rely heavily on the integrity of the prefrontal cortex. However, it remains contentious whether the sole locus for temporal order memory resides in the PFC (Fortin, Agster, & Eichenbaum, 2002; Howland et al., 2008).

There are several lines of investigation in dissociating the neural basis of temporal order deficits in IFN- α -treated animals. It is unknown if the dysfunction in prefrontal behaviour is 1) secondary to impairments of the hippocampus (data shown by (Fahey, 2005)) which would result in weakening of afferent input to the PFC, 2) occurring as a result of dysregulation in the HC-PFC pathway itself or 3) a result of pathological or chemical imbalance in the PFC. The neural basis of temporal order memory has been discussed in chapter II and PFC contribution verified. Notably, Howland et al. (2008) demonstrates the importance of the ventral hippocampus in some spatial temporal order memory paradigms (Howland et al., 2008). This would support the notion that these deficits arise from impairment in the hippocampus or hippocampo-PFC pathway.

Previous data from our lab demonstrate IFN- α -treated animals exhibit several hippocampal-dependent deficits, such as: 1) a decreased reactivity to spatial change in the object displacement task; 2) a delayed acquisition of spatial learning in the Morris water maze and 3) a significant block of the induction of LTP in the perforant path (Fahey, 2005). Global dysfunction of the hippocampus would lead to a weakening of the pathway (HC-PFC) connecting the hippocampus to the prelimbic cortex of the PFC. However, it is important to note that the hippocampal contributions interpreted from the aforementioned studies (Fahey, 2005) suggest a deficit in the dorsal hippocampus. The data presented here suggest that a ventral hippocampal dysfunction may also occur.

This may arise due to a global deficit in hippocampal function or may be secondary to dorsal HC impairments.

It may be possible that a disruption in PFC function such as hypometabolism, which has been demonstrated in IFN- α -treated patients (Juengling et al., 2000b) would lead to a disconnection of the HC-PFC pathway. It is also plausible that deficits in the ventral hippocampus may be the route of this prefrontal hypometabolism. The significance of a hypometabolism in the prefrontal cortex could lead to widespread problems including an inhibition of dynamic filtering (Shimamura, 2000). It is well known that the PFC, sometimes termed the central executive, orchestrates activity in subcortical regions. Dynamic filtering is required in order to inhibit unwanted activity in these regions, therefore a dysfunction in activity or integrity of the PFC may result in a global dysregulation of several subcortical regions.

An additional study indicating impairments in the PFC in IFN- α -treated animals was demonstrated by Ishikawa et al (2007). They found an alteration in serotonergic and noradrenergic axons in the rat brain with IFN- α -treatment. Degeneration of serotonergic axons was found in the ventral medial PFC and amygdala. Additionally, decreases in the density of noradrenergic axons were found in the dorsal medial PFC, ventral medial PFC and the dentate gyrus (Ishikawa et al., 2007). This suggests a role for the PFC and monoaminergic axons in the pathophysiology of depression associated with IFN- α . The behavioural results included herein complement Ishikawa's findings and it may be speculated that the deficits in behavioural performance arise due to a similar molecular basis. However, IFN- α -treatment in patients results in a constellation of adverse events that most likely arise from a dysregulation of a distributed network of structures.

We have collectively demonstrated in our laboratory that IFN- α -animals are deficient in both hippocampal-dependent tasks and prefrontal –dependent tasks suggesting that both these structures are damaged in some respect. It is unknown however, how these deficits arise? Does hippocampal damage precede prefrontal damage? or *vice versa*, or do the deficits arise from a dysregulation in the HC-PFC pathway, resulting in a disconnection of crosstalk between these two regions, which may in turn lead to specific atrophy of function? Additionally, is there a selectivity of dissociation in dysfunction in the septo-temporal axis of the hippocampus?

Many questions are formulated from this set of experiments that warrant further investigation. To address the nature of dysfunction it may be essential to assess plasticity in the HC-PFC pathway in IFN- α -treated animals. Jay et al 2004 demonstrated that there is a block of LTP in this pathway with the induction of stress (Jay et al. 2004). Stress may result in similar physiological responses and activate circulating cytokines similar to IFN- α -treated animals (A. H. Miller et al., 2009). Additionally IFN- α is structurally and functionally related to ACTH (Blalock and Smith 1980) and therefore may activate the HPA axis directly, although contradictory information surrounds this claim. The HC-PFC pathway has been implicated in the pathophysiology of neuropsychiatric disorders (Jay et al., 2004). Dopamine is vital for the functioning of this pathway, it is interesting to note, alterations of dopamine levels have been found in patients treated with IFN- α (Capuron & Miller, 2004). Therefore one could hypothesise that a similar block in LTP in the HC-PFC fibres may occur in IFN- α -treated animals leading to a limbic-cortical disconnection.

The impairment of IFN- α -animals in temporal order memory persists after the cessation of treatment. This suggests long term structural changes in either anatomical regions or persistent chemical imbalances in the molecular milieu. Deficits are evident 10 weeks after the cessation of IFN- α -treatment, this is supported by the persistence of cognitive deficits documented post-treatment in several clinical studies (Reichenberg, Gorman, & Dieterich, 2005). Interestingly, exercise was successful in recovering IFN- α -induced prefrontal impairments in this task. Enhancement of hippocampal function with exercise is well established (O'Callaghan et al., 2007; van Praag, 2008). The molecular basis behind this restorative benefit may reside in a neurotrophic rescue, or may be a result of the activation of neurogenesis. Neurogenesis in the dentate gyrus would provide a basis for plasticity related structural modification. Further studies addressing the impact of exercise on LTP induction in IFN- α treated animals would be beneficial. The obvious question arises; could exercise reverse the block in LTP observed in IFN- α treated animals?

Of note, the temporal order task demonstrates a paradigm which is easy to implement and may be used alongside affective tasks to identify frontal deficits in many disease states. Moreover, verification of a prefrontal deficit using this paradigm may be used as a tool for initial assessment of pharmacological agents which target the PFC circuitry. In light of the results gained from this series of experiments, translation to the clinic

may result in the implementation of exercise regimes in patients as a first approach for the prophylactic treatment of IFN- α deficits. This would hopefully aid in reducing the incidence of mood and memory dysfunction in this clinical population.

These data reflect the first time to our knowledge that prefrontal function has been examined in IFN- α -treated animals. Additionally, this is also the first time deficits in temporal order memory function have been addressed using a pharmacological intervention.

3. Chapter III – Impairment in Behavioural Flexibility

This experiment builds on the last chapter to assess another prefrontal function in order to qualify the previous findings that the integrity of the PFC is challenged. Additionally, clarification on the potential site of dysfunction in the PFC was sought using a task that relies on different subdivisions of the PFC. This clarification of the subregional localisation of behavioural impairment provides support for dysregulation in the HC-PFC pathway. IFN- α induced significant deficits in the acquisition of the variable goal direction task, specifically resulting in the presentation of perseverative behaviour thereby indicating an underlying impairment in behavioural flexibility.

In summary:

- IFN- α -treated animals took longer to complete a water plus-maze task that involves goal-shifting behaviours.
- IFN- α -treated animals demonstrate a delayed acquisition in the variable start session
- More perseverative errors were made by IFN- α -treated animals than their saline-treated counterparts in the initial goal switch session. This is likely attributable to an impairment of behavioural flexibility
- A mild correlation exists between learning deficits in the water plus-maze with anxiogenic traits exhibited on the EPM. This suggests co-morbidity in mood and neurocognitive symptoms.

These data support findings from the previous chapter that the PFC is compromised in IFN- α -treated animals. IFN- α animals demonstrate significant impairments in elements of the water plus-maze task. The impairments were specifically based on successfully finding the goal location when a variable start contingency was used. In a similar vein,

Fahey (2005) also demonstrated IFN- α -induced impairments in a spatial memory task (Morris water-maze) suggesting deficits in hippocampal representations of spatial information.

The deficit documented in these data resulted in an overtraining in the goal location to reach criterion therefore habit-forming behaviour was most likely introduced. With the imposition of a goal switch the IFN- α animals performed more perseverative errors to the old goal location. This perseverative behaviour is attributable to impairments in behavioural flexibility. The characteristics of this impairment reveal that IFN- α animals demonstrate reward devaluation, animals persist in visiting the old goal location, even though this behaviour is futile. Goal devaluation is suggestive of impairment in the prelimbic region of the ventral PFC (Killcross & Coutureau, 2003). The infralimbic PFC is spared considering habit formation is evident and the integrity of this region is unaffected by the valence of the reward.

This experiment aids to untangle the localisation of deficits arising from IFN- α . The contributions of the prelimbic and infralimbic subregions of the PFC have been investigated. It is evident that the infralimbic cortex is spared however we speculate that the prelimbic cortex is the site of dysfunction. Supporting evidence for a dysfunction in this region is three-fold in the context of these experiments; 1) connectivity of the ventral hippocampus with the PFC through the HC-PFC predominantly projects to the prelimbic cortex. 2) The prelimbic region is the site of lesion in several studies that suggests PFC involvement in temporal order sequencing. 3) Previous studies indicate a decrease in the density of noradrenergic and serotonergic axons in the ventral PFC, suggesting a molecular basis behind the deficit (Ishikawa et al., 2007). The three points here unify a plausible site of PFC dysfunction residing in the prelimbic cortex in IFN- α -treated animals.

It is possible however, that the orbitofrontal cortex (OFC) contributes to this task and may be impaired in IFN- α -treated animals. The orbitofrontal cortex is also vital for reward devaluation, however it is demonstrated with OFC lesions reversal learning is impaired (L. Clark, Cools, & Robbins, 2004), and in this experiment reversal learning remains intact. In light of this notion, it is unlikely that the OFC is impaired however there is a requirement for further studies to assess the specific involvement of this subregion.

An expansion of this task, utilising an 8 arm radial water-maze may reveal more subtle underlying features of IFN- α -treatment. This would allow more goal switches and determine a more sensitive measure of motor (egocentric) behaviour.

The water plus-maze paradigm is sensitive to both hippocampal- and prefrontal-dependent functions. This instils beneficial properties in this task for future use in drug screening applications.

4. Chapter IV – Rescue of Mood deficits with an SSRI in IFN- α -treated animals

This series of experiments aimed to build upon work previously conducted in our research group and assess the impact of SSRI antidepressant treatment on affective behaviour in IFN- α -treated animals. Additionally, the molecular mechanism underlying anxiogenic and depressive side effects of IFN- α were investigated in the context of neurogenesis.

