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Exploring the influence of reward mechanisms on the interaction between sickness and immunity: reasons to 'press-on'

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A life spent making mistakes is not only more honourable but more useful than a life spent doing nothing.

--George Bernard Shaw
Preface to *The Doctor's Dilemma, 1911*

As long as I have a want, I have a reason for living. Satisfaction is deadly.

--George Bernard Shaw
Overruled, 1912

A Little Note

A magician pulls rabbits out of hats while a behavioural neuroscientist pulls habits out of rats. *–Anonymous*

To my committee members, thank you for acknowledging my work and taking part in this document. An important lesson that I have learned over time is that good things are not often done alone; the accomplishment of attaining one's goal must be shared.

To this end, I would like to begin by expressing my gratitude to my Mom and Dad who loved and encouraged me over the years, but who had the foresight to be honest about my abilities (I now realize it's true that I cannot sing) and keep me grounded. Thank you for teaching me the importance of hard work and a sense of humour and for showing me that the best things in life aren't simply taken or given, but earned. I realize and appreciate the sacrifices you two have made for me throughout my life so that I could follow my dreams, and now I hope that you do the same. It is to you that I dedicate this dissertation. I love you both.

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Although I am now leaving your office grown-up, I know that the door is not closed. Thank you.

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Abstract

The clinical use of cytokines such as interleukin (IL)-2 and interferon (IFN)-α has been accompanied by reports of central nervous system disruptions, tempering their effectiveness as front line treatments of defense against cancer and hepatitis C. Along with the potential to change the course of disease, these cytokines often elicit an array of generalized non-specific side effects, known as sickness behaviours; these include disrupted febrile changes, lethargy, hypersomnia, anorexia, decreased mobility, and depression, all of which impact patient compliance in treatment plans. As of yet, an animal model has not been well developed to study the mechanisms underlying the sickness-inducing effects of these cytokines.

In the first three experiments, we evaluated the short- and long-term effects of a single systemic injection of either rat recombinant IL-2 (study 1) or IFN-α (studies 2 and 3) on a variety of physiological and behavioural indices. In the first experiment, a single IL-2 challenge increased thresholds for rewarding brain stimulation of the ventral tegmental area (VTA) in a progressive manner over the month long test period.

The next two studies were designed to investigate the sickness-inducing effects of IFN-α on temperature, body weight, food intake, sickness behaviours, and locomotor activity in both male and female rats. In the latter, thresholds for brain stimulation reward (BSR) were also chronicled. Unlike the first study, BSR thresholds were not compromised by the systemic cytokine challenge. Significant physiological disruptions such as elevated temperature and piloerection scores were observed; locomotor activity was only disrupted in male rats. Most of the behavioural and physiological effects of

IFN-α were observed at the lowest dose level (10 units) suggesting that this cytokine can induce long-term somatic changes without altering hedonic status.

In the third study, we observed that animals obtaining rewarding brain stimulation had attenuated signs of sickness consistent with previous work suggesting that BSR stimulates immunological processes, such as natural killer cell activity. Thus, in the fourth study, we further exploited this phenomenon and included, in addition to a BSR group, animals exposed to an environmentally enriched condition. In this study, the immune challenge consisted of 150 μg/kg i.p. of lipopolysaccharide (LPS). Both BSR and environmental enrichment diminished the sickness behaviours typically evoked by LPS. Real-time RT-PCR and a multiplex bead assay revealed that the mRNA and protein levels for several peripheral cytokines and their receptors varied according to environmental context. For example, BSR significantly influenced the cytokine profiles for IL-10 and natural killer cell receptor gene, whereas a trend of reduced IL-6 levels and a significantly elevated profile of IFN-γ was observed in the environmentally enriched group. The effect of LPS challenge on brain cytokine levels in the VTA was modest at best. Only IL-6 was significantly elevated in LPS control versus saline-treated animals while the environmentally enriched animals demonstrated a trend toward lower levels of this cytokine in the VTA. Together, these data suggest that environmental context may influence the physical demonstrations of sickness through redistributions of immunological parameters and status.

Table of Contents

Page

Quotes ii

A little note iii

Abstract v

Table of contents vii

Short-guide to acronyms ix

General Introduction 1

1. The Hitchhiker's Guide to the Immune System
    1.2. Road Map of the Immune System 1

2. An Overview of the Concept of Stress 4
    2.1. The HPA Axis: What it can do to you 7

3. Famous Henchmen of the Immune Response 9

4. Cytokines as Stressors 15

5. The Mechanics of Sickness 20
    5.1. A Shout out to Past Sickness Behavioural Investigators 25
    5.2. Sleep and Lethargy 25
    5.3. Piloerection and Temperature 27
    5.4. Ingestive Behaviours 31
    5.5. Motor Activity 35

6. Additional Behavioural Models to assess Sickness 42
    6.1. Forced Swim Test 42
    6.2. Food as Reward 45
    6.3. Brain Stimulation Reward 48

7. Further Considerations 52
    7.1. Reward and Immune Activation 56
    7.2. Goals: The Motivation of the Thesis 62

Experiment 1 – Short- an long-term effects of interleukin-2 on weight, food intake, and hedonic mechanisms in the rat.

1. Abstract 66
2. Introduction 66

3. Materials and Methods 70
4. Results 75
5. Discussion 78

Experiment 2 – Behavioural and physiological effects of a single injection of rat interferon-α on male Sprague-Dawley rats: a long-term evaluation.
1. Abstract 91
2. Introduction 92
3. Materials and Methods 95
4. Results 100
5. Discussion 104

Experiment 3 – Investigating the hedonic effects of interferon-α on female rats using brain stimulation reward.
1. Abstract 122
2. Introduction 123
3. Materials and Methods 126
4. Results 133
5. Discussion 137

Experiment 4 – The effects of environmental enrichment and brain stimulation reward on lippoplysaccharide induced sickness in the female rat.
1. Abstract 150
2. Introduction 151
3. Materials and Methods 155
4. Results 162
5. Discussion 166

General Discussion 181

References 195

Appendix A: Cognitive and Affective Disruptions Associated with Immunotherapy 234

Short-Guide to Acronyms

5-HIAA – Serotonin metabolite

ACTH - Adrenocoticotropic hormone

BSR - Brain stimulation reward

CCI - Cytokine and chemokine immunotherapy

CNS - Central nervous system

CRH - Corticotrophin-releasing hormone

DMBA - 9,10-dimethyl-1,2-benzanthracene

FST - Forced swim test

HPA - Hypothalamic pituitary adrenal axis

IFN- Interferon

IFN-α - Interferon-α, Intron A

IFN-δ – Interferon-δ

IL-1 - Interleukin-1

IL-1ra - Interleukin-1 receptor antagonist

IL-1β - Interleukin-1β

IL-2 - Interleukin-2, Proleukin

IL-6 - Interleukin-6

IL-18 - Interleukin-18

LPS - Lipopolysaccharide

MADRS - Montgomery and Asberg Depression Rating Scale

MIU - Million international units

NK - Natural killer cell activity

TNF- Tumor necrosis factor

TNF-α - Tumor necrosis factor alpha

VTA - Ventral tegmental area

General Introduction

1. The Hitchhiker's Guide to the Immune System

Since the emergence of living organisms, immunity, the ability to prevent destruction from infectious agents, has ensured the continuation of the species. This has been achieved by the ability of immune cells to distinguish between self, and non-self, in recognizing and destroying foreign pathogens that have infiltrated the organism (Janeway, et al., 2001). Emerging from this system are two branches: an innate or natural immunity, and an adaptive or acquired immunity.

The innate response is the only immune defense mechanism associated with invertebrate organisms (Thomson, 1995) and is initiated immediately upon recognition of a foreign agent (Sigal, 2002). Receptors of the innate system become alerted to pathogens via carbohydrate signals (Stahl, 1992). The structures that make up this branch of immunity either prevent entrance of a pathogen, or eliminate one that has infiltrated the organism. The epithelial layers such as skin and the mucosal layers prevent the access of a number of pathogens by acting as physical barriers (Podolsky, 1997). Molecules of the innate system such as macrophage, neutrophils, and dendritic cells have phagocytic properties. These molecules engulf the pathogen and actively destroy it through lysosomes. Protection by innate mechanisms is short-lived as there is no memory of the process involved, only passive recognition of foreign debris that is not self (Janeway et al., 2001).

Adaptive immunity is mediated by lymphocytes, or white blood cells, with receptors and memory cells. The adaptive response is divided into two components, the B cells that recognize peptides, carbohydrates, and other simple structures, and the T cells that identify

peptides (Fearon & Locksley, 1996). Overall, the main structures of the adaptive system are the major histocompatibility complex molecules, T cell receptors, and immunoglobulins. The antigen presenting cells such as macrophage, dendritic cells, and neutrophils are also very important and line the border between both the innate and adaptive systems via their phagocytic abilities (Bartl et al., 2004).

1.2. Road Map of the Immune System

Primary organs of the immune system, such as the thymus, have the ability to release classical neuroendocrine hormones such as growth hormone, prolactin, follicle stimulating hormone, and lutenizing hormone (Dardene & Savino, 1996). The thymus is one of the main sites of differentiation and proliferation of T cells that mediate hypothalamic and immune functioning (Dardene &Savino, 1996; Hadden, 1996). During the 1980's, histological studies revealed that both the thymus and the spleen are innervated by the autonomic nervous system (Bullock & Moore, 1981; Felten et al., 1987), indicative of a central nervous system influence. Additional innervations of the thymus stem from both the vagus nerve and the thoracic sympathetic chain. Disrupting either of these pathways results in impaired hypothalamic pituitary adrenal (HPA) axis hormone release (Pierpaoli & Sorkin, 1972), implicating the role of the endocrine system within these pathways.

Destruction of parts of the brain also affects immune activity. For example, disturbances in the neocortex compromise the production of T cell inducing factors (Renoux et al., 1983), and hypophysectomy, removal of the pituitary gland, affects spleen immunity in rodents (Berczi & Nagy, 1991). The relationship between the immune and central nervous systems is further supported through observations that the parasympathetic and sympathetic

nervous systems are anatomically similar to immune cells in that secreted neurotransmitters affect the activity of both T and B cells (see Coe et al., 2002).

The immune response is also mediated by the interplay of several hormones, which act through the HPA axis (described below). Pituitary hormones, such as growth hormone and prolactin, have both been associated with immune regulation, as have several others. For example, reduced growth hormone release has been implicated in compromised bone marrow, thymus, and T cell activity (Ader et al., 1990). Conversely, administration of growth hormone is able to reverse some of these deficiencies (Ader et al., 1990). The reproductive hormone, prolactin, is inhibited by dopamine and glucocorticoids. In addition, prolactin is inhibited by serotonin and estrogen which exert excitatory properties on many systems (Ader et al. 1990; Bernton et al., 1988). An inhibitory action on prolactin interferes with antibody responses (Bernton et al., 1988). Of interest is that melatonin, a hypothalamic hormone, acts as a transducer of photoperiod effects for both the neuroendocrine and immune systems (Guerro & Reiter, 1992). Melatonin also exerts control on the release of prolactin and growth hormone, resulting in endocrine and immune regulation (Lu & Meites, 1973).

During exposure to a stressor, there is an initial surge in immune activation (Sapolsky et al., 2000; Munck et al., 1984) followed by suppression when the HPA response is prolonged. The profile of natural killer cell (NK) activity in response to stress clearly demonstrates this pattern. Between the first 30 to 60 minutes after the stressor application, there is a surge of NK activity in the periphery (Benschop et al., 1996), followed by a decline in NK proliferation that may persist for days (Benschop et al., 1996). A reduction in NK activity, which impairs the killing of viruses and cancerous cells, can lead to

deficiencies in immunocompetence; however the link between stress-induced NK deficits and the development of cancer is still under debate (Sapolsky, 2002). Nonetheless, it has been suggested that long-term stress can alter the functioning of neutrophils and their phagocytic abilities, leading to the increased *likelihood* of an organism becoming sick, or having a pre-existing disease exacerbated (Melmed & Gelpin, 1996; Levenstein et al., 1995). These actions occur because of the influence of glucocorticoids that actively stimulate immune cells and recruit them to important immune sites, such as injured tissues, immediately after stress exposure (Dharbar & McEwen, 1996). Following this rise in immune activation, glucocorticoids suppress immune activity, presumably to bring levels back to baseline after stressor onset, thereby preventing the immune response from resulting in an autoimmune disease (Munck et al., 1984). Indeed, daily stressors are reported to induce worsened symptoms in patients with autoimmune diseases such as lupus erythematosus (Peralta-Ramirez et al., 2004), and rheumatoid arthritis (Affleck et al., 1997; Stone et al., 1997), while pharmacological levels of glucocorticoids help to alleviate these symptoms and maintain immunosuppression (Franchimont, 2004). Note: it appears to be the case that daily hassles worsen most diseases.

2. An Overview of the Concept of Stress

Stress is a common constituent of all living organisms. Borrowing from the framework offered by Newton's third law, that all actions have equal and opposite reactions, stress may be defined as a reactionary response to any manipulation exerted toward an organism,

whether negative or positive, in order to regain homeostatic balance. The reaction is typically a physiological one that helps the organism to regain equilibrium from the perception, or actual application of environmental, and/or physical challenges placed upon it (cited in Coe, 2002). Granted, this general concept that we understand today is more complex than Newton's third law articulates; similar organisms can respond to stress very differently; for example, some organisms' over- or even under-react to stressful stimuli depending on their coping strategies (Schouten et al., 1997).

Traditionally, to psychologists, stress has been primarily viewed as a response to negative stimuli. Usually the stress response is related to either chronic or acute emotionally negative experiences known as psychogenic stressors. Examples of stressors include, but are not limited to: social and interfamily conflicts, work pressures, death of a relative or friend, caregiver responsibilities, and the presence of illness in oneself or a close relation.

In the animal literature, the application of stressors such as those used in chronic mild procedures (Bielajew et al., 2003; Konkle et al., 2003; Bielajew et al., 2002), forced swim test (Bielajew et al., 2003; Porsolt et al., 1978, 1977a, 1977b), predator exposure (Anisman et al., 1997; Adamec & Shallow, 1993; Adamec, 1991) and social isolation (Baranvi et al., 2005) have all been designed to model the types of stressors that humans endure. This has resulted in the association of the term 'stress' with negative connotations; however not all stress results in a negative reaction. For example, when an animal is either under attack or in a predatory position, the 'fight-or-flight' response is initiated to help the survival of the organism; although the situation itself is negative, the response to the stress is a positive one (Cannon, 1929). In addition, similar physiological responses evoked by negative

stressors have also been shown to accompany either physical activity or novelty, even in positive contexts (Moncek et al., 2004; Konkle et al., in preparation).