In summary:

- IFN- α -treated animals displayed anxiogenic and depressive phenotypes on the EPM and FST respectively. Behaviour on these tasks was restored with co-administration of the SSRI, Fluoxetine.
- Correlations between the affective behaviours exist demonstrating that anxiety and depression are co-morbid in IFN- α -treatment.
- A decrease in cell proliferation in the subgranular zone in IFN- α animals was determined; this is suggestive of a decrease in neurogenesis. Cell proliferation was restored with SSRI treatment.
- Correlations are evident between cell proliferation and affective performance.

The recovery of anxiogenic and depressive side effects in this task reflect the potential for co-treatment with SSRIs for ameliorating neuropsychiatric adverse events in the clinic. It has previously been documented that the SSRI paroxetine may be of restorative benefit on these adverse events associated with IFN- α (Musselman et al., 2001). Additionally, other antidepressants have been demonstrated to be beneficial in the clinic (Schaefer et al., 2002). However, the mechanism behind recovery using SSRIs in this patient population remains ill-defined. Here, we demonstrate that a

contributing factor to IFN- α induced adverse events may originate in a decrease in neurogenesis. The results presented here reveal a decrease in cell proliferation in the subgranular zone, hilar region and molecular layer of the dentate gyrus. These decreases in cell proliferation and in particular those in the subgranular zone are indicative of a decrease in neurogenesis in the hippocampus. Fluorescent immunohistochemistry is required in order to determine the neuronal phenotype of these cells since the cells may be endothelial or glial in nature. However, It is important to note that glial and endothelial proliferation would also be supportive to the neuronal environment potentially increasing blood supply (endothelial) and aiding in trophic support and toxic scavenging of the neurons (glia) (Giaume, Kirchhoff, Matute, Reichenbach, & Verkhratsky, 2007; H. Song, C. F. Stevens, & F. H. Gage, 2002). Further confirmation is required to determine the fate of the cells in this experiment, this work is ongoing. Hypothesising that increased cell proliferation may indicate increased neurogenesis or neuronal support would suggest that the attenuated cell proliferation in the IFN- α -treated animals may contribute to the presentation of IFN- α -induced adverse events. The link between mood disorders, memory and neurogenesis is well established (Duman, 2005; Henn & Vollmayr, 2004). The neurogenic hypothesis of depression is a leading explanation of molecular events underlying depression (Thomas & Peterson, 2003).

Only one paper thus far has suggested the potential link between neurogenesis and IFN- α -induced deficits, however an acute dose of IFN- α was adopted in this experiment and administration was not therapeutically relevant (i.c.v). Here we provide a clinically relevant mid-high dose IFN- α -treatment over four weeks to reflect the time that is required to produce neuropsychiatric adverse events in an animal model of IFN- α treatment (Fahey et al., 2007). Although verification is required to determine the specific fate of the proliferating cells in this study, several lines of theory suggest that neurogenesis would be a plausible mechanism underlying the adverse events. The antiproliferative nature of IFN- α stems from its angiogenic property; this would suggest that a decrease in either endothelial or neuronal cell proliferation is likely in IFN- α -treatment. The delayed onset of cognitive adverse events, typically 3 weeks in IFN- α -treated animals is also indicative of a potential for neurogenic involvement since this coincides with the lifecycle of neuronal integration. Neuronal proliferation and integration into the network has been revealed to require a three week process

(Kempermann et al., 2004). IFN- α induced Neurovegetative symptoms are demonstrated soon after the initial receipt of treatment however, underlying mechanisms leading to the cognitive effects take longer to arise, therefore this reveals a mechanism behind the delay (3 week neurogenic time window) in presentation of neurocognitive symptomology. Interestingly, the Neurovegetative symptoms do not respond to antidepressant treatment (Capuron & Miller, 2004), this further indicates an alternative mechanism for symptom origin in this syndrome.

A correlation between cell proliferation and depressive side effects (as evidenced in the FST) is provided here, although correlation does not assume causation, this is suggestive of a mechanism underlying the adverse events. Additionally, in this study we have demonstrated that co-treatment with the SSRI Fluoxetine not only restores mood related adverse events but also aids in increasing cell proliferation. The potential for Fluoxetine to stimulate cell proliferation is well documented (Malberg, 2004; Santarelli et al., 2003). However, this is the first time, to our knowledge that a recovery of IFN- α -induced adverse events has been demonstrated alongside an increase in cell proliferation, suggestive of neurogenesis. Provided here is a plasticity-related mechanism for the recovery of IFN- α -induced adverse events. A decrease in cell proliferation may also explain the arrest in LTP that has been demonstrated in our lab (Fahey, 2005). Interestingly, LTP induction was successfully recovered with the co-treatment of the SSRI fluoxetine (Fahey, 2005). The mechanism behind this recovery of LTP function with an SSRI may stem from an increase in neurogenesis or glial and endothelial support. New born neuronal cells have been demonstrated to integrate into the neuronal network and exhibit electrophysiological activity (Snyder et al., 2001). An increase in cell population, or support from glial and endothelial recruitment may in turn increase the electrophysiological basis of LTP.

5. Chapter V – Hippocampal decrease in IFN- α treated animals

This chapter assessed MRI parameters (T1 and T2) as indirect measurements of physiological correlates of neuronal tissue modification. In addition, volumetric analysis was utilised to assess structural changes in the hippocampus with chronic IFN- α -treatment.

In summary:

- No differences were found in the saturation time of T1 or the relaxation time of T2 with IFN- α -treatment.
- A significant decrease in Hippocampal volume (7-8%) was observed with chronic IFN- α -treatment

There were no significant differences found in the T1 and T2 relaxation times. T1 and T2 times are very sensitive measures which are usually picked up with large pathological changes such as plaques or severe loss of tissue integrity (Duncan et al., 1996). To date, most of the T1 and T2 contrast changes have been documented in hippocampal sclerosis and aging (Woermann et al., 1998). We can deduce from this analysis that there is most likely no change in the gross integrity of the tissue of the hippocampus and cortex in terms of severe changes in density.

5. The data presented here demonstrate a significant decrease in hippocampal volume with IFN- α -treatment. The remarkable decrease in hippocampal volume in rodents treated with IFN- α is in line with volume changes that occur as a consequence of 4 weeks of chronic mild stress (Sapolsky, 2000). This is the first time known to the author that hippocampal volume has been assessed in IFN- α -treatment. These data reflect the stress and depression based decreases that have been demonstrated in both the animal (McEwen, 1998b) and human literature (Sheline et al., 1996).

A decrease in hippocampal volume could not only be suggestive of impaired mnemonic functions, but also explain mood related adverse events. A decrease in volume may result in deleterious effects in other anatomical subregions such as the prelimbic cortex, which receives a direct projection from the hippocampus (Jay et al., 1995).

Future directions in the context of this experiment would be to unravel the underlying reason for the significant decrease in volume. The mechanism for this significant decrease remains unknown however several reasons may be speculated. A decrease in volume may result from structural reorganisation (decrease in dendrite arborisation), may be a result of general cell shrinkage or programmed cell death (apoptosis), or a result of decreased hippocampal neurogenesis or gliogenesis. Clarification of the

basis of this substantial decrease in hippocampal volume need to be addressed, strategies are outlined below.

Assessment of dendrite arborisation and dendritic spine retraction would be beneficial in determining if the decrease in volume was a result of structural remodelling. This would fit in alongside the plasticity related change found in chapter IV (decreased cell proliferation in animals treated with IFN- α) and those previously found by Fahey (2005) in our lab demonstrating a block in LTP in IFN- α -treated animals (Fahey, 2005). The impact of apoptosis in the hippocampus could be addressed using TUNEL staining and may be a future avenue to explore. IFN- α activates TNF- α , therefore it is a plausible contributor to the stimulation of apoptosis and consequent decrease in volume. A decrease in cell proliferation in the subgranular layer has been determined in chapter IV. It is unknown if this is neuronal, glial or endothelial based, however a decrease in the proliferation of any of these cell could contribute to an overall decrease in volume. However it is disputed that a decrease in neurogenesis can be the sole cause for a decrease in hippocampal volume (McEwen, 1998a).

Another avenue that would hopefully be addressed using MRI imaging of IFN- α -treated animals would be to assess BBB integrity. Scans were performed using gadopentate, contrast agent that is used to assess BBB breaches. There are conflicting reports documenting both increases in the permeability and decreases in the permeability of the BBB in IFN- α -treated animals (de Boer & Gaillard, 2006; R. Milner & Campbell, 2006). To clarify the mechanism of action of IFN- α in the brain it is vital to address this question.

An additional experiment would look at co-treatment of IFN- α with SSRI (Fluoxetine) to determine if there is a rescue of volume change in the hippocampus. A restoration in hippocampal volume with Fluoxetine co-treatment would provide some insight into the suggestion that the mechanism behind restoration of IFN- α -induced adverse events stems from a global rescue of hippocampal integrity.

It is of note that classical (manual) volumetric analysis was conducted in this experiment; a future direction may be to assess global changes in the brain of IFN- α -treated animals using VBM (Voxel-Based-Morphometry). Manual volumetrics is sufficient when assessing volume changes in brain regions that are well defined such as the hippocampus or corpus callosum but limit the analysis of less well defined regions.

Although assessment of the PFC are documented using manual volumetric techniques (Cerqueira et al., 2005) it may result in misleading results. VBM is a high throughput method of analysing brain structure and would reveal differences in brain volume/integrity in regions that would be otherwise overlooked using the classical volumetric analysis. It would be of interest to determine if there are any substantial prefrontal decreases in the IFN- α animals. This would add weight to the theory of HC-PFC theory and determine if hypometabolism and the decrease in noradrenergic and serotonergic axons would produce an overall decrease in volume.

6. Chapter VI - Microarray and IFN- α -treatment

A preliminary study was conducted using microarray to assess differential gene expression in IFN- α -treated animals. Additionally gene expression was assessed with co-treatment with Fluoxetine. A number of interesting gene changes were found, these may be useful for future experimental design.

In summary:

- Expressions of IFN α stimulated genes (ISGs) demonstrating IFN- α activation in the brain.
- Differential expression in many pathways, of note, the Kynurenine, Calcium, tight junction and MAPK pathway are all modulated.
- Co-treatment of IFN- α with Fluoxetine restores gene expression on many genes back to saline baseline levels
- IFN α animals express up-regulated fibroblast growth factor (FGF), tryptophan and DBH (Dopamine Beta Hydroxylase).