Hans Selye viewed stress as a response, not only to psychogenic stressors, but to anything registered as a stressor, including physical demands (Selye, 1980). In his work (1980), he also differentiated between good, or 'eustress', and 'bad' stress arguing that both elicit the same physiological responses in a non-discriminate fashion. However, Selye also acknowledged that because there are different degrees of stress, the same response is not always produced (1980). Indeed, some studies are able to show physiological evidence of stress reactivity in response to enrichment (Moncek et al., 2004) whereas others do not (Belz et al., 2003; Morely-Fletcher et al., 2003; Schrijver et al., 2002). This difference may be particular to the methodology employed. For example, in some studies, rats are exposed to an enriched environment directly after weaning, and are therefore, relatively naïve to standard laboratory conditions (Morely-Fletcher et al., 2003; Schrijver et al., 2002). However, others (Moncek et al., 2004), including ourselves (Konkle et al., in preparation), report an increase in stress-related physiological activation following enrichment in animals previously housed in standard laboratory conditions; in this case environmentally enriched animals may represent an example of the 'eustress' that Selye proposed (Selye, 1980). These studies suggest that not only increased physical activity, but reward in the form of environmental enrichment, can be classified as a type of stressor. In short, the common underlying response to these 'good' and 'bad' stressors is activation of the HPA axis.

2.1. The HPA Axis: What It Can Do To You

The general adaptation response (Selye, 1936) is a physiological reaction to stress which results in the release of hormones and other chemical messengers that are mediated through the HPA axis. This axis represents the neuroendocrine link between the nervous and endocrine systems. The hypothalamic neuorsecretory neurons receive innervations from neurons that respond to both direct, and feedback effects of hormones present in the blood and in the cerebrospinal fluid (Coe, 2002). In addition, these neurosecretory neurons produce neurohormones that regulate pituitary secretion (Norris, 1997). The pituitary gland of adult mammals is located at the base of the brain, and is attached to the hypothalamus. Within this system is a portal that forms a neurovascular link between the hypothalamus and the pituitary gland. It is thought that blood containing neurohormonal regulators flows from the median eminence to the pars anterior, and that venous drainage then carries pituitary tropic hormones within the general circulation (Norris, 1997).

When an organism encounters a stressor, corticotropin-releasing hormone (CRH) is released from the hypothalamus, stimulating the anterior pituitary gland. This stimulation is one of many that cue the release of adrenocoticotropic hormone (ACTH) into the blood circulation which then acts on the adrenal cortex to release glucocorticoids. There are several analogues of glucocorticoids, but the two dominant forms are cortisol (hydrocortisone) in humans and primates, and corticosterone in rodents (Sapolsky, 2002; see also Lozovaya & Miller, 2003). Evidence suggests that different stressors result in varying patterns of hypothalamic hormone release depending on the response elicited; for example, responses to novelty and restraint stress may elicit oxytocin release whereas frustration and submission result in the secretion of both oxytocin and vasopressin

(reviewed in Scantamburlo et al., 2001). The important point is that both oxytocin and vasopressin have modulatory effects on CRH and the ACTH response to stress (Saccianoce et al., 1991; Wotjak et al., 1996; Neumann et al., 2000).

Some investigators attribute the variation in hormone release to stress specifically to the duration and intensity of the stressor (Ruisseau et al., 1978). This interpretation was based on the finding of similar hormone release in rats receiving cold stress, immobilization, or forced muscular exercise while different hormone secretions and patterns occurred in response to prolonged exposure to these stressors (Ruisseau et al., 1978). Moreover, long-term stress has been shown to induce physiological changes such as adrenal enlargement and thymus involution that parallel the corresponding alterations in growth hormone, prolactin, and lutenizing hormone levels that occur irrespective of the stressor protocol (Tache et al., 1978).

Activation of the stress response to negative and positive stimuli is important to survival in terms of its initiation of fight or flight reactions. The primary difference between a good and bad stress reaction appears to be related to the frequency and duration of HPA activation in that protracted physiological responses tend to change the survival benefit into a liability. This was clearly seen in Hans Selye's early work in which he quantified physiological compromises in both humans and animals exposed to prolonged stressors (Selye, 1936). The maladaptive consequences that Selye characterized were i) an increased level of endocrine activation and adrenal hormone titrations, ii) ulcerations in the stomach and small intestine, and iii) diminution in the size of immune glands, such as the thymus, as well as a decline in quantity of immune cells. It is particularly the latter consequence that is most detrimental to an organism's health.

3. Famous Henchmen of the Immune Response

Homeostatic maintenance is the physiological goal of living organisms; occasionally the mechanisms underlying this process fail resulting in sickness and disease. Cytokines are glycoproteins synthesized by immune cells and have pro- and anti-inflammatory effects upon other cells (Abbas & Lichtman, 2003; Janeway et al., 2001) and act in an autocrine and/or paracrine manner in addition to being endocrine signals (Plata-Salamán, 1998); chemokines are ligands that direct the migration of lymphocytes and phagocytic cells into the tissues from the blood (Abbas & Lichtman, 2003; Janeway et al., 2001). Consequences of both excessive and under activation of these mediators are affiliated with the etiology of cancer and autoimmune disease. Various treatment therapies, including cytokine and chemokine immunotherapy (CCI), have been developed to help restore the wellbeing of patients suffering from these diseases. Unfortunately, the reality of CCI is not as attractive as the theory because the positive aspects, such as recovery (Ghoreschi et al., 2003; Dutcher et al., 2000; Bernsen et al., 1998; Arenzana-Seisdedos et al., 1996), are accompanied by negative aspects including toxicity (Zhang et al., 2003; Dutcher et al., 2000). The reason for this is that the signalling pathways utilized via cytokine and chemokine communication are not direct, but are instead a complicated network of cells that mediate messages to other sources (Gaffen et al., 2001). Because of this, any single cell can simultaneously receive death and survival signals as a result of the actions of pleiotropy (the ability to act on several different cells), redundancy, and specificity in cytokine and chemokine therapy. On the one hand, the nature of these chemicals allow them to interact in sequence so that the release of a single cytokine, for example, will result

in the synthesis or release of additional cytokines that also exert regulatory capabilities (Plata-Salamán, 1999). Alternatively, cytokines can act 'in concert', that is, the physiological or behavioural end result is a function of the total stimulatory and inhibitory actions of multiple cytokines (Plata-Salamán and Ilyin, 1997).

Tumor-necrosis factor (TNF) is a cytokine family that has several biological implications, most likely because of its expression on all cell types (Aggarwal, 2003). Originally, TNF was thought to be an anticancer product; however its other actions, namely toxicity (including fever, chills, hypotension, and disrupted lipid metabolism), have negated its potential as a safe therapeutic agent (Feinberg et al., 1988). In addition, TNF-α up-regulates adhesion molecules and vascular growth factors which are involved in metastasis and invasion, implicating TNF as a tumour growth factor in addition to its anti-cancer effects (Aggarwal, 2003). To complicate matters, mice deficient in TNF-α are protected against the development of skin tumours (developing 5-10% the amount associated with control counterparts) when they receive either repeated exposure to the carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA), or when they receive both DMBA and 12-O-tetradecanoylphorbol-13-acetate (Moore et al., 1999), suggesting one mechanism underlying cancer prevention. However, due to the pleiotropic activity of TNF, this may not be a wise approach as the protein is necessary for haematopoiesis (formation of blood cells), (Adams et al., 2002) and protection from bacterial infections such as Listeria monocytogenes (Fontan et al., 2001). Despite its drawbacks, like other treatments that elicit adverse symptoms, TNF therapy is still considered useful in the treatment of Crohn's disease (inflammatory bowel disease) (Suenaert et al., 2002) and various types of cancers (Couriel et al., 2000).

Clinically, the anti-inflamatory cytokine, interleukin (IL)-2, also known as Proleukin therapy, offers encouraging results in the treatment of cancers, such as renal cancer and melanoma, which are typically non-responsive to standard treatment regimes (Hanish et al., 1997). Unfortunately, the high neurotoxicity affiliated with IL-2, such as behavioural deficits including aggressiveness and anhedonia, as well as cognitive deficits such as memory impairments (Caraceni et al., 1992; Denicoff et al., 1987), temper its efficacy. Each of these symptoms appears to be related to the dosage and treatment schedule (Denicoff et al., 1987). Indeed IL-2 associated molecules are located in neuronal bodies and astrocytes (Nistico, 1993), with the highest distribution within the hippocampus and significant levels being found in the locus coeruleus, caudate nucleus, and hypothalamus (Otero et al., 1995; Nistico, 1993; Lapchak et al., 1991; Araujo et al., 1989).

The vascular leak syndrome is another devastating symptom of IL-2 therapy and is thought to be induced by cytolytic effects on endothelial cells followed by cardiac myocytes (muscle tissue cells) resulting in lesions (Zhang et al., 1993).

Interferon-α (IFN-α), clinically known as Intron A (Schering-Plough, 2004) is another anti-inflammatory cytokine that has been shown to have considerable utility in the treatment of cancer and other diseases such as chronic hepatitis C, and multiple sclerosis (Schering-Plough, 2004; Nguyen, 2003; Arnaso et al., 1994). Accompanying IFN-α's treatment benefits are the risks of negative symptoms similar to those that may arise in conjunction with IL-2 therapy. Interestingly, the negative effects of IFN-α treatment have not been adequately explored in the literature although animal studies suggest that IFN-α gains entry into the brain via weakly protected areas of the blood brain barrier granting access to the pons and hypothalamus (Smith, 1986; Smith, 1985). In the clinical setting,

IFN-α is administered daily, or every other day (Fujioka et al., 1995) in the form of a subcutaneous, or occasionally, an intravenous injection. The negative effects that have been reported as a consequence of clinical IFN-α treatment seem to be a function of dosage. Low dose therapy (300,000 to 600,000 international units/m^2) (Beratis et al., 2005) is reported to result in fatigue, anorexia, and flu-like symptoms such as fever, chills, and somnolence (Myers et al., 1992; Adams et al., 1988; Quesada et al., 1986) while higher doses (1 million international units (MIU) /m^2 to 50 MIU/m^2) (Musselman et al., 2001) produce an increased likelihood of cognitive impairment including concentration disruptions in addition to lack of coordination and emotional disturbances such as anhedonia (Musselman et al., 2001; Myers et al., 1992; Goldstein et al., 1988; Iivanainen et al., 1985; Smedley et al., 1983). Disruptions in alertness are apparent, even after a single low dose (0.1 MIU/m^2) in healthy volunteers; this effect persists even ten hours later with slowed reaction time after one dose of 1.5 MIU//m^2 (Smith, et al., 1988).

Due to the overwhelming number of subjective accounts of depression resulting from immunotherapy, a substantial number of clinical studies have been undertaken to assess this aspect of IL-2 and IFN-α treatment. An example of one of these studies is the following by Maes and colleagues (2001) who assessed depressive symptoms in conjunction with IL-6 cytokine levels in patients treated with IL-2, IFN-α alone, or the two in combination. The Montgomery and Asberg Depression Rating Scale (MADRS) was administered and blood samples collected before, three, and five days after the start of treatment. Both the depression scores and IL-6 serum levels increased over time, but only in patients receiving IL-2 alone, or in combination with IFN-α – not in patients treated with IFN-α alone.

Overall, patients receiving IL-2 therapy score significantly higher on instruments such as the MADRS as early as three days following the commencement of drug administration (Maes et al., 2001; Capuron et al., 2000). Symptoms of depression typically occur in patients treated with IFN-α a few weeks after the start of treatment (Capuron et al., 2000), and these symptoms are often cited as reasons for non-compliance in therapy regimens (Musselman et al., 2001). In these studies (Maes et al., 2001; Capuron et al., 2000), depression scores were higher in patients receiving the combined IFN-α and IL-2 treatments than in those receiving either treatment alone, particularly at the beginning of the treatment regimen. This suggests that IL-2 and IFN-α have synergistic actions in the development of depression (Wichers and Maes, 2002). Interestingly, depression does not occur in all patients; the full scale disorder tends to occur in approximately 36% of those undergoing IFN-α therapy (Collier and Chapman, 2001), suggesting that there are underlying risk factors in the development of this symptom.

In a recent study Capuron et al (2004) explored suspected risk factors in patients who were scheduled to undergo immunotherapy. Prior to treatment, patients were evaluated for social support, sleep quality, somatic complaints, and depression using the MADRS. They were again evaluated on these measures one month after the commencement of treatment. Patients who had high to moderate MADRS scores at the end of the study had also scored higher scores in general at baseline (particularly on the sadness factor). At the cessation of the experiment, this corresponded to 50% of patients with mild, and 22% of patients with moderate depression (Capuron et al., 2004). See Appendix A for a list of selected articles that document in-depth the emotional and cognitive disruptions attributed to immunotherapy, specifically IL-2 and IFN-α. Overall, the pleiotropic effects of CCI

therapy elicit optimistic outcomes; however the associated negative side effects severely mar the positive effects of recovery.

The etiology of depression is likely pluralistic in nature, with several factors contributing to its onset and progression. Evidence tying the administration of cytokine agents used as immunotherapy to depression has propelled a new hypothesis that immune activation underlies the initiation of this disorder. It has long been recognized that immune functioning is compromised in depressed patients (Maes et al., 1994; Hickie et al., 1993), which makes an assertion such as the cytokine-hypothesis difficult to reconcile, although prolonged activation may impair the immune system (Maes et al., 1995; Maes et al., 1994). In addition, it is reported that infectious diseases such as influenza and herpes, as well as non-infectious disorders such as multiple sclerosis are all accompanied by immune activation and a higher propensity of depressive symptomology (Yirmiya, 1996). Granted, the depressive symptoms may alone be due to the psychological reaction to illness. Still, the pathogenesis of cytokine-induced depression may not be a simple under- or over-activation of the immune system, but relates perhaps to cytokine deregulation in general.

In favour of the cytokine over-activation perspective are some investigators who report increased plasma levels of IL-6 and IL-2 in patients with major depression (Trzonkowski et al., 2004; Kubera et al., 2000; Maes et al., 1997; Sluzewska et al., 1996; Maes, 1995; Nassberger and Traskman-Bendz, 1993), although others have observed depressed patients without elevated peripheral cytokine levels (Basterzi et al., 2005; Marques-Deak et al., 2005; Weizman et al., 1994). One group, who observed differences in IL-6 levels compared to healthy controls, reported IL-6 to vary as a function of the circadian rhythm with the highest plasma IL-6 levels being quantified in the morning (Alesci et al., 2005). Anisman

and colleagues (1998) measured increased IL-1β in the supernatant of mitogen (plant-derived protein that stimulates lymphocyte proliferation) stimulated lymphocytes from chronically depressed patients, but not in patients with major depressive disorder, suggesting that the severity of depression may be central to the level of cytokines (Anisman and Merali, 1999). There are several other examples of immune activation that accompany major depression such as increased quantities of peripheral neutrophils and leukocytes, activated T-cells, as well as elevated levels of prostaglandin secretion (Muller, 1995; Maes et al., 1992; Calabrese et al., 1986). Also, serotonin and norepinephrine, two neurotransmitters well known for their involvement in depression, have been identified as immunoregulators (Plata-Salaman, 1991). Cytokines affect both of these monoamines (Plata-Salaman, 1991) and antidepressants and cytokine antagonists can reverse immune disruptions and depression (Sammut et al., 2002; Yirmiya et al., 2001; Maes et al., 1999), although there is a paucity of research investigating the utility of anti-inflammatory drugs in the treatment of depression. Overall, these findings have resulted in a proposed cytokine model of depression, despite the uncertainty of immune activation as preceding depression, or merely as an epiphenomenon.

4. Cytokines as Stressors

The behavioural and cognitive consequences of cytokine therapy appear to be related to the stressful nature of cytokines on living organisms, as demonstrated through experiments showing that cytokines activate the HPA axis, thus eliciting the stress response. One of the first systematic observations supporting this contention occurred in 1986 when Besedovsky and colleagues showed the reciprocal relationship between cytokines and the HPA axis. In

a series of experiments, they demonstrated that *in vivo* production and action of interleukin (IL)-1 was inhibited by glucocorticoids, in addition to showing that glucocorticoid blood levels increased in response to factors released by Newcastle virus, and that this action was inhibited by the application of an IL-1 antibody (Besedovsky et al., 1986). Furthermore, they demonstrated that administration of IL-1 increased ACTH and glucocorticoid levels in both rats and mice (Besedovsky et al., 1986). Additional work by the same group corroborated these findings by showing that an intraperitoneal injection of human recombinant IL-1 in rats increased peripheral blood levels of corticosterone and ACTH, as measured via pituitary adrenal activation (Berkenbosch et al., 1987). In the same study, they observed that when corticotropin releasing factor was neutralized, the IL-1 affiliated increase in ACTH blood levels was attenuated (Berkenbosch et al., 1987).

Historically, this was an exciting time because the field of psychoneuroimmunology had not yet been validated in the scientific community. It was still a widely held belief that the immune system was its own entity, functioning separately from the endocrine and central nervous systems (CNS). Although some early studies had investigated the relationship between stress and disease, the work failed to attract any notable attention (Ader, 2000).

The first series of these investigations took place in the 1950's when Rasmussen documented the effects of avoidance conditioning, physical restraint, electric shock, and social crowding on mice that had been inoculated with the herpes virus (Rasmussen et al., 1957); the findings revealed that the progression of the viral infection was dependent on the nature of the stressor (Rasmussen et al., 1957). Some of this work even examined the suppressing effects of stressors on the production of cytokines such as IFN in virally infected animals (Chang and Rasmussen, 1965). Even a decade later, Ader's serendipitous

observations using taste aversion tasks, that the immunosuppressive effects of cyclophosphamide could be classically conditioned (1974) went by relatively unnoticed, but the gradual culmination of studies showing the direct impact the immune system had on the CNS and endocrine systems could not be ignored (Ader, 2000). With respect to the specific work with cytokines, it was the repeated demonstrations, which still continue today, that these glycoproteins, particularly IL-1, can be exogenously administered and activate the HPA response, that eventually certified them as stressors (Anisman et al., 1993; Dunn, 1990).

Contemporary work provides ample evidence that this relationship between cytokines and the stress response exists, not only with the administration of IL-1 (Dunn, 1990), but of other cytokines as well. Recombinant murine TNF-α administered intracerebrally to male Wistar rats stimulates corticosterone levels as measured via microdialysis; this increase peaks between 30 and 60 minutes following injection, with both the 50 ng and 100 ng doses eliciting the described pattern (Pauli, et al., 1998). Intracerebral ventricular injection of recombinant human IL-2 in male Wistar rats activates the HPA axis in a dose dependent manner; free corticosterone measures reached maximum levels between 2 and 2.5 hours following IL-2 administration, particularly in response to the highest dose (500 ng) (Pauli, et al., 1998). Systemic IL-2 injection does not result in these hormone changes, although there is an increase in hypothalamic norepinephrine turnover (Zalcman et al., 1994), and decreased dopamine release in the nucleus accumbens (Anisman et al., 1996).

Interestingly, on its own, IL-6 administration in mice failed to elicit any measurable changes in corticosterone concentrations in the periphery (Brebner et al., 2000); however, co-administration with IL-1 resulted in synergistically induced increases of ACTH and

corticosterone in rat (Zhou et al., 1996) which surpassed the HPA activation evoked by IL-1 administered alone (Zhou et al., 1996). Moreover, IL-6 challenge, in conjunction with exposure to a novel stressor synergistically activated the stress response in rats, in comparison to vehicle controls, or animals exposed to the novelty stressor, or IL-6 alone (Zhou et al., 1996). However, this effect appears to be stressor specific in that restraint stress, combined with IL-6, failed to elicit comparable synergistic actions (Zhou et al., 1996). Synergistic activity, between IL-1β and a neurogenic stressor (air puff to the eyes), has also been demonstrated via a pronounced increase in monoamine variations within the nucleus accumbens, hippocampus, and prefrontal cortex, as measured by microdialysis (Merali et al., 1997).

In this work, the elucidation of changes in monoamine neurotransmission that immune challenges evoke is partly due to the action of stressors on monoamine activity in the brain, particularly that of norepinephrine and serotonin. This finding begs the notion that cytokines, if they are stressors, should induce similar changes. Indeed, systemic and intracerebral administration of IL-1α and IL-1β significantly increase norephinephrine turnover within the hypothalamus and paraventricular nucleus, in addition to the serotonin metabolite 5-HIAA in the hypothalamus (Dunn, 1992). Both cytokine administration and acute stressor exposure have also been reported to increase serotonin levels in the hippocampus (Pauli et al., 1998; Kalen et al., 1989). Kalen et al (1989) have demonstrated monoamine influences in response to acute stressors while monitoring norepinephrine and serotonin release over a 24 hour period in freely moving rats; when animals received a tail pinch, the two monoamines each increased by approximately 50%. In another microdialysis experiment however, restraint stress evoked a decrease of norephinephrine and serotonin in

the locus coeruleus of both rats and mice, whereas serotonin was decreased in the hippocampus of rats, but not mice, suggesting a stressor and species specificity in neurochemical responses to stress (Konstandi et al., 2000). Still, by employing microdialysis techniques, investigators have demonstrated that both IL-1β and TNF-α increased monoamine activity in the mouse locus coeruleus, and central amygdala (Brebner et al., 2000); of interest, the neuronal firing pattern in these two structures has been shown to be dependent on the length of stress exposure, at least with respect to restraint stress (Valles et al., 2006; Crane et al., 2005).

Curiously, systemic injections of IL-2 increased norepinephrine levels within the central amygdala and median eminence, but resulted in lowered activity in the locus coeruleus (Lacosta et al., 2000). Perhaps the difference in neurotransmitter release patterns is dependent on the duration of action, and/or intensity of individual cytokines. This may be comparable to the diverse physiological effects reported in response to more traditionally accepted stressors such as tail pinch and restraint stress. Still, there is a difference between immune challenges and acute stressors, in terms of neurotransmission in the raphe nucleus; in general, immune challenges decrease, while acute stressors tend to increase the serotonergic activity in this brain site (Kennett and Joseph, 1981; Bliss et al., 1972).

An additional point to mention is that the effects of cytokine challenge may differ as a function of dose and route of administration, as well as the specific outcome being measured (Pollmacher et al., 2002; Petitto et al., 1997). Receptor specificity is another element primary to the effects of cytokine administration and any neurochemical alterations in discrete brain areas. Returning to the work done by Pauli and colleagues (1998), IL-2, as opposed to TNF-α administration, was shown to increase serotonin efflux in the

hippocampus of Wistar rats. This effect was attenuated by pre-treatment with an Il-1 receptor antagonist providing evidence that the influence of IL-2 on hippocampal serotonin transmission is mediated through IL-1 mechanisms (Pauli et al., 1998). Despite the differences in neurochemical changes in response to cytokine challenge, and other stressors, there appears to be a general consensus that the consequences of stressors are mediated via serotonergic receptors (Shimizu et al., 2000; Grignaschi et al., 1993).

5. The Mechanics of Sickness

Considering the cytokine and central amine variations that occur in response to an immune challenge, it may come as no surprise that such changes are 'accompanied' by a number of disturbances. In fact, many of these such as anorexia, REM sleep changes, decreased sexual motivation, and emotional upheavals, for example, are mediated by serotonin (von Meyenburg et al., 2003; Imeri et al., 1999). These disturbances, which also include decreased motor activity and cognitive dysfunction, are known to be associated with sickness in both humans and animals. It was Hart (1988) who first recognized that these behaviours, as a group, also known as the acute phase response, centered on inducing and maintaining fever as an organized strategy to help the organism fight viral, bacterial, and parasitic infections. This idea incited an array of studies focusing on the role of the immune system in the activation of these behaviours, now more widely known as sickness behaviours (Kent et al., 1992).

Bennett and Beeson (1953) first delineated the role of leukocytes in releasing a fever-inducing factor. However, it was the work by Kluger and associates (1978; 1975) that demonstrated that fever, once thought to be a detriment to sick individuals, actually

suppressed bacterial and viral growth in the body (reviewed in Hart, 1988). The first of these was a study investigating the survival rates of lizards infected with the bacterium *Aeromonas hydrophilia*; lizards kept in cold environments had significantly lowered survival rates compared to those kept in warm environments (Kluger et al., 1978). Furthermore, lizards treated with an antipyretic were likely to die, whereas the lizards permitted to develop a fever in response to infection had significantly higher survival rates (Bernheim and Kluger, 1976). This same effect was seen in the mammalian system when Kluger and Vaughn (1978) infected rabbits with *Pasteurella multocida.*

Febrile responses help organisms fight infection by initiating the non-specific immune system as demonstrated through increased neutrophil killing of bacteria (Sebag et al., 1977), for example. The acquired arm of the immune system is also activated by fever in that lymphocyte proliferation is augmented (Smith et al., 1978). It is important to note, however, that fever is not always induced in response to infection and disease (Atkins and Bodel, 1979) and significant individual variability in the febrile response has been reported (Stitt, 1985).

The introduction of the fever response occurs when activated immune cells such as macrophages, lymphocytes, and phagocytic cells synthesize endogenous pyrogens, such as IL-1, that lower iron and zinc levels in plasma which in turn elevate sodium excretion leading to pathogen resistance (Dinarello, 1984). Most bacteria require iron in order to self-multiply. The increase in fever that reduces available iron levels therefore stalls the reproduction of pathogens which otherwise grow optimally at levels either equivalent, or slightly below regular body temperature (Weinberg, 1984). During the febrile response, the hypothalamic set point is raised, possibly through the increased synthesis of prostaglandins

(Milton, 1982) so that the organism perceives cold, initiating heat conservation. It was Hart's contention that alongside this physiological mode of fever, in response to sickness, was a behavioural component, including piloerection and shivering in addition to other heat maintenance and energy conserving behaviours that potentiate thermoregulatory actions (1988).

The field of psychoneuorimmunology is now largely focused on the question of how the immune system communicates with the brain to produce these behaviours, and the literature suggests that cytokines are the primary mediators.

Tumor necrosis factor and IL-6 are cytokines that do not reliably induce sickness behaviours (Swiergiel et al., 1997); however the action of IL-1 alone does not explain all sickness behaviours (Larson and Dunn, 2001). For example, peripheral administration of IL-1β did not elicit learning or memory impairments in the Morris water maze in the assessment of acquisition, consolidation, or retention of spatial information, despite cognitive disruptions being accepted as sickness behaviours (Thomson and Sutherland, 2006). The administration of IL-1 has also been shown to suppress sexual behaviour, but only in female rats (Yirmiya et al., 1995). In another study, endotoxin challenge suppressed sexual receptivity in female rats, presumably because of the synergistic actions of IL-1 and TNF-α. Interestingly, simultaneous administration of antagonists associated with both of these cytokines reinstated sexual behaviour, but not when administered separately (Avitsur and Yirmiya, 1999). Peripherally administered IL-1 receptor antagonist is able to block most of the sickness effects produced by endotoxin administration such as decreased social exploration and body weight; however intracerebral administration of IL-1 receptor antagonist is not able to prevent these effects (Bluthé et al., 1992). This suggests

that it is the combined breakdown of metabolites and their actions across the blood brain barrier that prevents sickness induced by endotoxin.

The bacterial endotoxin, lipopolysaccharide (LPS), is frequently used in studies investigating cytokines because its administration results in a well-mapped release of the glycoproteins (Schotanus et al., 1993; Zuchermann et al., 1989) that induce the basic pattern of behavioural sickness (Kent et al., 1992).

This endotoxin is a component of the cell wall of gram negative bacteria and activates monocytes and macrophages to release cytokines, particularly IL-1α, Il-1β, TNF-α, and IL-6 in addition to the IL-1 receptor antagonist, IL-1ra (Laye et al., 1994; Gatti and Bartfai, 1993; Shotanus et al., 1993), resulting in elevated norepinephrine and serotonin levels (Linthorst et al., 1995; Dunn, 1992) in the mouse hypothalamus and rat hippocampus, respectively.