This preliminary microarray study has revealed some most interesting results. However, no conclusive results can be delineated to date without verification of the expression using rtPCR and in-situ-hybridisation.

However, some pathways are implicated in the pathogenesis of IFN- α -induced adverse events, such as the kynurenine enzyme expression and tight junction protein expression. The IDO/Kynurenine shunt has been implicated in neuropsychiatric disorders in particular those associated with IFN- α (Wichers, Koek, Robaey, Verkerk

et al., 2005). IFN- α stimulates the production of IDO which results in the kynurenine shunt, stealing tryptophan from the production of 5-HT, in effect depleting these stores. Two of the main enzymes are altered in the IDO pathway with IFN- α -treatment. One of the enzymes stimulates oxidative stress which can contribute to microglial activation and cell strength.

Of interest, the decrease in tight junction proteins in IFN- α -patients may result in a compromise in the BBB increasing the infiltration of IFN- α into the CNS. Several studies have found conflicting results regarding the impact of IFN- α on the BBB (de Boer & Gaillard, 2006; R. Milner & Campbell, 2006).

Interestingly, indications of the restorative mechanisms of Fluoxetine in IFN- α results in some highlighted pathways such as a general increase in FGFs, tryptophan, decrease in Stat1, and many more genes are modulated. A large decrease (34 fold) in the expression of Mx2 (an interferon stimulatory gene (ISG)) with Fluoxetine treatment may result in inhibition of the activation of the immune system via the Jak-stat pathway. This raises the question of efficacy of disease treatment in patients treated with Fluoxetine. Would a decrease in the IFN- α induction although alleviating the neuropsychiatric disorders reduce the efficacy of Hepatitis C treatment? Further research in this area is required.

Few pathways have been discussed here, however many more may be of interest; these are included in the main body of chapter VI. Prior to in depth investigation of these specific pathways validation is required of the pathways of interest here. It is proposed the expression changes included in this microarray may highlight future research avenues.

7. Suggested Model

The results that are revealed in this thesis provide insight into the molecular mechanisms underlying IFN- α -treated adverse events. We speculate that a decreased cell proliferation may contribute to a decrease in hippocampal volume. This decrease in volume substantially impacts on many cognitive processes, resulting in memory related deficits and mood disorders. In addition a decrease in hippocampal volume and activity may result in a dysregulation of the HC-PFC pathway, which in turn results in a compromise of the PFC. The compromise in the integrity of the PFC further impacts on other subcortical regions by resulting in a decrease in dynamic filtering. The inefficient regulation of specific subcortical regions leads to other neurovegetative symptoms. Preliminary analysis of differentially regulated genes suggests 5-HT dysregulation as a potential basis of IFN- α -related mood changes. The IDO/Kynurenine pathway may be integral to the pathophysiology and tight junction protein alterations may provide a route of entry for IFN- α into the brain other than the leaky regions.

8. Future Directions

The following work is outlined that would aid in furthering our understanding of the mechanisms underlying IFN- α induced neurocognitive adverse events.

LTP in the HC-PFC pathway

Assessment of HC-PFC LTP induction would aid in the clarification of the model that has been proposed in this thesis that IFN- α adverse events arise from a limbic-cortical disconnection. Jay et al (2004) suggest that the dysregulation of plasticity in this region may be characteristic of many neuropsychiatric disorders (Jay et al., 2004). Given the data presented here and those data from Fahey 2005, it is plausible that IFN- α -treated animals exhibit a dysregulation in this pathway which would result in an impairment of plasticity.

Serotonin transporter (Ragozzino et al.) knockout rat model

The serotonin transporter knockout rat (SERT $-/-$) characterisation has just been completed (Homberg et al., 2007). This knockout rat may be useful in elucidating the role of serotonin in IFN- α -induced adverse events. The rat has a 9 fold decrease in extracellular 5-HT levels in the hippocampus and no compensatory mechanisms were produced for monoamine turnover. This experimental model system would be useful for studying the affective disturbances that occur as a consequence of 5-HT depletion. These rats exhibit an anxiogenic phenotype that may be used comparably with IFN- α -animals to determine dissociations of impairments. Alternatively, IFN- α may be given on top of this animal model to elucidate mechanisms that may occur that are not attributable to 5-HT. Some clues to the susceptibility of IFN- α -induced animals to adverse events may be determined from such a study as SERT knockout rats would have an increased vulnerability to anxiety and depression.

Cognitive Behavioural Therapy (CBT) for IFN- α -treated rats

Admittedly, the use of antidepressants to treat depression although effective is symptom-suppressive rather than curative (DeRubeis et al., 2008). Therefore their use may actually pose a greater challenge for the individual for potential relapses after treatment cessation. An attractive alternative to medication is to treat the underlying causes with behavioural intervention techniques.

Pollak and colleagues (2008) use a learned safety paradigm as a model of behavioural antidepressant in rodents. In this paradigm animals learn to seek safety away from aversive environments. They find that learned safety models antidepressant action well with increase in neurogenesis resulting from an increase in BDNF. This paradigm is the first attempt to model cognitive behavioural therapy in rodents (Pollak et al., 2008). In light of the beneficial impact of a forced exercise regime in IFN- α -treated rats alternative behavioural modifications may be of interest. This learned safety paradigm may provide more insight into the benefits of CBT in patients taking IFN- α .

9. Conclusions

Several interesting findings have been discovered here that are beneficial in furthering our knowledge in the area of IFN- α research. The data presented here unravels the anatomical basis of IFN- α -induced adverse events. Research in our lab previously reported hippocampal-dependent deficits, here we extend this research and report prefrontal impairments. Additionally the molecular basis underlying the hippocampal deficits was explored and we speculate that the impairments may arise from decrease in cell proliferation indicating neurogenesis in the dentate gyrus and hippocampal atrophy. A compromised hippocampus and prefrontal cortex may suggest a dysregulation in the hippocampo-prefrontal pathway, which incidentally has been linked with the occurrence of neuropsychiatric disorders. We propose that the basis for these deficits arises from a limbic-cortical dysregulation.

Additionally a preliminary microarray study reveals some target that may be useful for future studies.

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Appendix 1

Included here are details provided by Almac describing the RNA extraction methodology

RNA Extraction

Snap-frozen or RNA Later Tissue

All snap-frozen or RNA-later preserved cells or tissue were homogenised and processed using a standard phenol/guanidine isothiocyanate-based organic extraction method (e.g. TRI-Reagent, TRIZOL, or RNA STAT-60). This method combines a robust lysis/denaturant extraction with ethanol precipitation for recovery of high-quality total RNA. In most cases, this total RNA was further purified by incubation with DNaseI followed by a silica-gel based membrane filter purification to yield pure RNA that is free of residual proteins, lipids, and excess salts.

RNA Quality Control

All total RNA samples were analysed for concentration, purity and integrity by using spectrophotometric methods in combination with the Agilent Bioanalyzer.

Eukaryotic Target Preparation

All eukaryotic target preparations using the project-appropriate NuGEN™ RNA Amplification System in combination with the FL-Ovation™ cDNA Biotin Module V2 were performed in accordance with the guidelines detailed in the corresponding NuGEN™ technical manual.

Amplification of Total RNA

Total RNA was amplified using the NuGEN™ WT-Ovation™ FFPE RNA Amplification System / NuGEN™ Ovation™ RNA Amplification System V2. First-strand synthesis of cDNA was performed using a unique first-strand DNA/RNA chimeric primer mix, resulting in cDNA/mRNA hybrid molecules. Following fragmentation of the mRNA component of the cDNA/mRNA molecules, second-strand synthesis was performed and double-stranded cDNA was formed with a unique DNA/RNA heteroduplex at one end. In the final amplification step, RNA within the

heteroduplex was degraded using RNaseH, and replication of the resultant single-stranded cDNA was achieved through DNA/RNA chimeric primer binding and DNA polymerase enzymatic activity. The amplified single-stranded cDNA was purified for accurate quantitation of the cDNA and to ensure optimal performance during the fragmentation and labelling process. The single stranded cDNA was assessed using spectrophotometric methods in combination with the Agilent Bioanalyzer.

Fragmentation and Labelling of Amplified Single-Stranded cDNA

The appropriate amount of amplified single-stranded cDNA was fragmented and labelled using the FL-Ovation™ cDNA Biotin Module V2. The enzymatically and chemically fragmented product (50-100 nt) was labelled via the attachment of biotinylated nucleotides onto the 3'-end of the fragmented cDNA.

Hybridisation, Washing, Staining and Scanning of Labelled cDNA

The resultant fragmented and labelled cDNA was added to the hybridisation cocktail in accordance with the NuGEN™ guidelines for hybridisation onto Affymetrix GeneChip® arrays. Following the hybridisation for 16-18 hours at 45°C in an Affymetrix GeneChip® Hybridisation Oven 640, the array was washed and stained on the GeneChip® Fluidics Station 450 using the appropriate fluidics script, before being inserted into the Affymetrix autoloader carousel and scanned using the GeneChip® Scanner 3000.

Appendix 2 – Almac Stringency Criteria for Differential Gene Expression

This filter is typically used with Rosetta Error Model summarised data.

For each summarised and normalised intensity value, a confidence measure (p-value) is provided as an indicator as to whether the transcript/gene is present in the transcription. The underlying null hypothesis for the P-value is that mRNA from a gene is not present in the GeneChip microarray hybridization sample. The lower the p-value is, the more confidence can be associated with the presence of the transcript. The p-value threshold is selected based on the level of stringency required and the constraints of the data.

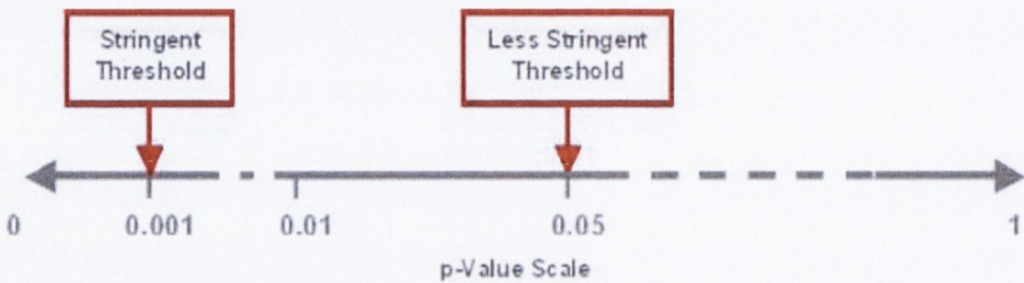


Figure 1. Example intensity p-value thresholds

This filter is typically used with Rosetta Error Model summarised data.

Background is a measure of signal intensity caused by auto-fluorescence of the array surface and non-specific binding of the target molecules. While data pre-processing corrects for the absolute background level, this filter accounts for the background noise fluctuation. The average standard deviation (σ) of the background noise, overall all arrays, is used as a threshold to filter out those transcripts affected by the background noise fluctuation. The background threshold is selected based on the level of stringency required with 2 times the average standard deviation of the background **AND** 3 times the average standard deviation of the background being the recommended defaults for less stringent and stringent criteria, respectively.

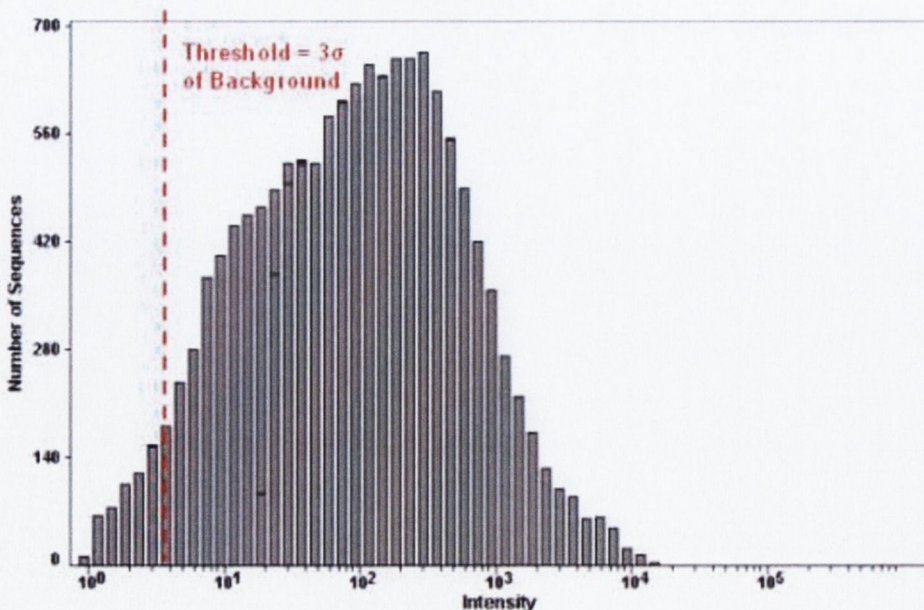


Figure 2. Example average standard deviation of the background threshold for stringent criteria

For each intensity ratio between two arrays or conditions (baseline & experiment), a signature p-value is provided as an indicator of the confidence associated with the differential expression.

The underlying null hypothesis for the P-value is that the transcript is not differentially expressed. The lower the p-value is, the more confidence can be associated with the differential expression. Similar to the intensity p-value filter, the p-value threshold for significance of difference is selected based on the level of stringency required and the constraints of the data.

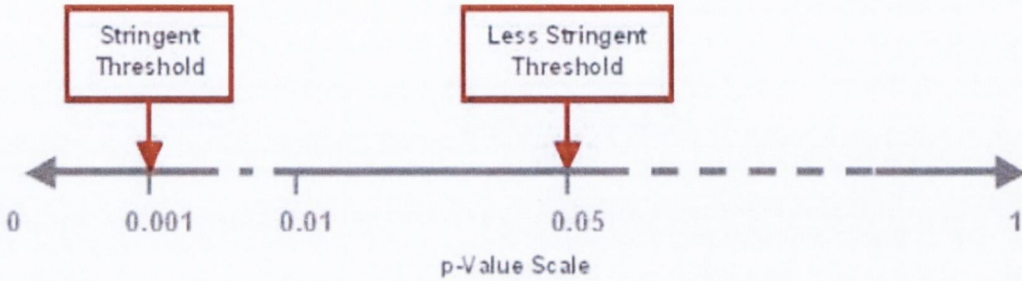


Figure 5. Example signature p-value thresholds

The genes are also examined based on fold change, i.e. the difference in expression between the experimental case and the baseline case.

Selecting genes based on an arbitrarily chosen fold change threshold can produce misleading results, especially when examining lower signal intensities, where fold change variance is greater. These genes will then tend to numerically meet any given fold change cut-off even if the gene is not truly differentially expressed (false positives). The inverse is also true, where highly expressed genes, having less error in their measured levels, may not meet an arbitrary fold-change cut-off even when they are truly differentially expressed (false negatives).

Rather than set an arbitrary fold change threshold, the coefficient of variation (CV) of the background noise level is used to estimate a suitable fold change threshold. For the less stringent criteria the fold change threshold is set as two times the coefficient of variation of the background level (with a floor of 1.3):

$$\max(1.3, 1+2\langle CV(Bg) \rangle)$$

For the more stringent criteria this threshold is increased to three times the coefficient of variation of the background level (with a floor of 1.5):

$$\max(1.5, 1+3\langle CV(Bg) \rangle)$$

The calculated fold change threshold value will depend on the quality of data. Noisy data will have high thresholds and consequently a limited number of genes will pass the filter criteria. High quality data will have low thresholds and as a result a lot of genes may pass the filter criteria.

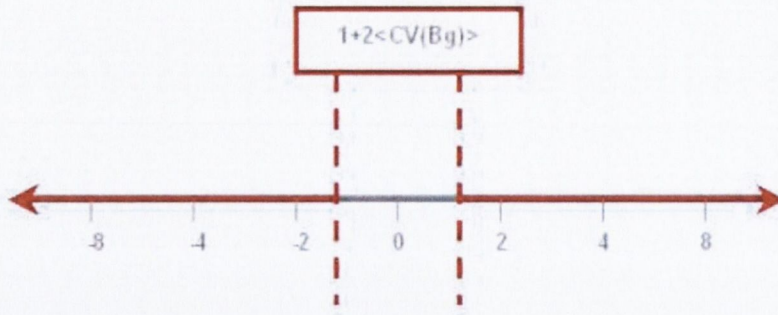


Figure 6. Fold Change thresholds based on less stringent selection criteria. Those genes with fold changes below threshold are removed. The remaining differentially expressed genes (red area) will comprise the 'Less Stringent' Gene List.

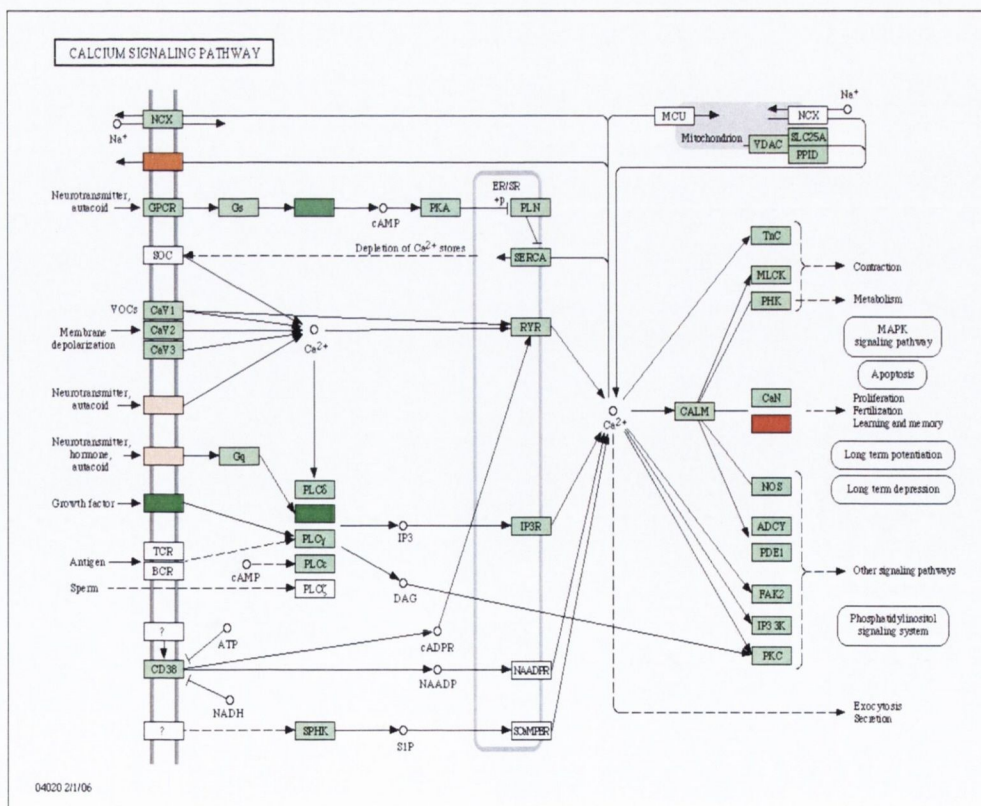
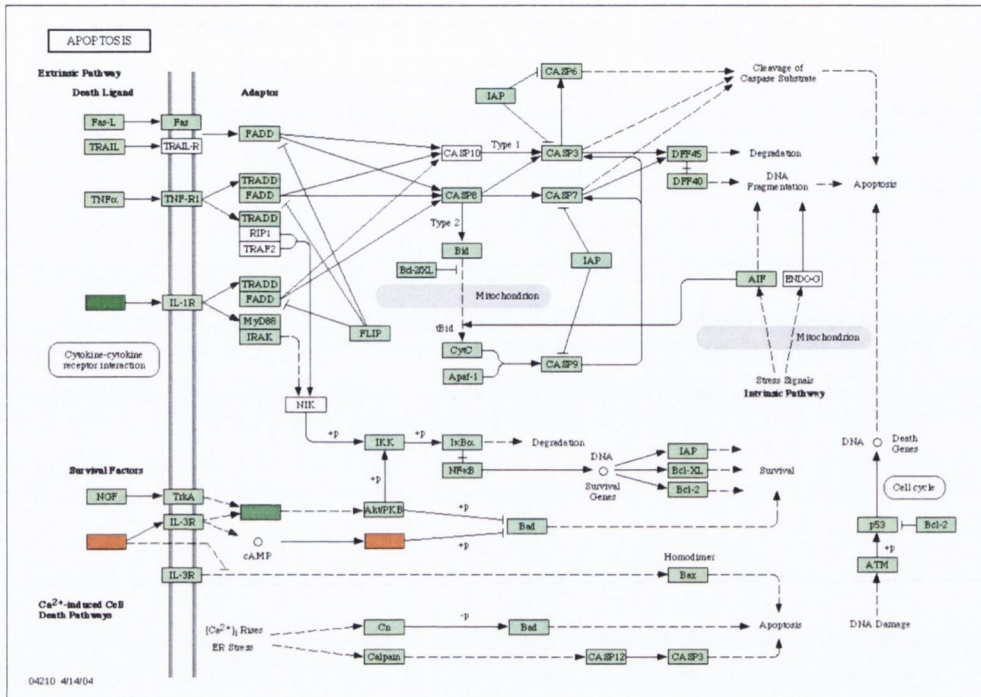
Statistical testing (t-test, ANOVA) is applied to determine whether any transcripts exhibit mean expression intensities that are significantly different between experimental groups.