Since cytokines are large hydrophilic proteins (Il-1β is 17.5 kD, human IL-2 is 15.5 kD, IFN-α is between 17.5 and 23 kD depending on the subtype), they cannot passively cross the blood brain barrier (Dantzer et al., 2000). This suggests that cytokines may be synthesized in the brain. Indeed some work shows this to be the case, at least with respect to IL-1 (Konsman et al., 1999; Tada et al., 1994; Bandtlow et al., 1990). In the mouse brain, investigators utilizing immunohistochemical methods detected IL-1 receptors in the hippocampus, cerebellum, and choroid plexus (French et al., 1999) as well as in the anterior pituitary (French et al., 1996), corroborating evidence that cytokines are synthesized from glial cells within the brain (Konsman et al., 1999). In addition to this, transport systems have been identified in the movement of IL-1, Il-6, and TNF across the blood brain barrier (Plotkin et al., 2000; Banks et al., 1995) although there is some doubt as

to whether the amount transferred into the brain is adequate to exert any neurochemical or behavioural changes (Larson and Dunn, 2001; Watkins et al., 1995). Nonetheless, physiological consequences of cytokine challenge, such as fever, have been reported in response to unquantifiable serum amounts of LPS, or other cytokines such as IL-1β (Kluger, 1991; Long et al., 1990; see also Watkins et al., 1995). From this perspective, cytokines have been reasoned to instead act on circumventricular organs that lack the blood brain barrier, stimulating the release of prostaglandins to induce both corticotropic and pyrogenic cytokine activity (Katsuura et al., 1990; Stitt, 1995).

Conversely, based on Fos protein distribution studies, the general route of activation for LPS following systemic administration is presumed to begin with vagus nerve projections to various brain regions including the nucleus tractus solitarius, parabrachial nucleus, hypothalamic paraventricular and supraoptic nuclei, and amygdala (described by Dantzer, 2001). Injection of LPS results in IL-1 expression within the circumventricular organs followed by the brain parenchyma where it is synthesized by microglia cells (Konsman et al., 1999). Lipopolysacharide induced expression of rat IL-1ra mRNA is at its height following the IL-1 peak (Licinio et al., 1991), and most of the effects induced by LPS are thought to be mediated via these IL-1 mechanisms. Delineation of this pathway was important because it illustrates a route by which peripherally synthesized cytokines have an influence on brain and behaviour.

Further evidence points to vagal activation as being integral to the behavioural effects induced by peripheral cytokines, particularly that of IL-1. This interpretation arises from work in rats and mice demonstrating that both the LPS- and IL-1- mediated decreases in social exploration (Bluthé et al., 1994) and food-intake (Bret-Dibat et al., 1995), in addition

to LPS induced fever (Watkins et al., 1995) are abolished following vagotomy. Interestingly, IL-1 receptors are undetectable on the vagus nerve; however they have been located on nearby terminals, known as paraganglia (Goehler et al., 1997). Maier and Watkins (1998) proposed that IL-1 binding onto the paraganglia releases a signalling transmitter that synapses onto the vagus nerve, initiating the afferent transmission to the brain, specifically to the nucleus tractus solitarius and the area postrema (see Watkins et al., 1995; Maier and Watkins, 1998).

5.1. A Shout out to Past Sickness Behavioural Investigators

Pro-inflammatory cytokines such as IL-1 and IL-6 are not used in clinical treatments, whereas the anti-inflammatory cytokines, IFN-α and IL-2 are. Interestingly, these two cytokines appear to elicit behavioural effects similar to those induced by IL-1, LPS, and less frequently IL-6, and TNF, although IFN- α and IL-2 have not been investigated as thoroughly. Some laboratories are now attempting to develop, and validate, animal models of IFN- α and IL-2 immunotherapy in order to understand the mechanisms underlying the negative symptoms, including depression, in the clinical population; however the work completed so far has been rather conflicting in comparison to the robust sickness behaviours induced by the pro-inflammatory cytokines.

5.2. Sleep and Lethargy

There are several measures used to assess the behavioural profile of a sick animal. Some investigators evaluate a variety of physical behaviours to assess general health status. In particular, ptosis (droopy eyelids), piloerection (raised fur), lethargy, and sleep are

observed in order to create a composite score that indexes the overall health consequences of an animal in response to TNF-α and IL-2 (Sudom et al., 2004; Anisman et al., 2003; Hayley et al., 2002; Hayley et al., 1999). For example, these investigators employ an overall sickness score that encompasses the ratings: 1 = normal looking, 2 = slightly lethargic, slow movement, slightly ruffled fur, eyes slightly drooping, 3 = lethargic, fur ragged, eyes drooping, breathing altered, and 4 = very sick appearance, ptosis, ragged fur, curled body posture, difficulty breathing, general non-responsiveness. From this scale, Anisman's group (Sudom et al., 2004; Anisman et al., 2003; Haylery et al., 2002; Hayley et al., 1999) has been able to distinguish the sickness behaviour effects of TNF-α and IL-2 in CD-1 mice.

Sleep and lethargy are particularly important to assess because maintaining a quiet state is integral to the recovery process. This is due to the increase in energy stores that accompany reduced activity, which are then used to produce fever and conserve heat (Maier and Watkins, 1998; Hart, 1988). Somnolent-related behaviours are well documented, even during the wake period, in response to cytokine exposure, particularly with respect to IL-1. Intracerebral injection of IL-1 into rabbits and rats produces the classically known increase in slow wave activity (Krueger et al., 1984; Tobler et al., 1984). Although IL-6 appears to only modulate sleep (Hogan et al., 2003), the administration of other pro-inflammatory cytokines, such as TNF-α, IL-18, also increase the amount of non-rapid eye movement (nREM) sleep as characterized by heightened amplitude on the electroencephalogram (Kubota et al., 2001; Kruger and Majde, 1995; Kapas and Krueger, 1992). This appears to be related to cytokines precipitating their own effects on transmitter systems, specifically through the inhibition of serotonin in the dorsal raphe nucleus, the activity of which is known to induce wakefulness (reviewed in Portas et al., 2000).

Microinjections of 0.5 ng of IL-1β into the dorsal raphe nucleus of conscious rats increased nREM sleep for two hours following administration (Manfridi et al., 2003). In the same study, electrophysiological recordings of serotonin neurons in the dorsal raphe of the guinea pig were observed to decrease by 50% following infusion of IL-1β into the brain site (Manfridi et al., 2003) suggesting that IL-1β has a significant impact on serotonin transmission related to sleep induction.

The anti-inflammatory cytokine, IL-2 is also known to elicit sleep behaviours in rats and rabbits. In their study, Kubota and associates (2001) found that rabbits administered an intercerebral injection of IL-2 showed a dose dependent increase in nREM sleep during the beginning of the dark period (Kubota et al., 2001). Intracerebral administration of either the human or rabbit source of IFN-α produced a similar sleep profile to that induced by IL-2 and pro-inflammatory cytokines in rabbits - an increase in nREM sleep (Kimura et al., 1994). In rats, both the rat and the human subtype of IFN-α administered to the third ventrical or the locus coeruleus, resulted in sleep (De Sarro et al., 1990); again sleep is presumed to help the organism in recovery against infection (Maier and Watkins, 1998; Hart, 1988). It is believed that sleep during the recuperative process compels the organism to remain in a still or heat conserving position, such as curled up, or to remain in a sought out warmer environment, to help raise body temperature (Hart, 1988).

5.3. Piloerection and Temperature

Piloerection is another important behaviour employed by animals to increase body temperature. This is known as 'goose bumps' in humans and is a reflex under control of the sympathetic nervous system in response to the re-direction of blood from the periphery to

the internal organs (Hart, 1988). When an organism becomes cold, muscles in the skin contract to pull the hair upright, creating a cover of insulation, preserving heat. The insulation properties of the rat coat is fundamental to heat-maintenance; depilation (hair removal) of the rat increases heat loss from 30 to 50% in an environment maintained between 24 and 26°C (Roussel and Bittel, 1979).

In rats, piloerection is known to be readily induced by IL-1, TNF-α, IL-2, and LPS challenges (Hayley et al., 1999, 2002; Lacosta et al., 1999; Pauli et al., 1998; Linthorst et al., 1994), although the magnitude of this effect is not typically evaluated. Instead, the behaviour is assessed alongside other physical attributes, and a composite score is used as an overall indicator of the animal's health.

As mentioned, each of the above behaviours contributes to thermoregulation and fever induction in order to slow the growth of pathogens and stimulate immune cell proliferation (Dinarello et al., 1984; Kluger et al., 1978; Smith et al., 1978; Sebag et al., 1977; Kluger et al., 1975). The neural-pathway by which fever is induced is located in the pre-optic region of the anterior hypothalamus (Myers et al., 1994), mediated through prostaglandin E2 activation (Konsman et al., 2002; Dinarello et al., 1984). Heat gain behaviours such as shivering and piloerection have been proposed as being under the control of an inhibitory mechanism in the pons (Amini-Sereshki and Zarrindast, 1984) and electrical stimulation of the pons has been reported to affect activity in the pre-optic anterior hypothalamus (Eisenman, 1974). Together, this supports the idea that there is reciprocal communication between these two structures that regulates temperature and heat-maintenance behaviours.

In the laboratory rat, core temperature is generally recorded using rectal thermometers, or else by abdominally implanted telemetry devices monitored by radio-frequencies

(Lomax, 1966). In the animal literature, brain temperature has often been considered comparable to core temperature; however this has been shown as incorrect. Brain temperature values can fluctuate over 1 °C across daily rhythms (Abrams and Hammel, 1965). Moreover, significant changes in brain temperature take place between periods of inactivity and activity (such as feeding) (Abrams and Hammel, 1964).

The typical core temperature range over a nycthemeron, as measured by rectal probe in an unrestrained rat, is 37 to 38°C when ambient temperature is between 20 to 24°C. As expected, this range has been shown to deviate when animals are administered cytokines. For example, both IL-1 and TNF-α induce a curled body posture and fever. Intracerebral injection of IL-1β into conscious rats produces an average 1.8°C increase from baseline rectal temperature (Dascombe et al., 1989) while intraperitoneal injection of murine TNF-α into rats elicits values 0.9°C higher than in control animals (Stefferl et al., 1996). Comparable temperature elevations have been recorded in mice that received an intravenous injection of IL-1 (Duff and Durum, 1983). In that study, rectal temperature was chronicled while mice were individually housed in an incubator set to 33°C until temperature was stabilized for one-hour. Following cytokine administration, recordings continued over 15 minute intervals. Baseline values held fast at 37.28 ± 0.1°C; however IL-1 induced fever that ranged from a 0.4 to 1.9°C increase above normal values (Duff and Durum, 1983).

Changes in temperature also accompany administration of IFN-α and IL-2, particularly with high doses. Patients treated with these two cytokines separately (Schwinger et al., 2005; Eton et al., 2002; Cotler et al., 2000), or in combination (Culine et al., 2006) often experience feverish responses (>39°C) (Schwinger et al., 1995).

The animal literature also includes demonstrations of temperature being altered by IFN-α and IL-2 cytokine challenge. Intracerebral administration of IL-2 in the Zucker rat elicited a modest increase in core temperature as measured by radiotelemetry (Plata-Salamán et al., 1998), and the same fever response profile was noted in rabbits (Kubota et al., 2001). With respect to IFN-α, there is controversy regarding both the magnitude and direction of this change. Some investigators report elevated temperatures in guinea pigs (Blatteis et al., 1991), cats, mice, and rabbits (Dinarello et al., 1984), while others observe small increases (Plata-Salamán et al., 1992) or decreases in the temperature of rats (Sammut et al., 2001). Procedural variations appear to be the reasons underlying these inconsistent results, including differences in the animal models used between studies. For example, each group employed a different dose range of IFN-α and administration route. One group gave an intraperitoneal injection of the rat subtype of IFN-α to Listar Hooded rats (10, 100, or 1000 units), observing the highest dose to produce the largest hypothermic effect (-0.3°C) 20 minutes following injection; this effect dissipated 40 to 80 minutes later (Sammut et al., 2001). The other group (Plata-Salamán, 1992) gave intracerebral injections of rat IFN-α (15 – 225 units) to Wistar rats and observed a small increase in rectal temperature. Hyperthermic effects of IFN-α were also reported by Blatteis and colleagues (1991) who injected the human form of the cytokine intraventricularly at a dose of 1×10^7 units in guinea pigs. A pyrogenic peak (0.5°C) was noted 75 minutes following cytokine administration, with a second increase (1.2°C) occurring 210 minutes later. Dinarello and associates (1984) also demonstrated a hyperthermic effect of human IFN-α with a peak (0.7°C) between 75 and 110 minutes following intravenous injection in rabbits.

Methodological differences, such as those described above, may also account for differences between studies with respect to the anorectic effect of cytokines, particularly IFN-α (see below) on food and water intake, and body weight.

5.4. Ingestive Behaviours

A disruption in ingestive behaviour can be elicited by cytokine interactions. Inhibition of feeding due to these glycoproteins has been observed in both humans and animals. Anorectic effects are common symptoms of cancer, autoimmune diseases, viral sickness, and immunotherapy (Plata-Salamán, 1996). In a rat tumor model, IL-1 levels in the cerebrospinal fluid have been found to be inversely correlated with the level of food consumption, and associated with increased hypothalamic serotonin neurotransmission (Laviano et al., 1996) as well as diminished release of neuropeptide Y (Chance et al., 1995). Additionally, LPS can induce short-term anorexia in rats and mice (Dunn and Swiergiel, 2005; Plata-Salamán, 1996 et al., 1998b; Swiergiel et al., 1999; Swiergiel et al., 1997a). Intraperitoneal injection of IL-1 in male Wistar rats significantly reduced food intake in conjunction with an increase in the release of corticotropin releasing factor (Uehara et al., 1989); this effect, inhibition of feeding, was prevented when corticotropin releasing factor was neutralized in the brain, suggesting an additional mechanism of food intake suppression (Uehara et al., 1989).

In addition to its effect on food intake, cytokine challenge is also associated with a decrease in fluid consumption. Chance and Fischer (1991) reported diminished food and water intake, as a consequence of an intrahypothalamic injection of IL-1, that persisted from 4 to 8 hours. Injection of IL-1β into the third ventricle of rats, that had been water

deprived or placed in other thirst-inducing conditions, reduced water intake unless the animal was pre-treated with a central administration of naloxone, suggesting that opiods interact with IL-1 challenge, at least with respect to fluid intake (Luz et al., 2006). Other investigators have noted changes in water intake in combination with a decline in food consumption following intraventricular injection of TNF (Mahony and Tisdale, 1988). The intake of sweet fluids such as chocolate milk, that have a high caloric count, have also been reduced after intraperitoneal IL-1β and TNF-α cytokine administration (Hayley et al., 1999; Brebner et al., 1998). Similar effects have been observed on the consumption of flavoured non-caloric fluids subsequent to LPS administration (Yirmiya, 1996). However, reduced fluid intake is not a prerequisite of hypophagia; it has been shown that cytokines, such as IL-1β, suppress feeding without interrupting water intake (Plata- Salamán et al., 1988).

Not surprisingly, these changes in consumption are frequently accompanied by reductions in body weight (Reyes-Vazquez et al., 1994; Cesario et al., 1991; Mahony and Tisdale, 1988). For example, daily intravenous treatment of high dose recombinant IL-2 (900,000 units) in Wistar rats produced a dramatic reduction in both food intake and body weight over a nine day monitoring period (Cesario et al., 1991).

In terms of IFN-α administration, some investigators report a decline in food-related behaviour (Crnic and Segall, 1992; Reyes-Vazquez et al., 1994; Segall and Crnic, 1990) in conjunction with loss of body weight (Reyes-Vazquez et al., 1994). However, others fail to observe any fluctuations in weight even though there is a significant IFN-α-induced reduction of food intake (Crnic and Segall, 1992), suggesting a more efficient metabolic response to IFN-α challenge (Boyle et al., 1978).

Reyes-Vazquez and colleagues (1994) monitored food and body weight changes in Wistar rats receiving daily injections for ten days of either intraperitoneal (1500 units), or intracerebralventricular (1500 or 150 units) infusion of IFN-α into the lateral hypothalamic area. Administration of each IFN-α dose resulted in a prominent reduction of food intake and body weight, but no significant decline in water intake. Weight loss appeared on day four in the group receiving the lowest intracerebral dose of IFN-α; the highest dose resulted in weight loss being exhibited on the first drug injection day. The intraperitoneal group demonstrated the most substantial weight loss over the course of the study. Following the completion of drug administration, each of these effects were reversed in a dose dependent manner (the group receiving 1500 units intraperitoneally retained lower body weight values) 20 days later. Interestingly, the reductions in food intake were associated with a depression in lateral hypothalamic neuronal activity (Reyes-Vazquez et al., 1994) suggesting that IFN-α has inhibitory effects on hypothalamic nuclei, thus influencing food intake and body weight.

Conversely, Crinc and Segall (1992) observed significant reductions in food and water intake that were not accompanied by any change in weight 23 hours after intraperitoneal administration of IFN-α. Employing a 1600 unit dose of either mouse IFN-α, or recombinant human IFN-α, Crinc and Segall (1992) monitored food and water intake using an automated food delivery system and lickometer, reporting that the most apparent effects on these measures occurred within the first six hours following IFN-α administration. In another study, Segall and Crinc (1990) again investigated the anorexic and adipsic effects of mouse IFN-α, but over a longer duration in order to examine the presence of sensitization effects using four different doses (400, 800, 1600, and 400 units) in the same

animals. The investigators reported no evidence of sensitization effects, but did corroborate their other findings of decreased food and water intake, with no change in body weight (Crinc and Segall, 1992; Seagall and Crnic, 1990).

In the studies described above, food intake was consistently suppressed following IFN-α. However this is not always the case. For example, in Segall and Crinc's (1990) work, the 1600 and 800 unit doses of IFN-α affected food intake whereas the lower 400 unit dose did not. Additionally, Sammut and associates (2001) gave intraperitoneal injections of either recombinant human IFN-α (10, 100, 1000 units) or rat IFN-α (1, 10, 100) to Lister Hooded rats and reported a decreased drinking rate, as well as a corresponding reduction in sucrose consumption, interpreted as an anhedonic effect (addressed at a later point in the thesis). Body weight was not altered implying that IFN-α did not elicit anorexia (Sammut et al., 2001), although food intake specifically was not investigated. To complicate matters, daily administration of 1000 units of recombinant human IFN-α increased water intake in Sprague-Dawley rats compared to animals receiving daily injections of saline solution (Bethus et al., 2003).

In general, IFN-α has been considered to have an influence on food intake, particularly with respect to meal patterns. Wistar rats, centrally infused with rat IFN-α (between 15 - 225 units), displayed significant reductions in food intake in the short term (2 hours) (Plata-Salamán, 1992). Interestingly, these animals did not diminish their frequency of meal initiation, but instead reduced their meal size and feeding duration; animals given equivalent doses of intraperitoneal IFN-α did not show any fluctuations in food consumption (Plata-Salamán, 1992). On this basis, investigators suggest that the central nervous system impact of IFN-α on food intake is not anorexia per se, but instead a change

in satiety onset (Plata-Salamán, 1992), a profile demonstrated by other cytokines such as IL-1β and IL-8 (Plata-Salamán, 1996) .

The neural control of this cytokine-induced food suppression is presumed to be under the influence of glucose-sensitive neurons within the ventromedial hypothalamus whereas food-intake activation is located to glucose-sensitive neurons in the lateral hypothalamic area (Plata-Salamán, 1996; Reyes-Vazquez et al., 1994). Cytokines such as IFN-α, TNF-α, and IL-1β likely excite ventromedial hypothalamic neurons to diminish food intake, while concomitantly reducing activity within the lateral hypothalamic area (Plata-Salamán, 1996; Reyes-Vazquez et al., 1994). The idea of cytokine-mediated food suppression does not seem intuitive to the recovery process. Hart (1988), a pioneer theorist in the area of sickness behaviours, proposed that these behaviours centre on the development and maintenance of fever; he also noted that it is the release of the cytokine IL-1 that produces anorexia, as opposed to fever, in the acute phase response. This was supported by evidence that administration of both E. coli endotoxin and an antipyretic still resulted in suppressed food-intake (McCarthy, 1986). Hart (1988) also suggested that a sick animal demonstrates reduced food seeking activity in order to conserve energy and heat loss, as well as to avoid the risk of predation. In addition, an animal is less likely to increase plasma iron concentrations, which prevents the growth of pathogens, if it does not take part in ingestive behaviours.

5.5. Motor Activity

Motor activity is also an index of the physical health of an animal. Ambulatory behaviours are under the control of circadian rhythms, and a sick animal shows a reduction

in movement compared to a healthy one, this being most evident in the active phase of the nycthemeron. In the laboratory, activity levels in animals are most often measured using the open field test, evaluations of social interaction, or the forced swim test.

The open field test, or a comparable locomotor test, is typically carried out in an open area separated into equal regions, or grids. Animals are assessed in this structure on several measures of motility such as rearing, and passage from one grid into another, in addition to total distance travelled, speed, and time spent in specific areas of the field (centre versus perimeter). Using this latter measure, Song and colleagues (2004) found decreased activity in animals that had received central administration of IL-1β (Song et al., 2004). Dunn and Swiergiel (2005) observed that intraperitoneal administration of IL-1β in CD-1 mice diminished the amount of rearing and number of line crossings (into different areas) in the open field. Interestingly, animals pre-treated with central infusion of naloxone prior to IL-1β administration did not display the typical reduction in locomotor activity reported following injection of this cytokine, suggesting a role for opioids in cytokine-induced motor suppression (Luz et al., 2006).

In one study, this depression in open field activity following systemic IL-1 injection did not appear to be gender dependent in rats (Yirmiya et al., 1995); however other work by the same laboratory demonstrated a gender difference dependent on the estrous cycle of rats (Avitsur et al., 1995). For example, following administration of IL-1β, open field test activity was decreased in males and in female rats during their estrus phase whereas females in their non-estrus phases had little or no reduction in activity after the higher and lower IL-1β doses respectively (Avitsur et al., 1995). This is interesting since female rats in estrus tend to have higher activity levels than rats in the non-estrus phase. This suggests the

role of ovarian hormones such as estrogen and progesterone in activity level, particularly the latter hormone which is at its peak during estrus (Avitsur et al., 1995).

Interferon-α is another cytokine that depresses locomotor activity. Balb/c mice receiving intraperitoneal daily injections (1600 units) of IFN-α were tested six hours following cytokine administration and demonstrated a lower number of passages between grids than animals receiving mouse albumin buffer (Dunn and Crinc, 1993). Kumai and associates (2000) observed a decrease in locomotor activity in male Wistar rats given daily subcutaneous administration of IFN-α (300,000 U) over seven days. Another laboratory systematically investigated both the human and the rat source of IFN-α, IFN-β, and IFN-γ (6000 units) administered into the tail vein of male Wistar rats; neither a single injection, nor a chronic protocol of seven administrations disrupted crossings between divisions in the locomotor test within 45 minutes of cytokine injection (Makino et al., 2000). The same result was reported by Bethus and colleagues (2003) who monitored locomotor activity over 24 hour periods for one week following daily intraperitoneal administration of 1000 units of recombinant human IFN-α in male Sprague-Dawley rats. However, Segall and Crinc (1990) monitored 24 hours of horizontal activity in mice prior to and following an order of 400, 800, 1600, and 400 units of mouse IFN-α. Each dose was given once with eight days of behaviour stabilization between subsequent injections. Following the 800 unit IFN-α dose, mice exhibited decreased horizontal activity. Mice administered the 1600 unit dose displayed a reduction in locomotion and a decrease in the number of head pokes into a chamber for food delivery (Segall and Crinc, 1990). The same effects were observed two years later when these investigators probed into the utility of the mouse subtypes of IFN-α and IFN-γ (1600 units each); however these reductions in behaviour were not detected

when Crinc and Segall (1992) employed the identical dose of the human form of either cytokine.

Systemic injection of human IL-1α has been shown to induce a general non-significant increase in open field activity in rats. This increased activity was notably evident 24 hours after IL-1α administration (Broderick, 2002). An elevation in activity level subsequent to immune challenge has also been reported following administration of other cytokines. For example, Zalcman and colleagues (1998) showed an increased level of ambulatory behaviours in mice following injection of IL-6 and IL-2 although Dunn and Swiergiel (1998) reported no change in motor activity following IL-6 administration. A single intraperitoneal injection of murine IL-2 (0.05 to 0.80 μg/mouse) also increased activity in the locomotor test. This effect persisted through five additional daily injections of the cytokine (Zalcman, 2001).

It has been suggested that the behavioural outcome in locomotor tests may be dependent on the familiarity of the new environment (Simmons et al., 2005). In two experiments that demonstrated increased ambulatory behaviour, animals received either two hours of habituation in the test cage (Broderick, 2002) or were placed in a test cage similar to their home cage in order to reduce anxiety (Zalcman et al., 1998). The concern is that locomotor tests introduce an anxiety element. However, Kumai et al (2000) gave their Wistar rats an opportunity to acclimatize to the locomotor activity box prior to data collection and proceeded to observe a reduction in division crossings in the IFN-α treated rats.

In spite of the different measurement protocols used to assess locomotor activity, the general pattern tends to be one of decreased activity following cytokine administration. Pauli and colleagues (1998) tracked behavioural activity of male Wistar rats by using a 3-

point scale such that 1 indicated that the animal was engaging in active behaviours for two minutes or less, a 2 indicated activity for more than two minutes, but less than 15 minutes, and a 3 represented an active animal for more than 15 minutes of a 30 minute monitoring period after cytokine challenge. Using this scale, the investigators were able to show that ventricular injection of human IL-2 or IL-1 receptor antagonist decreased behavioural activity level; the same was observed for intraventricular administration of murine TNF-α, but this was only apparent during the dark period (Pauli et al., 1998) when rodents are generally more active. Another group of experimenters measured locomotor activity in CD-1 mice as follows: 1 = no movements, 2 = slow, lethargic movements, 3 = normal locomotion, and 4 = hyperactive, continuous movements (Hayley et al., 1999). The employment of this scale revealed that a single systemic administration of TNF-α did not disrupt motor activity, whereas a second administration of the cytokine 14 or 28 days after the original injection elicited a significant sensitization effect as demonstrated by a reduction in ambulatory behaviours (Hayley et al., 1999). Interestingly, Larson and Dunn (2001) note that changes in this open field type behaviour may signify a depression of either motor activity in general, or exploratory behaviour; they caution that the typical open field and locomotor tests are not designed to assess the latter. Because of this limitation, novelty and social exploration tests have often been employed as an alternative means of evaluating exploratory behaviour.

Some investigators have adapted open-field and locomotor tests in order to measure exploratory behaviour by adding a novel stimulus for the animal to explore. For example, Lacosta and colleagues (1999) placed a cylindrical container in the middle of the test cage and measured latency to make contact with the stimulus and total contact time in mice. In

addition, total time displaying locomotor activity, number of rearing behaviours, and time spent quiescent was also assessed. When CD-1 mice received a single systemic injection of IL-2, there were no disruptions in locomotor activity or exploratory behaviour. However, when mice were administered the cytokine over a period of seven days prior to tests, mice displayed less locomotor activity than their saline-injected counterparts, although rearing behaviour was not altered in either group. Interestingly, exploratory behaviour, as assessed by time spent with the novel container, was significantly reduced in mice given the cytokine (Lacosta et al., 1999). This group of investigators then proceeded to examine the anxiety-inducing properties of IL-2 by measuring behaviours in the elevated plus maze – a test used by some to assess locomotor activity because of its dependency on motor ability (Larson and Dunn, 2001; Petitto et al., 1999). The elevated plus maze comprises four perpendicular arms in the shape of a plus sign. Two of the arms have high walls in which animals may contain themselves, and the other two arms are not enclosed. The entire apparatus is elevated above the ground. It is assumed that animals that spend longer periods of time in the open arms are less fearful than those that remain within the closed arms (Lister et al., 1987). Locomotor activity is evaluated by the number of alternations between the arms (Petitto et al., 1999). Neither a single, nor repeated administration of IL-2 disrupted performance in the elevated plus maze suggesting that this cytokine does not result in anxiogenic behaviours or reduced motor activity (Lacosta et al., 1999). Deletion of the IL-2 gene in knock-out mice also failed to influence behaviour in the elevated plus maze (Petitto et al., 1999). Since some cytokines are able to disrupt locomotor activity, and not elevated plus maze performance, this measure is perhaps more appropriate as an indicator of anxiety than motor performance.

Another method used to assess motility includes observations of social interaction. This is measured by placing an adult animal (usually male) into contact with one or more animals of the same sex, species, and strain. The following four point scale has been used to evaluate social exploration: 1 = animal huddling with conspecifics, 2 = occasional interaction with conspecifics, 3 = mostly staying away from conspecifics, and 4 = animal mostly isolated from conspecifics (Hayley et al., 1999). The most common method to measure social interaction is to record the duration of exploration that the animal of interest engages in over a five-minute period. Social exploration is usually identified by behaviours including anogenital sniffing, grooming, and following the conspecific (Bluthé et al., 1997; Bluthé et al., 1992; Bluthé et al., 1991). Overall, both of these protocols have been employed to demonstrate a reduction of social exploration and activity as a consequence of cytokine administration, particularly IL-1β, and TNF-α (Hayley et al., 1999; Bluthé et al., 1997; Bluthé et al., 1991) and endotoxins such as LPS (Bluthé et al., 1992).