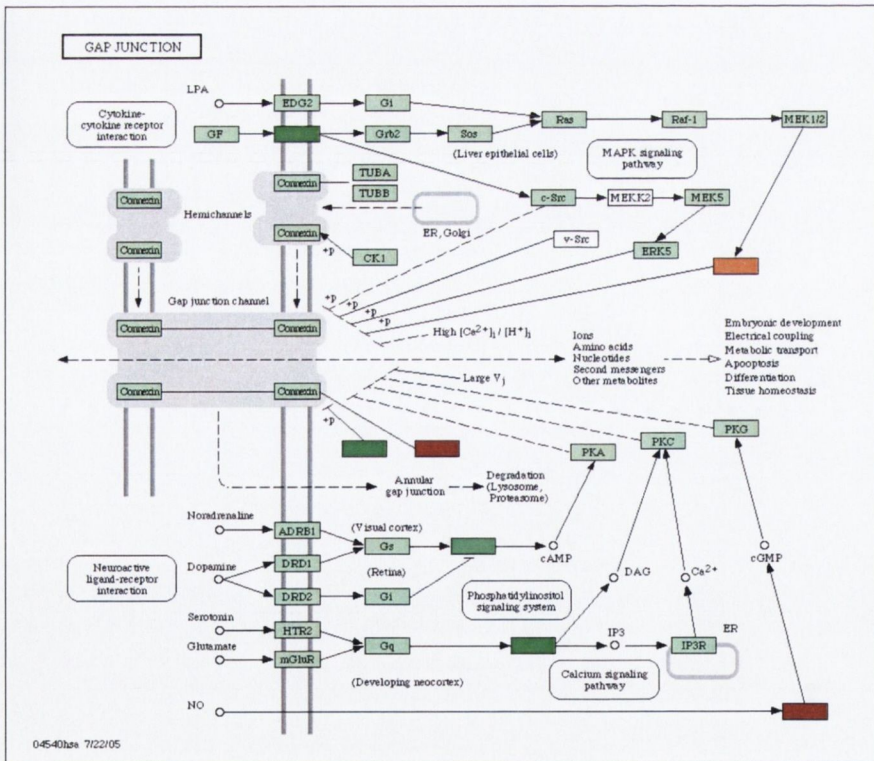
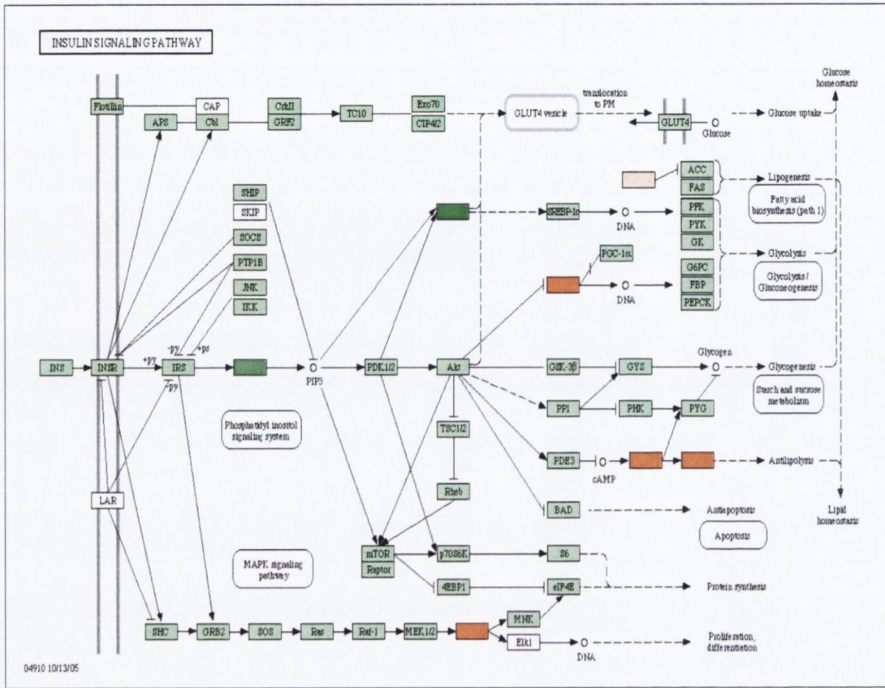
When conducting multiple tests, a P-value threshold of 0.01 means that we accept one false positive for every 100 tests (Type I error). When working with microarray data where there are many genes tested simultaneously, the null hypothesis for each gene is that there is no differential expression. Given the large number of genes tested in a particular experiment, the probability measure of significance can result in an unacceptable level of false positives over the entire set of comparisons. Multiple test corrections (default Benjamini and Hochberg False Discovery Rate) are used to adjust the P-values derived from the multiple statistical tests applied to the data and improve the false positive rate.

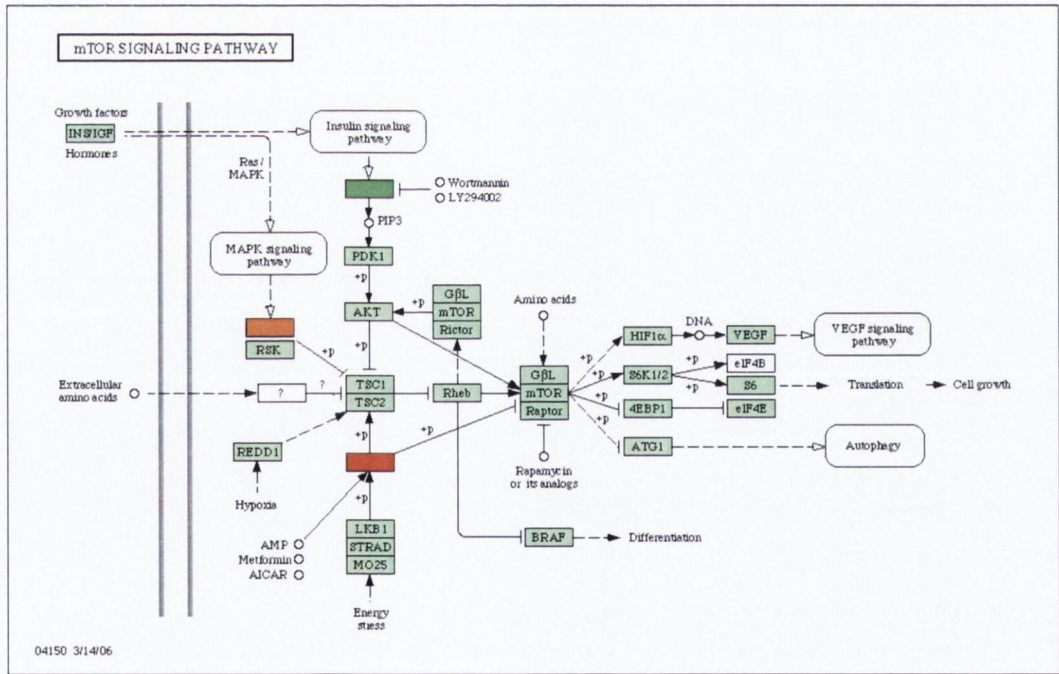
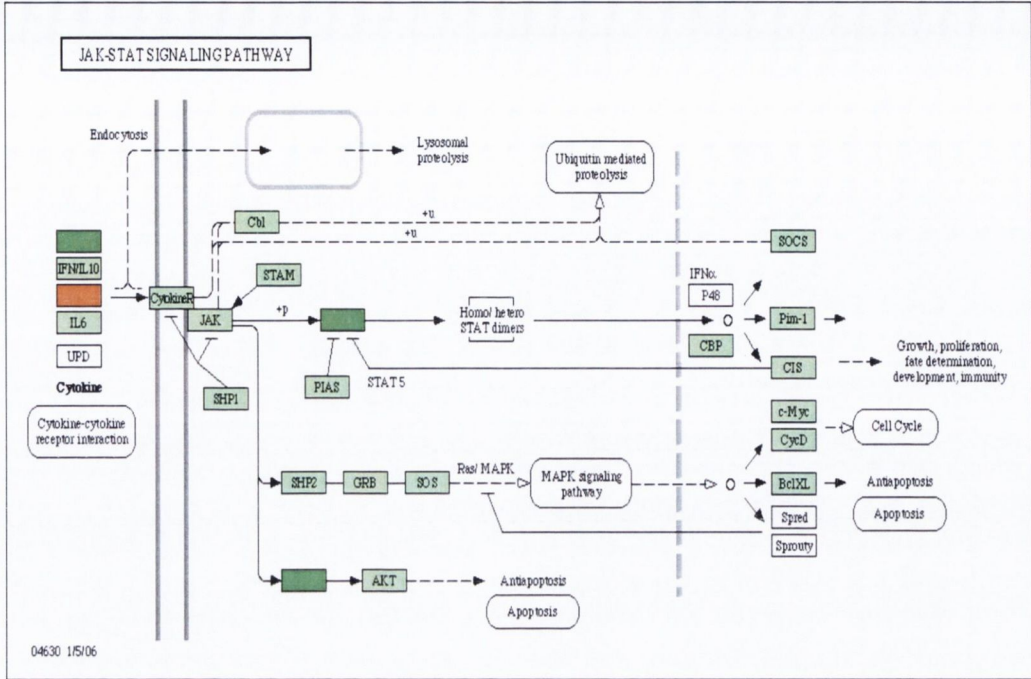
The p-value threshold is selected based on the constraints of the data. Should the multiple testing correction adjustment prove overly stringent, the correction will be removed and the p-value threshold reduced, to control the number of false positive results.

Appendix 3 – Genes Changed with IFNFlx Vs IFN

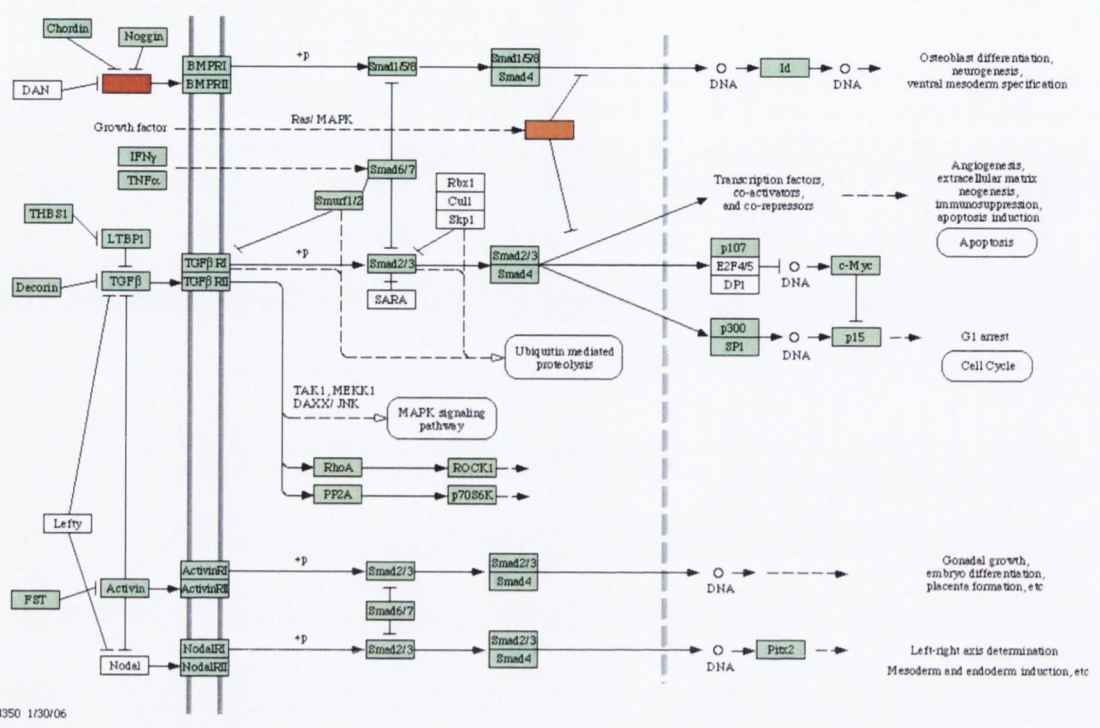
Kegg pathway maps depicting gene changes with Fluoxetine treatment (red is up-regulation, green down-regulation)





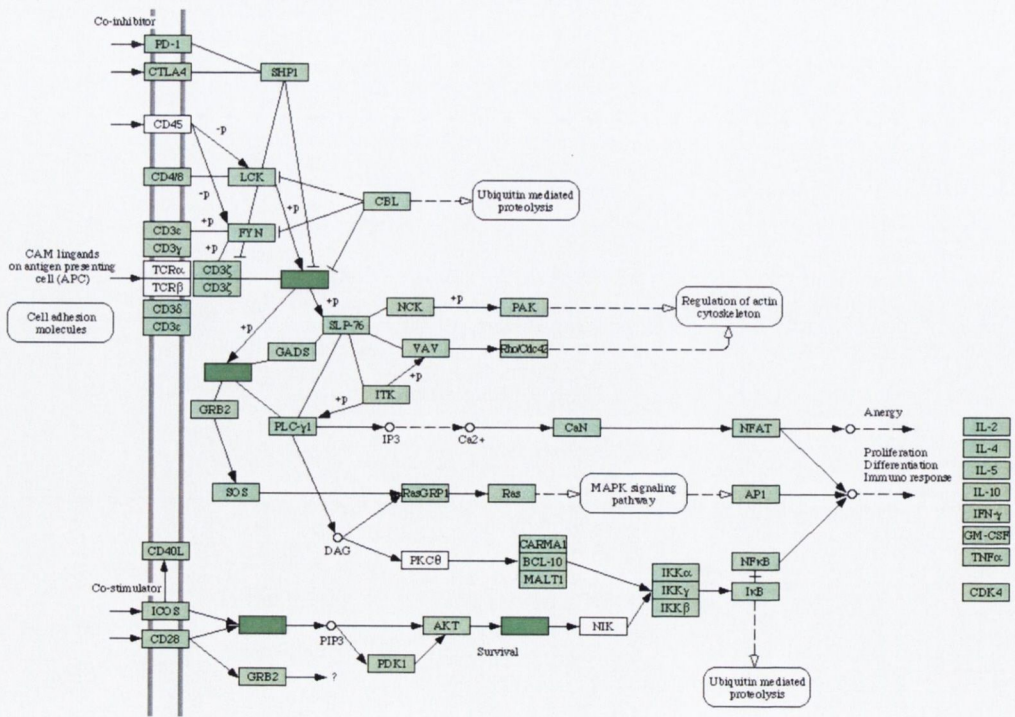


TGF-BETA SIGNALING PATHWAY

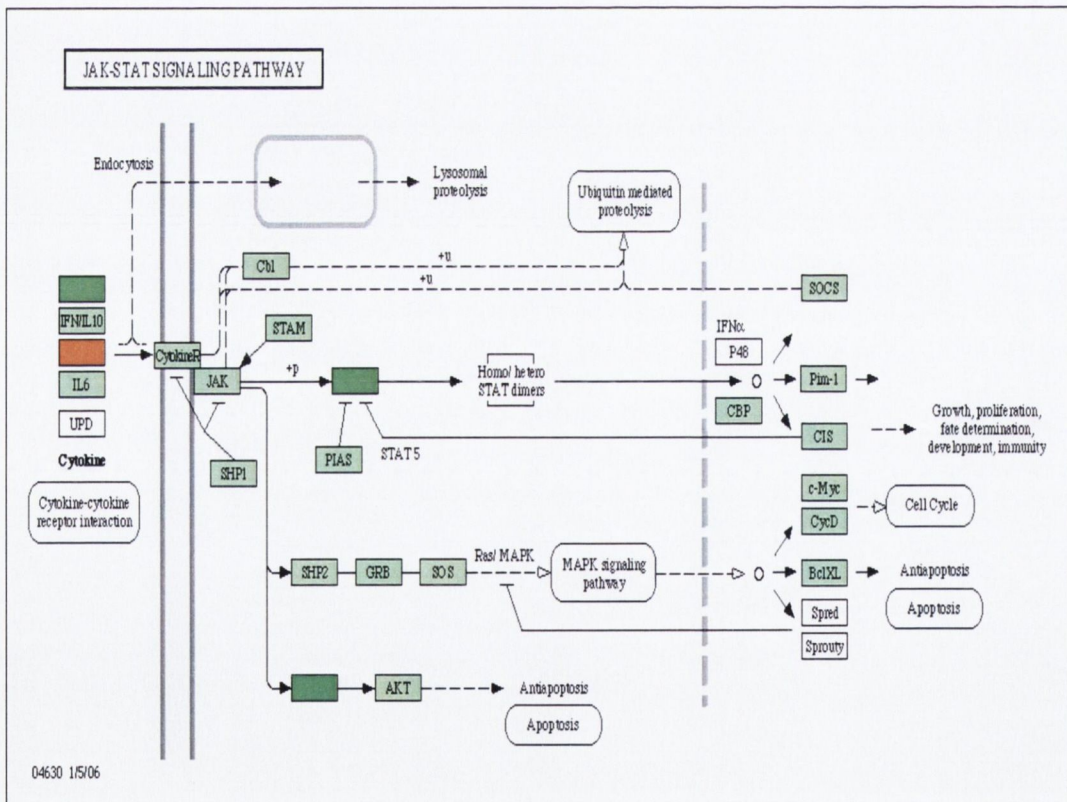
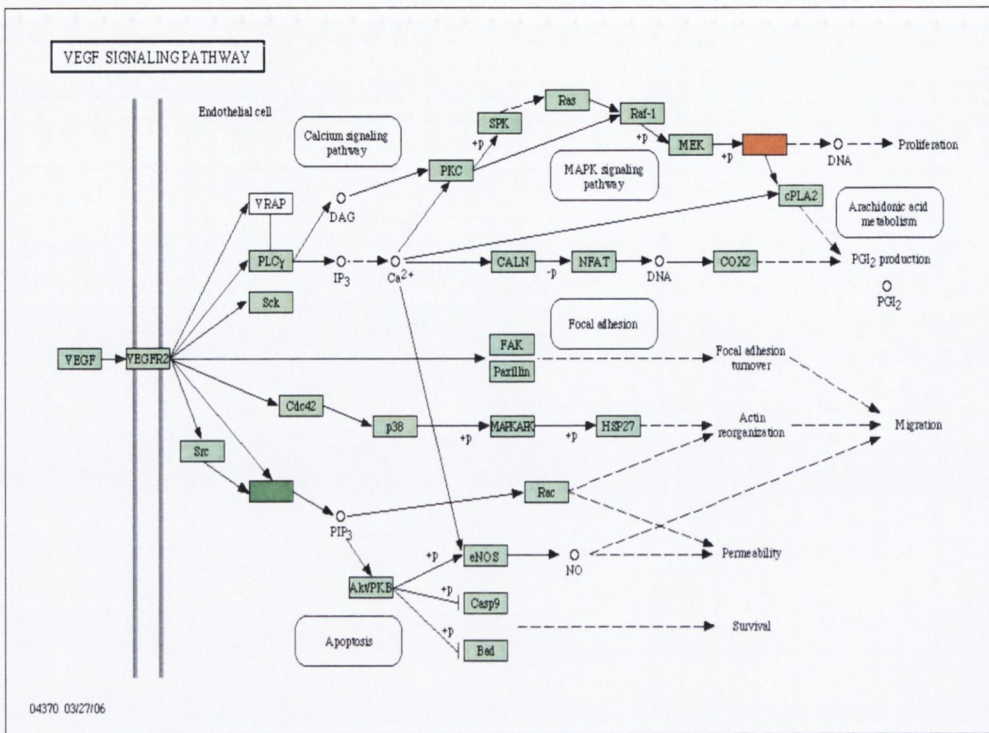