Social interaction is also assessed via observations of sexual behaviour. For example, Yirimya and associates (1995) administered IL-1β systemically to both regularly cycling and ovariectomized rats being treated with ovarian hormones. In both cases, IL-1β depressed sexual behaviours including lordosis, proceptive (initiating-like) activity, and mating. This was not the case for male rats that essentially demonstrated an increase in sexual receptivity at the highest cytokine dose (Yirimya et al., 1995). Ultimately, these changes likely correspond to an alteration in motivation. For example, the depressing motor effects of LPS were over-ridded when lactating dams had to retrieve their young that had been scattered about the cage (Aubert et al., 1997). In the same study, mothers treated with LPS ceased nest building behaviour when they were housed in regular temperatures

(22°C); however, when the room temperature was reduced to 6°C, nest building behaviour resumed (Aubert et al., 1997); granted, this may also be due to a different underlying set of mechanisms that are activated during a crisis situation.

6. Additional Behavioural Models to Assess Sickness

6.1. Forced Swim Test

Traditionally, the forced swim test (FST) has been used to assess immobility, which in this paradigm is considered to be an indicator of lowered mood and poor coping ability (Porsolt et al., 1978a; Porsolt et al., 1978b; Porsolt et al., 1977a; Porsolt et al., 1977b). When an animal is placed into a tall cylinder filled with water from which they cannot escape, they initially display several active behaviours, until adopting an immobile, passive state. This form occurs more readily following the second exposure to the FST. Chronically stressed animals tend to demonstrate passive behaviours much sooner than control animals, a phenomenon particularly observed in the Long Evans strain of rat as compared to the Sprague-Dawley (Bielajew et al., 2003). As mentioned, when re-exposed to the FST, most animals will quickly revert to the immobile state; however it has been observed that with the administration of antidepressants, this state can be delayed and even prevented presumably due to the anxiolytic property of the drugs (Porsolt et al., 1978a; Porsolt et al., 1978b). Dunn and Swiergiel (2005) gave systemic injections of IL-1β and LPS to male CD-1 mice 90 minutes before exposing them to either the FST or open field test. Both the cytokine and the endotoxin diminished active behaviours in the FST, but also did so in the open field suggesting that any changes in the FST were a consequence of motor

suppression, and not learned helplessness or other changes in mood (Dunn and Swiergiel, 2005). For this reason, some investigators employ the FST, or a similar protocol, as an indicator of motor ability following a cytokine challenge. Dunn and Crnic (1993) systemically administered 1600 units of a recombinant hybrid of IFN-α for five days to Balb/c mice and reported that the animals displayed reduced float time in a swim test. In another study, Wistar rats received either a single, or repeated intraventricular administration of human IFN-α which significantly increased immobility in the FST. Interestingly rat IFN-α did not have an impact on FST activity (Makino et al., 2000).

Despite these findings, there are some concerns that the FST may not be an appropriate measure of the effects of IFN-α on motor ability. One indicator is the observation that neither single nor repeated administration of the human nor rat forms of the cytokine affected locomotor activity in the home cage, although immobility in the FST was increased (Makino et al., 2000). Further inconsistencies in the effects of IFN-α administration on FST mobility were demonstrated by De La Garza II and colleagues (2005b). In their study, male Wistar rats failed to decrease mobility to any discernable amount following repeated intraperitoneal injections of recombinant human IFN-α (100,000 units) for 14 days. These observations were reminiscent of ones observed earlier in which male Wistar rats, administered a single intracerebral injection of recombinant human IFN-α (1000 units), did not show a decrease in motor ability, although there was large variability between the test groups (De La Garza II and Asnis, 2003). Together, these findings suggest that the FST may not be a reliable indicator of IFN-α induced motor disruption. This underscores why the FST is most widely accepted as a measure of anxiety and other mood disruptions.

Cytokine administration has been shown to induce what is recognized as 'behavioural despair' in the FST by increasing immobility or 'float time'. This effect is believed to model depression. Rats that received intracerebral administration of recombinant rat TNF chronically for 14 days displayed prolonged immobility in the FST and intraperitoneal injection of the antidepressant desipramine attenuated this effect (Reynolds et al., 2004). Noteworthy, IL-6 has not been found to influence FST behaviour (Swiergiel and Dunn, 2006).

In the LPS literature, the data with respect to the FST have not been consistent. For example, Deak and colleagues (2005) observed that neither 10 nor 100 μg of systemic LPS increased FST float time in Sprague-Dawley rats, despite the fact that these same doses elicited disruption in social behaviour tests. However, Dunn and Swiergiel (2006) reported that male CD-1 mice given systemic IL-1β (100, 300 or 1000 ng) or LPS (1 or 5 mg) increased immobility in the FST. The same doses also decreased open field activity, suggesting a decline in overall activity level as opposed to a disruption in affective functions. This is an important conclusion since it seems inappropriate to attribute lowered activity alone to depression.

The inconsistent findings in FST behaviour, as a consequence of LPS challenge, cross over into the literature examining IFN-α (De La Garza et al., 2005; De La Garza and Asnis, 2003; Makino et al., 2000). Makino et al (2000) reported dose-dependent increases in float time in male Wistar rats receiving either a single, or chronic intravenous administration of human recombinant IFN-α (60, 600, or 6000 units), with the highest dose corresponding to the greatest immobility. These doses did not affect locomotor activity suggesting that the immobility was an indicator of despair and not general motor disruption (Makino et al.,

2000). Using a different injection route, the investigators observed that a single intraventricular injection of the rat source of IFN-α did not induce immobility in the FST (Makino et al., 2000). Contrary to the above, De La Garza II and Asnis (2003) failed to observe any changes in FST behaviours as a consequence of a single intraventricular injection of human recombinant IFN-α (200 units) in male Wistar rats. In subsequent work, De La Garza II and associates (2005b) investigated daily administration of recombinant human IFN-α (100,000 units) injected intraperitoneally over 14 days. Wistar rats that were administered the cytokine displayed similar FST behaviours to their saline control counterparts (De La Garza II et al., 2005b) indicating that the FST may not be a reliable measure of cytokine-induced depression.

One difficulty with this measure is its dependency on activity level. Another is the concern that FST may not necessarily reflect anxiety or depression. To avoid these potential confounds, many investigators opt to employ tests of food motivated behaviours in order to assess anhedonia.

6.2. Food as Reward

In most studies investigating the effects of chronic mild stressors on sucrose consumption, intake level is the predominant measure, although in some cases sucrose preference is also determined. For example, Willner et al. (1987, 1996) conducted a series of studies designed to evaluate the effectiveness of chronic mild stressors on these measures. Using male hooded Lister rats, they observed significant decreases in sucrose and saccharine intake and preference in stressed animals; this was interpreted as anhedonia. Interestingly, non-stressed control animals actually increased their intake and preference

over time. This work also showed that baseline reward sensitivity could be reinstated by the administration of a tricyclic antidepressant, desmethylimipramine (Willner et al., 1987).

Similar affective disruptions have also been elicited through administration of cytokines including IL-1β, TNF-α, and IFN-α (Sammut et al., 2002; Sammut et al., 2001; Brebner et al., 20000; Hayley et al., 1999) as well as the endotoxin LPS (De La Garza II et al., 2005a). Palatable intake of chocolate milk was significantly and dose-dependently reduced in CD-1 mice administered IL-1 or TNF-α in isolation. One-hour decreases in consumption were larger in mice administered the largest dose of each cytokine, although reductions were most prominent when both cytokines were administered together (Brebner et al., 2000). In the case of TNF-α, a 4.0 μg followed two weeks later by a lower (1.0 μg) TNF-α injection elicited remarkable sensitization effects as demonstrated by decreased consumption of chocolate milk recorded over a 1 hour monitoring period (Hayley et al., 1999). Interestingly, reduced rates of lever pressing for a sucrose pellet reward elicited by subcutaneous LPS (20 μg) injection was prevented by the non-steroidal anti-inflammatory drug, diclofenac (2.5 μg), in male Wistar rats (De La Garza II, 2005a), suggesting that these cytokine-induced anhedonic effects involve the HPA axis (De La Garza II et al., 2005a). However, it has also been shown that a single systemic administration of recombinant human IFN-α (100,000 units) does not disrupt the rate of lever responding for sucrose pellets, or number of reinforces consumed (De La Garza II et al., 2005b).

Sammut and colleagues (2001) systematically assessed the anhedonic potential of both human (10, 100, and 1000 units) and rat (1, 10, 100 units) IFN-α injected intraperitoneally using a three bottle (1, 8 and 32%) sucrose test. Intake of the 1% sucrose concentration was greatly inhibited following the middle and higher doses of both sources of IFN-α in Lister

Hooded rats (Sammut et al., 2001). A follow-up study to their findings confirmed that 1000 units of recombinant human IFN-α induced a significant reduction in 1% sucrose intake, alongside an increased consumption of the 32% sucrose solution (Sammut et al., 2002). At the end of the 15 day cytokine treatment period, administration of either the antidepressant desipramine (7.5 mg) or fluoxetine (7.5 mg) had each resulted in a decreased intake in the 32% sucrose solution. This drug-induced decrease returned intake of the 32% sucrose solution to near baseline values in IFN-α treated rats (Sammut et al., 2002), suggesting that hedonic status was reinstated to levels equivalent to those present prior to cytokine challenge.

One issue is that palatable-intake paradigms employ food and water restriction for a period preceding the test which in itself adds a stressful component. However, one group has shown that latency to consume a food reward is reliable without using food restriction procedures (Merali et al., 2003). In the case of fluid-intake, Hagan and Hatcher (1997) have discussed issues surrounding the 24 hour measure of saccharine solution concluding that food deprivation is necessary in order to observe a decrease in intake. For this reason they argue that the 24 hour measure of saccharine intake is not an adequate measure of anhedonia. Even when food and water restriction is applied, sucrose intake and preference disruptions are not reliably seen across laboratories (Konkle et al., 2003; Baker et al., 2006; Hatcher et al., 1996; Willner, 1997). Overall, the difficulty of food motivated paradigms is the inability to discriminate between the suppression of fluid and food intake due to an anhedonic influence, or as a consequence of anorexia, particularly with cytokine-induced sickness. This has incited the use of tests that do not rely on food intake as the primary motivator.

6.3. Brain Stimulation Reward

Since its serendipitous discovery in 1954 (Olds and Milner, 1954), BSR has been long employed as a model to study the biological basis of motivation (Milner, 1989). Surgically implanted electrodes are aimed at the medial forebrain bundle, or other regions of the brain and electrical stimulation of the neurons at the tip of the electrode probe an animal to perform operant behaviours to obtain stimulation. The assumption is that the animal is responding to receive stimulation that is rewarding. This phenomenon has been effectively demonstrated in a large variety of organisms from humans (reviewed in Jacques, 1979) to fish (Boyd and Gardner, 1962).

Originally BSR was measured by tracking changes in the *rate* of the operant task performed. However, this method of scaling was based on the unwarranted assumption that rate of response is linearly related to the rewarding value of the stimulation. It quickly became evident that a number of performance factors could influence the absolute rate of responding. The stimulation threshold, based on either rate-frequency or current-frequency functions proved to be more reliable and reflect the behavioural weight of the stimulation. Scaling methods based on thresholds have been demonstrated to be independent of motor effects, and therefore have an advantage over other animal models measuring anhedonia.

Thresholds obtained from BSR responding are typically determined by holding the current constant while decreasing the frequency (50, 40, 32, 20, 16 Hz) in equal steps from a value of maximal responding to one of little or no responding; the threshold is the frequency at which half the number of maximum responses occurs

Once established, BSR thresholds are strikingly reliable. However rodents exposed to chronic mild stress schedules demonstrate an increase in threshold values, indicating a

reduction in the rewarding value of the stimulation or evidence for anhedonia (Moreau et al., 1992; Moreau et al., 1993; Moreau et al., 1994 Moreau et al., 1995; Moreau et al., 1996). In reference to chronic mild stressors, some investigators have examined the effects of antidepressants on animals exposed to these procedures and found that the drug administration prevented or reversed the increase in stimulation thresholds (Moreau et al., 1993; Moreau et al., 1994; Moreau et al., 1996). However, it is important to note that some laboratories have been unable to replicate this finding, that is, they do not observe changes in thresholds in response to chronic mild stressors (Baker et al., 2006; Bielajew et al., 2003; Lin et al., 2002; Nielsen et al., 2000).

Interestingly, both cytokines and the endotoxin LPS have been reported to induce anhedonia, as measured by BSR (Anisman et at., 1998; Borowski et al., 1998; Hebb et al., 1998; Anisman et al., 1996). Similar consequences were associated with a rat model of heart failure in which responding for BSR was reinstated following administration of etanercept, a TNF antagonist (Grippo et al., 2003). Interleukin-2 was one of the first cytokines to have its anhedonic potential assessed using the BSR paradigm (Anisman et al., 1996). For example, male Wistar rats were trained to respond to rewarding lateral hypothalamic stimulation with discrimination nose-poke behaviours, and the resulting rate-current thresholds were determined. Animals were then administered an intraperitoneal injection of saline, or recombinant human IL-2 (0.5 or 1.0 μg) and acute (same day) and chronic (over 7 days) BSR thresholds were then collected. In this work, the thresholds associated with the acute phase in IL-2 challenged rats did not change when the current was at its maximum or minimum, although response rate was significantly lower in rats receiving the higher dose of IL-2 at the midrange current intensities (Anisman et al., 1996).