04350 1/30/06

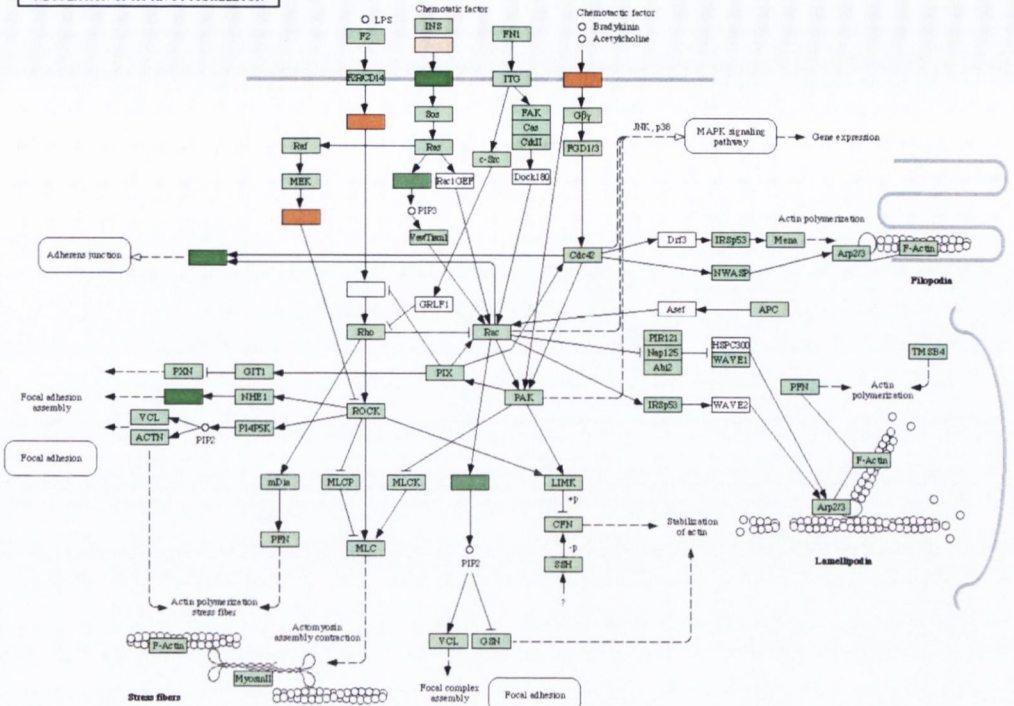
T CELL RECEPTOR SIGNALING PATHWAY



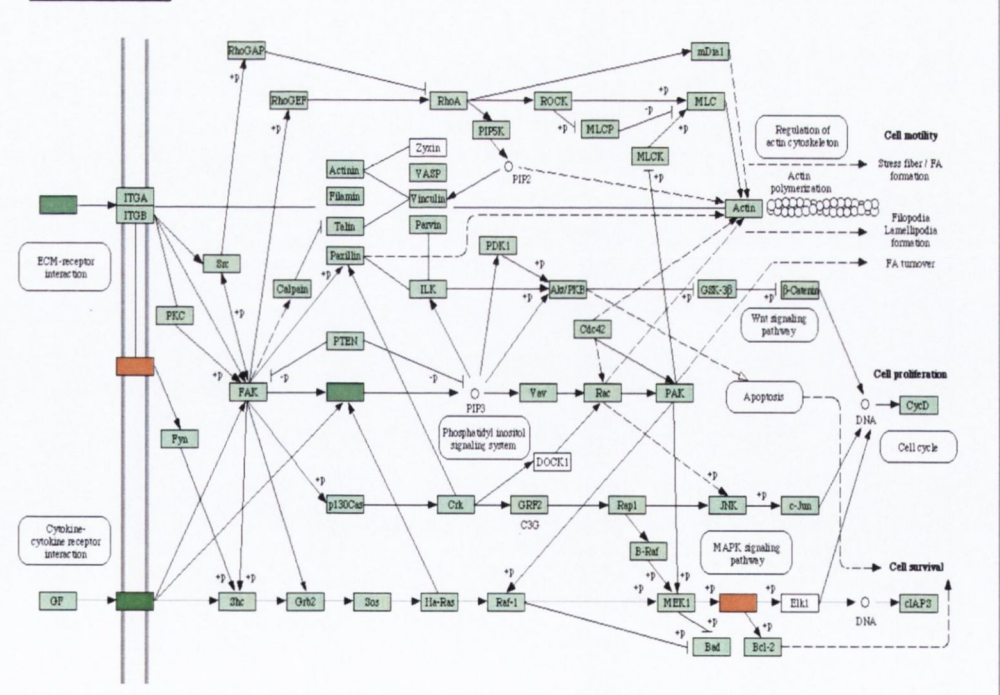
04660 9/2/04



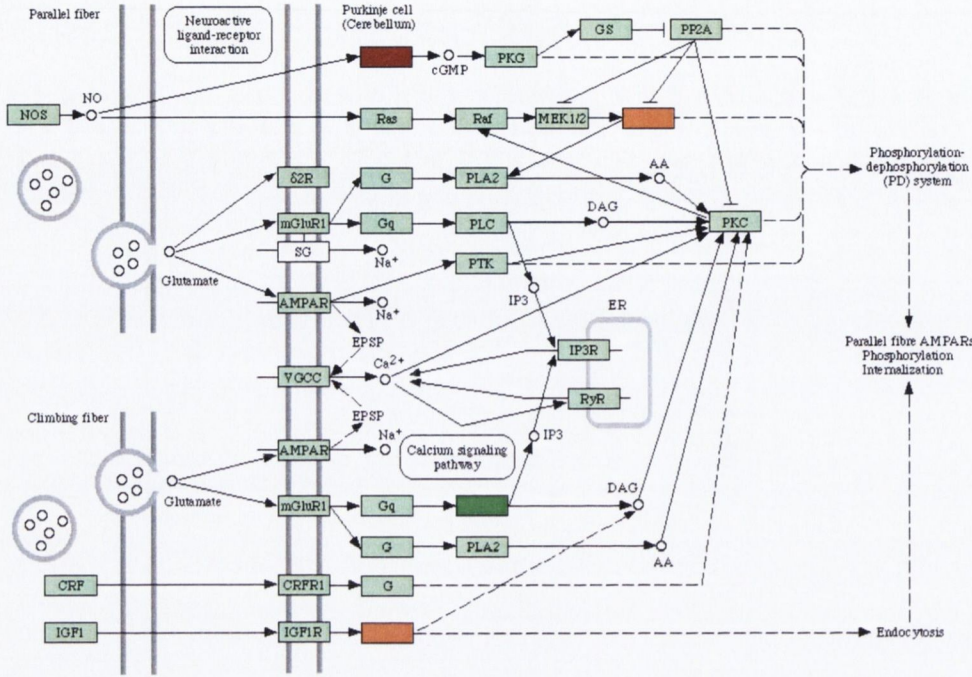
REGULATION OF ACTIN CYTOSKELETON



FOCAL ADHESION

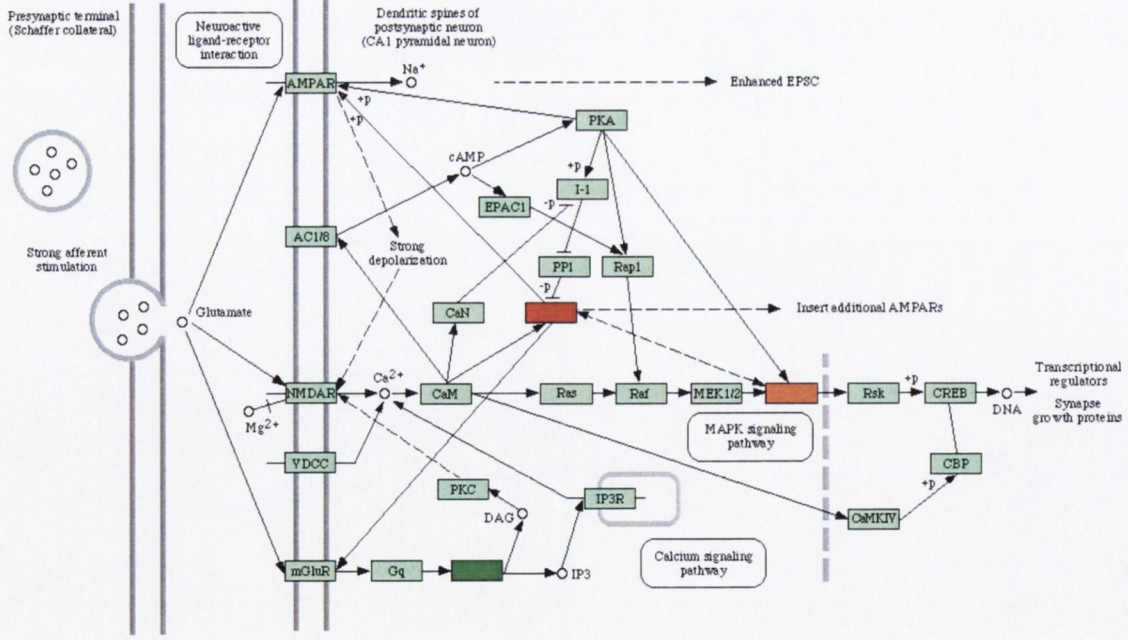


LONG-TERM DEPRESSION



04730 2/09/06

LONG-TERM POTENTIATION



04720 1/30/06

Appendix 4

Table 1. Differentially Expressed Genes in all treatment groups (More detailed list)