Following the acute period was the first observation that disrupted thresholds following IL-2 administration may not easily be reinstated in rats, alluding to long-term disruptions from cytokine challenge (Anisman et al., 1996). In a second experiment, the same methods were repeated but rats were tested every 24 hours for an additional 7 days following the single IL-2 injection. In this case, a similar, but more pronounced pattern to that observed in acute tests was seen (Anisman et al., 1996). In a separate series of experiments, Anisman and colleagues (1998) again employed the discrimination nose-poke procedure to determine rate-current thresholds in male Wistar rats and replicated the same increase in BSR thresholds seen previously as a consequence of IL-2 injection (Anisman et al., 1998; Anisman et al., 1996); this effect had dissipated two weeks following injection (Anisman et al., 1998). In addition, Anisman and associates (1998) did not observe threshold changes from the lateral hypothalamic stimulation following systemic injection of either human IL-1 (1.0 μg) or IL-6 (1.0 μg). This finding was contrary to previous work showing that IL-1, in particular, had anhedonic consequences as observed by a reduction in palatable solution intake (Brebner et al., 2000). One suggestion is that the 'anhedonic' effect in the food-related paradigm was most likely a reflection of cytokine-induced anorexia. Anisman et al (1998) did observe some animals to display disrupted responding for BSR following a high dose of IL-1 (2.0 μg), although this disruption was likely due to the illness associated with IL-1, thus the inability of the rats to respond to the operant task rather than anhedonia per se (Anisman et al., 1998). As of yet, there has been no investigation of the potential changes of BSR thresholds as a consequence of IFN-α challenge.

A surprising observation is that cytokines have variable effects, at least with respect to current thresholds derived for BSR, that are dependent on the source of the cytokine (one

supplier versus another) and within that, differential dose effects according to supplier and cytokine activity (Anisman et al., 1998). This leads one to speculate whether there are other cytokine-induced effects reported in the literature that may be attributable to such factors. A final remark is that different studies employ different forms of each cytokine. Whether it is the human, murine, or rabbit subtype, the main point is that the specific forms are not necessarily applied to the correct species, which may induce an antigenic, as opposed to a cytokine effect.

Along this vein, it is reasonable to question how a known antigen influences responding for BSR. Intraperitoneal administration of sheep red blood cells (10^6 cells) produced an antigenic response in male CD-1 mice that corresponded to a site-specific reduction in BSR performance rate at the peak time of immune activation (3-4 days later). This effect was only elicited from the nucleus accumbens, whereas BSR disruption was not seen for responding from the substantia nigra (Zacharko et al., 1997). Systemic injection of another antigen, the endotoxin LPS (50, 100, or 200 μg), significantly increased current thresholds for BSR obtained from the lateral hypothalamus in male Wistar rats. This effect had entirely dissipated 24 hours later (Borowski et al., 1998). Interestingly, both the administration of sheep red blood cells and LPS elevated concentrations of dopamine from the nucleus accumbens (a site believed to mediate BSR) corresponding to the timing of decreased BSR rates (Borowski et al., 1998; Zalcman et al., 1998). However, the systemic administration of cytokines results in cytokine-specific changes in accumbal dopamine levels, with TNF-α, and IL-1 slightly elevating, and IL-6 and IL-2 decreasing dopamine levels (Hayley et al., 1999; Song et al., 1999; Anisman et al., 1996). Anisman and Merali (1999) note the importance of exploring dose response curves which may explain the

dopamine response to IL-2 reasoning that this cytokine may have biphasic responses on dopamine activity as a function of dose.

In another study, the role that recombinant human IL-2 (5 ng in 1 μl volume) may play in mediating anhedonia was investigated by assessing site-specific threshold changes in response to intraventricular cytokine administration (Hebb et al., 1998). Stimulating electrodes were implanted at different ventral and dorsal positions in the ventral tegmental area (VTA), and CD-1 mice administered IL-2 demonstrated increased frequency thresholds that were specific to the dorsal VTA (Hebb et al., 1998).) Note that their electrodes were also scattered within the longitudinal plane which might have obscured any differences due to VTA depth (Hebb and colleagues, 1998). These studies allude to the idea that there is a differential distribution of IL-2 receptors within the medial forebrain bundle.

7. Further Considerations:

A large issue surrounding animal models of sickness behaviour, particularly those that investigate the onset of depression or anhedonia as part of the overall sickness profile, is why these behaviours occur in some cases, but not in others in response to the same cytokine. Combinations of factors such as gender and genetic differences, subtypes of the cytokine employed, as well as divergent drug doses, and administration routes are likely in part responsible for the inconsistent effects between cytokine challenges; this is in addition to individual immune phenotypes and circadian cycles. Although many of the listed considerations are addressed above, they deserve further discussion in order to understand their importance in animal models of immune challenge.

In humans, hormonal changes have been attributed to psychiatric disruptions leading to the proposal that reproductive hormones and their cyclical nature may indeed play an important role in depressive disorders. In spite of this, animal models designed to investigate these affective states have been dominated by results based on male subjects. The motive for studying male responses has been largely to avoid the potential effects that the estrous cycle may have on behaviours and as a result ignoring a potentially important population. The influence of reproductive hormones may be very relevant given that the incidence of clinical depression is at its height following the onset of menarche, with a second tier evident at menopause, two stages of female life in which there are large fluctuations in reproductive hormones.

In the case of the immune system, some of its constituents, such as NK activity, are known to respond according to hormonal variations across the estrous cycle of the rat (Ben-Eliyahu et al., 1996). For example, the quantity and activity of NK cells are at their lowest during proestrous when estradiol is at its peak (Ben-Eliyahu et al., 1996). There is another proposed relationship between hormones and immunity in that autoimmune diseases tend to be more prevalent in the female population. Granted, this is dependent on the disease and the role of hormones in this context is not entirely clear (Coe, 2002).

Even mobility is cycle dependent with female rats displaying their highest activity levels during estrus than during the non-estrus phases. This implicates progesterone as a behavioural influence (Avitsur et al., 1995) because levels are at their highest during the estrus phase.

Following an intraperitoneal injection of IL-1β, a protracted elevation of temperature was observed in female Sprague-Dawley rats with regular cycles, most evident during the

proestrus phase (Mouihate et al., 1998). In ovariectomized rats, treatment with both progesterone and estradiol 17β resulted in a higher febrile response following IL-1β challenge, than in females treated with estradiol 17β alone (Mouihate et al., 1998). Overall, the variations within the female cycle may play an important role in attenuating immune challenges, although this proposal requires further elucidation.

Genetic factors have also been implicated in differences between cytokine-induced physiological and behavioural changes. For example, Sprague-Dawley and Fawn Hooded rats administered an acute systemic injection of IL-1α demonstrated increased activity levels. Particularly in the Fawn Hooded strain of rats (Simmons and Broderick, 2005) activity behaviours included grooming and sniffing in the locomotor test. In BALB/c mice, an immune challenge led to the release of IL-2, IL-3, and IL-6, one to eight hours after injection. This resulted in severe sickness effects such as piloerection, hypothermia, and decreased motility. The same challenge given to NZW, CBA/J, and C3H/HeJ mice resulted in varying cytokine release patterns and sickness behaviours (Ferran et al., 1991). Additionally, following the administration of IFN inducers, C57BL/6 mice showed increased slow-wave sleep levels, whereas BALB/c mice did not exhibit such effects (Toth, 1996).

With respect to the febrile response, Fischer 344 rats had attenuated temperature values compared to Sprague-Dawley rats, following LPS challenge. The Fischer 344 rats had higher corticosterone and IL-1β serum levels (Taylor et al., 2005). Individual variability in the febrile response has also been reported, at least in rabbits (Stitt, 1985).

Additional concerns that need to be addressed include the subtype of cytokine employed, as well as the dose and administration route to elicit various sickness responses.

The first issue, that of subtype, relates to the fact that most studies exploit the human source of cytokines as opposed to the respective species-specific form. Although there is homology between the human and murine sources of IFN-α (De Sarro et al., 1990), one example of the importance of species-specific cytokines lies in TNF-α (Stefferl et al., 1996). This is because the diverse subtypes of TNF-α each elicit a different physiological effect in terms of fever induction (Stefferl et al., 1996). Contrary to this, the IFN-α forms induce similar behavioural effects (De Sarro et al., 1990), but the murine source of IFN-α is thought to be more potent (Crnic and Segall, 1992; Saphier et al., 1994). Findings such as these calls into question the appropriateness of the human form in animal models because is possible that any illness induced may be a consequence of an antigenic response as opposed to a true cytokine one, as suggested by De La Garza et al. (2005b).

Reviews of the literature indicate that the proper dosages and administration routes of many cytokines have not been sufficiently identified in rat models of disease. This is based on observations that different doses of the same cytokine have large behavioural variations. For example, high doses of IL-1β reduce locomotor activity, while low doses induce short term increases in exploration of a novel stimulus, and increased anxiety-related behaviours in the elevated plus maze (reviewed in Anisman and Merali, 1999). However the pattern of dose-responses for IL-2 and IFN-α, for example, are not as well known since many studies use different dose ranges and administration routes making comparative analyses difficult.

Another element to consider in the mechanics of cytokine action is the role of pre-existing endogenous cytokine levels. It is possible that unique variations of such immune factors may provide an explanation for individual differences in anhedonic, and other sickness consequences of immunotherapeutic drugs via additive, or synergistic

mechanisms. Another tie to the idea of individual variation is the relationship of the immune system to the circadian cycle. In the laboratory, photoperiod has been shown to have an impact on thymus size. Rats housed in complete darkness for a four-week duration had an increased thymus size of over 300% compared to animals living in standard housing. Animals kept in constant light conditions had a 50% reduction in thymus weight (Mahmoud et al., 1994). Interestingly, when challenged with a pathogen, the immune response of rats is most effective in the evening, corresponding to the animals' active phase (Ucar et al., 1983). Indeed, endogenous levels of cytokines, particularly IL-6, are well known to vary across the light-dark cycle in mammals (Alesci et al., 2005; Guan et al., 2005).

7.1. Reward and Immune Activation

There have been some assertions in the literature that positive outlook and emotions may improve immunocompetence and survival rates, or at least quality of life, in cancer and AIDS patients (Spiegel et al., 1998; Levy et al., 1990; Fawzy et al., 1993; Greer, 1991), combined with proper medical attention and treatment. In the case of breast cancer patients, elevated NK activity has been tied to disease control in that the levels of this lymphocyte are correlated with predictive factors such as positive perceptions of social support and willingness to seek out such support in cancer populations (Levy et al., 1990). In contrast, events related to negative stress reactions were associated with lowered NK activity (Gerra et al., 2003; Zisook et al., 1994; Irwin et al., 1987) and higher rates of morbidity in widowed spouses (Irwin et al., 1987) and family members. Lower NK activity is also

linked to faster disease progression in AIDS patients who have inefficient coping mechanisms and report lack of social support (Leserman et al., 2000). Together, these examples of immunocompromised states can be related directly to the depressive symptoms that accompany illness (Zisook et al., 1994; Irwin et al., 1987). However, there are some who criticize this perspective on grounds of no such relationship between immune and psychosocial factors in breast cancer survival (Osborne et al., 2004). In addition, there are several criticisms of the research examining the clinical relevance between psychosocial determinants and survival length in cancer on the basis of methodological flaws. For example, in the literature, sample sizes tend to be quite limited (Carlsson and Harim, 1994), and the patient demographics between studies in terms of cancer stage and type are not comparable (De Boer et al., 1999; Mulder et al., 1992). Measurement tools employed are usually different between investigators, and extraneous factors, such as smoking, are not always controlled for which may account for inconsistencies between studies (De Boer et al., 1999; Carlsson and Harim, 1994; Mulder et al., 1992). Despite this, there are many empirical studies linking reward directly to immunocompetence.

Studies employing lesion techniques and BSR methods were some of the first to purport an existing pathway between the brain and the immune system. Through these studies it was demonstrated that the hypothalamus plays a fundamental role in immunity. For instance, hypothalamic lesions offer protection against anaphylaxis (Luparello et al., 1964) and a reduced delayed hypersensitivity response to antigenic challenge (Janković and Isaković, 1973). Fessel and Forsyth were the first to use electrical brain stimulation in order to affect immune functioning in 1963. In their work, they showed that rat immunoglobulin-γ increased following lateral hypothalamus stimulation (cited by Vlajković et al., 1993).

Šakić and Vlajković (1990) later showed that male Wistar rats permitted to self-stimulate for lateral hypothalamic stimulation (one hour a day for nine days, five of which were prior to a systemic challenge with sheep red blood cells), had an augmented humoral response, measurable by increases in sheep red blood cell antibody levels. In addition, peripheral blood levels of lymphocytes were also elevated (Šakić and Vlajković, 1990). Site-specific effects of these immune changes were also demonstrated; Wistar rats passively receiving electrical stimulation either to the dorsomedial hypothalamus or sensorimotor cortex had larger delayed hypersensitivity skin reactions and antibody secretions to bovine serum albumin, whereas electrical stimulation of the ventromedial hypothalamic nucleus did not elicit comparable immune changes (Janković et al., 1988).

The protective effects of BSR against antigenic challenges appear to be time dependent in that BSR must occur prior to the challenge for any benefit to occur. In one study, rats were trained to self-administer either lateral hypothalamic, or VTA stimulation for 30 minutes a day, four days prior to either sheep red blood cells or bovine serum albumin administration. These animals displayed an increase in antibody levels for the respective challenge compared to sham controls; however animals responding for stimulation following antigenic injection did not develop equivalent increases in antibody titres (Vlajković et al., 1993). In addition, the brain site receiving stimulation determined the magnitude of these humoral immune responses. For instance, animals responding for VTA stimulation displayed larger delayed hypersensitivity reactions to bovine serum albumin 14 days after challenge, although lateral hypothalamic stimulation resulted in the largest immune responses overall (Vlajković et al., 1993).

Natural killer cell activity also appears to be modulated by the rewarding, and even aversive elements of electrical brain stimulation. Peripheral blood levels of NK activity were significantly suppressed in Sprague-Dawley rats following one session of stimulation aimed at the dorsal periaqueductal grey area for 30 minutes (one simulation train per minute) (Demetrikopoulos et al., 1994). The periaqueductal grey region is a brain site known for its aversive properties (Diotte, et al., 2001). Moreover, electrical stimulation of the ventromedial hypothalamus suppressed immune activation of both spleen and peripheral blood NK activity, increased plasma prolactin and testosterone levels, and had accompanying changes in corticosterone and growth hormone. The magnitude of the NK immunosuppressant effect and direction of corticosterone and growth hormone changes were dependent on the behaviours induced by the stimulation (Wrona and Trojniar, 2005). For example, Wistar rats that did not demonstrate behavioural reactions to 30 minutes (30 second stimulation trial followed by 30 seconds of rest) of ventromedial hypothalamic stimulation (but instead fell asleep during the test period) had greater decreases in NK activity, corticosterone, and growth hormone than animals that displayed aversive reactions over the 21 day test period; this latter group actually had increases in plasma cortiosterone levels (Wrona and Trojniar, 2005). Similar immunodepressive effects elicited by ventomedial hypothalamic stimulation were observed by Okamoto and associates (1996) and were prevented by systemic administration of chlorisondamine (3 mg/kg), a ganglionic blocking agent, or proranolol (10 mg.kg), a β-adrenergic receptor antagonist, but were not prevented by adrenalectomy.