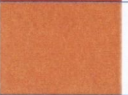



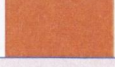
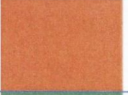
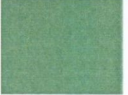
Pathway Name	Gene Name	Accession	IFN Vs Sal	IFN Vs IFNFlx	Sal Vs SalFlx
Cytokine-cytokine receptor interaction					
Thyroid Peroxidase	Tpo	NM_019353			
tumor necrosis factor (TNF superfamily, member 2)	Tnf	AA819227			
tumor necrosis factor (ligand) superfamily, member 9	Tnfsf9	BF394106			
Fas (TNFRSF6)-associated via death domain	Tnfrsf6	BE108106			
chemokine (C-X-C motif) receptor 3	Cxcr3	NM_053415			
interleukin 2 receptor, beta chain	Il2rb	NM_013195			
interleukin 3	Il3	NM_031513			
interleukin 1 alpha	Il1a	NM_017019			
chemokine (C-C motif) receptor 3	Ccr3	NM_053958			
growth hormone receptor	Ghr	NM_017094			
Neuroactive ligand-receptor interaction					
5-hydroxytryptamine (serotonin) receptor 1A	Htr1a	NM_012585			
5-hydroxytryptamine (serotonin) receptor 2B	Htr2b	NM_017250			
5-hydroxytryptamine (serotonin) receptor 2C	Htr2c				
growth hormone releasing hormone receptor	Ghrhr	NM_012850			
growth hormone releasing hormone	Ghrh	NM_031577			
growth hormone receptor	Ghr	NM_017094			
gamma-aminobutyric acid (GABA-C) receptor, subunit rho 2	Gabbr2	NM_017292			
gamma-aminobutyric acid (GABA) B receptor 1	Gabbr1	NM_031028			
gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 3	Gabra3	NM_017069			
gamma-aminobutyric acid (GABA-A) receptor, subunit delta	Gabrd	AI228492			

dopamine receptor D3	Drd3	NM_017140	█
galanin	Gal	NM_033237	█
opioid receptor, delta 1	Oprd1	NM_012617	█
cholinergic receptor, muscarinic 2, cardiac	Chrm2	NM_031016	█
Gap junction			
5-hydroxytryptamine (serotonin) receptor 2B	Htr2b	NM_017250	█
tight junction protein 1	Tjp1_predicted	AW141192	█ █
guanylate cyclase 1, soluble, beta 2	Gucylb2	NM_012770	█
platelet derived growth factor receptor, beta polypeptide	Pdgfrb	AI071374	█ █
adenylate cyclase 4	Adcy4	NM_019285	█
phospholipase C, beta 2	Plcb2	NM_053478	█
guanylate cyclase 1, soluble, alpha 2	Gucyl1a2	NM_023956	█
mitogen activated protein kinase 1	Mapk1	NM_053842	█
adenylate cyclase 4	Adcy4	NM_019285	█
cell division cycle 2 homolog A (S. pombe)	Cdc2a	NM_019296	█
MAPK signaling pathway			
tumor necrosis factor (TNF superfamily, member 2)	Tnf	AA819227	█
Fas (TNFRSF6)-associated via death domain	Tnfrsf6	BE108106	█
neurofibromatosis 1	Nfl	NM_012609	█ █
calcium channel, voltage-dependent, alpha 1I subunit	Cacna1i	NM_020084	█
platelet derived growth factor receptor, beta polypeptide	Pdgfrb	AI071374	█ █
fibroblast growth factor 8	Fgf8	NM_133286	█
fibroblast growth factor 12	Fgf12	AW252096	█
fibroblast growth factor 18	Fgf18	NM_019199	█

fibroblast growth factor 6	Fgf6	NM_131908	Orange	
mitogen activated protein kinase kinase kinase 1	Map4k1_predicted	AI237423	Green	Red
mitogen activated protein kinase 1	Mapk1	NM_053842	Orange	
mitogen-activated protein kinase kinase kinase 8	Map3k8	NM_053847	Green	
interleukin 1 alpha	Il1a	NM_017019	Green	
guanine nucleotide binding protein, alpha 12	Gna12	NM_031034	Orange	
Calcium signaling pathway				
5-hydroxytryptamine (serotonin) receptor 2B	Htr2b	NM_017250	Orange	
calcium channel, voltage-dependent, alpha 1I subunit	Cacna1i	NM_020084	Orange	
platelet derived growth factor receptor, beta polypeptide	Pdgfrb	AI071374	Dark Red	Green
adenylate cyclase 4	Adcy4	NM_019285	Orange	Green
troponin C2, fast	Tnnc2	BG663128	Orange	
phospholipase C, beta 2	Plcb2	NM_053478		Green
	P2rx11	NM_012721		Green
cholinergic receptor, nicotinic, alpha polypeptide 7	Chrna7	NM_012832		Dark Red
purinergic receptor P2X, ligand-gated ion channel, 7	P2rx7	NM_019256	Orange	
prostaglandin E receptor 3 (subtype EP3)	Ptger3	NM_012704		Green
calcium/calmodulin-dependent protein kinase II gamma	Camk2g	BF390092		Red
ATPase, Ca ⁺⁺ transporting, plasma membrane 2	Atp2b2	NM_012508	Orange	
cholinergic receptor, muscarinic 2, cardiac	Chrm2	NM_031016	Orange	

Protein phosphatase 3, catalytic subunit, alpha isoform	Ppp3ca	AI14615 8									Green
Cell adhesion molecules (CAMs)											
Poliovirus receptor	Poliovirus	BE10242 6	Orange			Green					
cadherin 22	Cdh2	AW4346 31		Green		Red					
RT1 class II, locus Db1	RT1-Db1	BI27952 6	Orange								
CD22 molecule	Cd22	AI40828 9	Orange			Dark Green					
claudin 1	Cldn1	AI13764 0					Orange				
F11 receptor	F11r	NM_053 796					Orange				
poliovirus receptor	PVR	AI54885 6									Green
neuroligin 3	Nlgn3	BF41371 5									Orange
Jak-STAT signaling pathway											
thyroid peroxidase	Tpo	NM_019 353	Orange			Green					
interleukin 2 receptor, beta chain	Il2rb	NM_013 195		Green							
signal transducer and activator of transcription 3	Stat3	BE11392 0		Green							
signal transducer and activator of transcription 4	Stat4	AA9553 54	Red			Green					
	Il3	NM_031 513					Orange				
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053 481					Green				
growth hormone receptor	Ghr	NM_017 094									Green
Apoptosis											
tumor necrosis factor (TNF superfamily, member 2)	Tnf	AA8192 27	Red								
Fas (TNFRSF6)-associated via death domain	Fadd	BE10071 5	Orange								
Fas (TNFRSF6)-associated via death domain	Tnfrsf6	BE10810 6	Orange								
protein kinase, cAMP dependent regulatory, type II alpha	Prkar2a	AW9190 85					Orange				
interleukin 3	Il3	NM_031 513					Orange				
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053 481					Green				
interleukin 1 alpha	Il1a	NM_017 019					Green				

platelet derived growth factor receptor, beta polypeptide	Pdgfrb	AI07137 4		
moesin	Msn	NM_030 863		
Rho guanine nucleotide exchange factor (GEF) 12	Arhgef12	BI30305 3		
fibroblast growth factor 8	Fgf8	NM_133 286		
fibroblast growth factor 12	Fgf12	AW2520 96		
fibroblast growth factor 18	Fgf18	NM_019 199		
fibroblast growth factor 6	Fgf6	NM_131 908		
mitogen activated protein kinase 1	Mapk1	NM_053 842		
IQ motif containing GTPase activating protein 3	Iqgap3	AI07139 8		
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053 481		
	LOC31645 7	BE09886 0		
cholinergic receptor, muscarinic 2, cardiac	Chrm2	NM_031 016		
guanine nucleotide binding protein, alpha 12	Gna12	NM_031 034		
Axon guidance				
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	Nfatc4	AI17532 0		
mitogen activated protein kinase 1	Mapk1	NM_053 842		
TGF-beta signaling pathway				
tumor necrosis factor (TNF superfamily, member 2)	Tnf	AA8192 27		
activin receptor IIB	Acvr2b	AI54879 9		
bone morphogenetic protein 2	Bmp2	NM_017 178		
	Mapk1	NM_053 842		
ABC transporters - General				
ATP-binding cassette, sub-family A (ABC1), member 1	Abca1	AW9183 87		
ATP-binding cassette, sub-family C (CFTR/MRP),	Abcc6	NM_031		

member 6		013			
ATP-binding cassette, sub-family G (WHITE), member 2	Abcg2	AI17561 6			
Toll-like receptor signaling pathway					
tumor necrosis factor (TNF superfamily, member 2)	Tnf	AA8192 27			
Fas (TNFRSF6)-associated via death domain	Fadd	BE10071 5			
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053 481			
Long-term potentiation					
phospholipase C, beta 2	Plcb2	NM_053478			
calcium/calmodulin-dependent protein kinase II gamma	Camk2g	BF39009 2			
mitogen activated protein kinase 1	Mapk1	NM_053842			
Protein phosphatase 3, catalytic subunit, alpha isoform	Ppp3ca	AI14615 8			
Long-term depression					
guanylate cyclase 1, soluble, beta 2	Gucy1b2	NM_012 770			
Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), gamma isoform	Ppp2r2c	BF41457 5			
phospholipase C, beta 2	Plcb2	NM_053 478			
mitogen activated protein kinase 1	Mapk1	NM_053 842			
guanine nucleotide binding protein, alpha 12	Gna12	NM_031 034			
phospholipase A2, group V	Pla2g5	NM_017 174			
Antigen processing and presentation					
RT1 class II, locus Db1	RT1-Db1	BI27952 6			
VEGF signaling pathway					
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	Nfatc4	AI17532 0			
mitogen activated protein kinase 1	Mapk1	NM_053 842			
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	Pik3cb	NM_053 481			

Protein phosphatase 3, catalytic subunit, alpha isoform	PPP3CA	AI14615 8			
phospholipase A2, group V	PLA2G5	NM_017174			
Hedgehog signaling pathway					
serine/threonine kinase 36 (fused homolog, Drosophila)	STK36	BE10889 7			
wingless related MMTV integration site 10a	WNT10A	AI029140			
bone morphogenetic protein 2	BMP2	NM_017178			
Circadian rhythm					
period homolog 2 (Drosophila)	PER2	BG3735 24			
Insulin signaling pathway					
protein kinase, cAMP dependent regulatory, type II alpha	PRKAR2A	AW919085			
protein kinase, AMP-activated, alpha 1 catalytic subunit	PRKAA1	NM_019142			
mitogen activated protein kinase 1	MAPK1	NM_053842			
protein kinase, AMP-activated, gamma 2 non-catalytic subunit	PRKAG2	AI579640			
lipase, hormone sensitive	LIPE	AW533397			
forkhead box O1	FOXO1A	BI295511			
phosphatidylinositol 3-kinase, catalytic, beta polypeptide	PIK3CB	NM_053481			
histidine triad nucleotide binding protein 1	HINT1	NM_022192			