The rewarding element of brain stimulation is elicited most robustly from sites within the medial forebrain bundle including the VTA, the main source of dopamine cell bodies.

From this perspective, the elevated level of NK activity following BSR derived from the medial forebrain bundle suggest a role for reward in cellular immunity. For instance, Sprague-Dawley and Wistar-King-Aptekman rats administered electrical stimulation to the lateral hypothalamus had increased splenic NK activity compared to sham controls and animals that received comparable stimulation to the frontal cortex, suggestive that it was the rewarding aspect of the stimulation, and not the stimulation itself that altered immune activity (Wenner et al., 1996). The major criticism of this work is whether the stimulation was interpreted as rewarding given that the animals were anesthetised during the 30-minute test period (Wenner et al., 1996). In addition, not all medial forebrain sites support self-stimulation and some sites in the frontal cortex do (Bielajew and Trzcinska, 1998).

In another study, Wenner and colleagues (2000) observed NK activity to be higher in fully conscious animals receiving non-contingent stimulation (30 minutes a day for 14 days) in the lateral hypothalamus compared to sham controls. Although the BSR animals had been trained to self-stimulate prior to the test period, it is not clear that the non-contingent stimulation applied to the same sites is necessarily rewarding (Tsang, Stutz, 1984). Other experimenters have investigated forced lateral hypothalamic or VTA stimulation on immunomodulation in rats using a 5-8 second feeding or locomotor latency as a criterion in order to interpret the stimulation as rewarding (Wrona et al., 2004; Wrona and Trojniar, 2003). In their study, increased splenic and peripheral NK and large granular lymphocyte activity was observed following 21 days (30 minutes per day) of the stimulation, with splenic NK activity higher than that of blood (Wrona and Trojniar, 2003). In contrast, animals receiving thalamic stimulation did not show any changes in immune activation according to these measures (Wrona et al., 2004; Wrona and Trojniar, 2003). It

is remarkable that increased peripheral NK cytotoxicity was observed as early as 3.5 hours following the first acute 30-minute test. In both the chronic and acute experiments, Wistar rats that primarily responded to stimulation by eating had higher NK levels than those that responded with locomotor activity (Wrona and Trojniar 2003). Stimulation arising from 60 minute daily periods of VTA stimulation, over 14 days, elicited comparable, albeit slightly lesser increases in NK activity than that observed from lateral hypothalamic stimulation in Wrona and Trojniar's (2003) previous work. This increase in NK activity following forced VTA stimulation occurred without increasing large granular cell number or plasma hormone levels (Wrona et al., 2004). From these studies, it appears that brain stimulation does have immunomodulatory effects; however the rewarding contribution of this control has not been properly validated.

Natural killer cells are important when looking at immunomodulation because of their role in virus containment and thwarting cancer development (Abbas and Lichtmen, 2003). Overall, NK activity is used as a marker of immunocompetence (Levy et al., 1990) and proliferation of these cells is induced by cytokines such as IFN-α and IL-2. It is noteworthy that IFN-α is well recognized as an activator of NK cells mediated through IL-2-dependent proliferation (Herberman, 1997).

Other systems are also believed to influence NK activation (Wrona and Trojniar, 2005), specifically β-endorphin which increases its activity (Matthews et al., 1983). This is interesting considering that the opioid system is also tied to both rewarding and aversively motivated behaviours (Wrona and Trojniar, 2005; Okamoto et al., 1996; Fanselow et al., 1989). Additionally, the lateral hypothalamus and the VTA, both of which mediate BSR, have large quantities of opioid binding sites (Moskowitz et al., 1984; Kuhar et al., 1973).

Cytokines such as IFN-α and IL-2 are also mediated by opiates. The effects (i.e. sleep-induction) of these cytokines are prevented by naloxone administration (De Sarro et al., 1990). However, as interesting as these effects and connections between NK activity and rewarding stimulation may be, the question remains whether they are functionally relevant. In favour of this argument is the demonstration that intermittent application of forced lateral hypothalamic stimulation in animals previously trained to self-stimulate prevented and reduced the formation of gastric ulcers in Long Evans rats following 6 hours of tail shock compared to their control counterparts (McCutcheon et al., 1986). This corresponds to earlier reports that electrical stimulation attenuates stomach ulcers (Freimark, 1973; Marshall McCutcheon, 1976).

The above findings are curious in the context of sickness and the possibility that BSR may alter cytokine release in the brain and periphery, changing the acute phase response reaction to challenge. This also ties nicely with the theory that the phenomenon of increasing immune activity via BSR is comparable to the relationship between positive attitudes and quality of life in diseased populations (Wrona & Trojniar, 2003). Discerning the mechanisms underlying this relationship has great implications for understanding health and recovery from illness.

7.2. Goals of the Thesis: The Motivation

The following outlines the purpose of the four major studies presented in the thesis.

Study 1: The rationale behind this work was to re-assess the acute and chronic anhedonic potential of a single injection of IL-2 by employing BSR and a scale

measurement based on rate-frequency thresholds. In addition, we wanted to determine if the effects of IL-2 challenge were specific to particular VTA loci.

Studies 2 & 3: In the clinical literature, the sickness behaviours induced by IFN-α immunotherapy have been validated using the Sickness Impact Profile, which assesses health-related quality of life factors such as sleep, eating, recreation, vigilance, emotional behaviour, social interaction, and communication (Bergner et al., 1976; Iorio et al., 1997).

In the present thesis, we were guided by the quantitative procedures used by Hayley et al. (2002; 1999) and Sudom et al. (2004) to evaluate the acute sickness behaviours elicited by TNF-α and IL-2 (described above) in male rats. However, we modified their methods to discriminate the contribution of individual sickness behaviours to the overall sickness score, as opposed to creating a composite score based on the individual behaviours. To document a sickness behaviour profile in our studies, we selected piloerection, lethargy, ptosis, and sleep due to their importance in the adaptive recovery of an organism in response to illness. On this basis, we scored the appearance of each behaviour on a 3 point scale as either none, mild, or severe, except for sleep which was scored as either present or absent. Overall, we wanted to establish a more resolved sickness evaluation scale in order to discriminate sickness behaviours in response to cytokine challenge, specifically IFN-alpha, using similar doses and administration routes as others.

Study 3: In addition to evaluating the sickness behaviours induced by IFN-α, the purpose of this study was to investigate the anhedonic consequences of IFN-alpha using BSR elicited from the VTA in female rats. The decision to focus on female responses was rooted in the well-established fact that clinical depression is more prevalent in the female population, yet the contribution of females is largely ignored in animal models. This also

gave us the opportunity to examine the role of estrous cyclicity in response to IFN-α administration.

Study 4: The results of the third study suggested that BSR offers a protective effect against the sickness induced by systemic IFN-α administration. Continuing from this perspective, we decided to further investigate the potential protective role of BSR and another form of reward, namely environmental enrichment, on sickness behaviours in response to LPS. In addition, we chose to measure the endogenous levels of cytokines between the groups in order to delineate their abilities, if any, in offsetting the effects of endotoxin challenge.

It is known that IL-2 and IFN-α stimulate NK cell activity. Stemming from this, we wish to determine if BSR is activating NK cell activity directly or indirectly via IL-2, IFN-α, or other cytokines. Investigating the differences in cytokine variations within and between animals is also of interest considering the individual anhedonic consequences of immunotherapy, likely acting through additive or synergistic modes. We will address these questions using Polymerase Chain Reaction (PCR) techniques in order to amplify DNA, for the purpose of isolation and quantification.

Experiment 1

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1. Abstract

In the present work, we investigated the short- and long-term effects of a single systemic injection of rat recombinant interleukin-2 on weight, food intake, and brain stimulation reward thresholds elicited from the ventral tegmental area. An inverted U-shaped dose-function was obtained with 0.5 μg producing the greatest increases in the threshold for rewarding brain stimulation which were sustained during the month long tests. No differences between groups in terms of maximum response rates, a measure of performance, were observed. Although all injected groups showed a minor decline in the rate of weight gain over time, % efficiency of food utilization (% weight gain/food intake) was the same across groups, suggesting that metabolic function was not affected by the cytokine. In animals with bilateral ventral tegmental area implants, there was no consistent correspondence between the threshold change obtained from ipsilateral stimulation and that associated with the contralateral site; side-to-side differences ranged from 0 to 100%, suggesting a specific interaction between cytokine activity and the locus of rewarding brain stimulation. These data suggest that peripheral IL-2 significantly modifies hedonic processes arising from medial forebrain bundle stimulation in a long-term manner. We further suggest that since this modulation appears to be notably site-specific, IL-2 receptors or its metabolites may not be evenly distributed within the medial forebrain bundle.

2. Introduction

It is now recognized that cytokine activity influences brain function and gives rise via peripheral and central mechanisms to a variety of behavioural disturbances. For instance,

pro-inflammatory cytokines released in response to infection have been shown to reduce ingestive behaviour (Kent et al., 1994; Swiergiel et al., 1999) and exploration (Kent et al., 1994; Lacosta et al., 1999), alter cognitive function (Pugh et al., 2001), hinder female sexual conduct (Avitsur et al., 1998), and produce anhedonia (Borowski et al., 1998). A dynamic form of communication thus appears to occur between immune and central nervous systems, representing an important homeostatic mechanism for the organism (see Larson & Dunn, 2001).

While most of this work has concentrated on investigating mediators of sickness behaviour, especially interleukin-1β, tumor necrosis factor, and interleukin-6, the non-proinflammatory family of cytokines has been the object of less scrutiny. Of particular interest is interleukin-2 (IL-2), due to its growing use in experimental and clinical immunotherapeutic models of a variety of pathologies, including autoimmune disorders, chronic infectious diseases, AIDS, cancer, and organ transplant rejection (see Nistico, 1993).

Interleukin-2 plays a crucial role in the initiation and modulation of the immune response. Produced mainly by T cells, this cytokine has direct effects on the growth and differentiation not only of these but also of B cells, natural killer cells, monocytes, and macrophages (Nistico, 1993). For instance, IL-2 contributes to the process of differentiation of naive B lymphocytes into antibody-producing cells, and to the proliferation and increase of activity of natural killer and lymphokine-activated cells (Hanisch & Quirion, 1996; Hanisch et al., 1997).

Clinically, IL-2 therapy has had positive results in the treatment of many types of cancers that do not respond to standard treatments, such as renal cancer and malignant melanoma, where success rates of other types of treatment are estimated to be at about 20% (Hanisch et al., 1997). One of the major problems with IL-2-based cancer immunotherapy, however, is that of its high neurotoxicity. Investigations have found mild to severe behavioural (combativeness, aggressiveness, intense agitation, anhedonia), and cognitive changes (disorientation to time, place, or person, perceptual hallucinations, paranoia, delusion, and memory impairments) in cancer patients receiving IL-2-based immunotherapy. All of these effects appeared to be related to the dose and time of treatment administration (Denicoff et al., 1987; Caraceni et al., 1992).

The troublesome neurotoxic side-effects that result from IL-2-based therapy clearly suggest that this cytokine is not only an immunoregulatory molecule, but that it also participates in a communication pathway linking the immune and central nervous systems. There is solid neurochemical support for this view. Molecules and receptors akin to the IL-2 family of chemical mediators have been detected in rodent and human central nervous system tissues, both in neuronal cell bodies and in astrocytes (Nistico, 1993).

In animal models of immune function, one paradigm that has been used to assess anhedonic consequences of IL-2 administration is that of brain stimulation reward (BSR). For example, Anisman et al. (1996, 1998) have shown that peripheral administration of IL-2 can significantly influence motivational levels in rats, indexed by an increase in the current thresholds for BSR, and thus interpreted as a decrease in the rewarding value of the stimulation. The effects of IL-2 on BSR do not appear to be restricted to the lateral

hypothalamus. Hebb et al. (1998) extended this exploration to the ventral tegmental area (VTA) and similarly found that intraventricular injections of IL-2 in mice caused a marked reduction of the rewarding efficacy of BSR, as measured by frequency thresholds, suggesting that this cytokine produced anhedonic effects. Since a subset of both lateral hypothalamic and VTA fibers form a common bundle (Bielajew & Shizgal, 1986), one interpretation of these data is that the production of anhedonic effects within the medial forebrain bundle is a general characteristic of this cytokine. Another plausible explanation, however, is that group effects obtained in earlier studies obscured site-specific differences that the cytokine may have had on different loci of the medial forebrain bundle.

The purpose of the present study was first to resolve this question by looking at the effects of IL-2 on reward thresholds from different medial forebrain bundle sites within the same animals. Thus, we compared bilateral placements in a subset of animals to determine if the systemic effects of IL-2 stem from a general capacity of this cytokine to alter hedonic processes or rather, if they are tied to the stimulation site. Additionally, we wished to investigate the effects of rat recombinant IL-2 on thresholds for BSR from the medial forebrain bundle, as opposed to the work discussed above, which used a human source of IL-2.

3. Materials and methods

3.1. Animals and Surgery

Forty-two male Sprague-Dawley rats (Charles River Canada, St-Constant, Québec, Canada) were individually housed in a temperature-controlled environment and kept on a 12 h light/12 h dark cycle with light onset at 0700 hour. Purina rat chow and water were available at libitum. All handling and procedures were done in accordance with the guidelines of the Canadian Council on Animal Care and approval of our institution's Protocol Review Committee. Surgery was performed when rats achieved a weight of more than 300g which occurred approximately one week after arrival to the animal facility. Thirty minutes before surgery, each rat received a 0.05 cc subcutaneous injection of atropine sulfate to reduce bronchial secretions. General anesthesia was induced by either an intraperitoneal injection (65 mg/kg) of sodium pentobarbital (Somnotol), the inhalent fluoroethane (Halothane), (Sigma-Aldritch, Canada) or a combination of the two. Using standard stereotaxic surgical techniques, a bipolar stimulating electrode was aimed at the medial forebrain bundle at the level of the VTA; the coordinates, based on the Paxinos and Watson (1998) atlas were the following: 4.8 mm posterior from bregma, 0.7 mm lateral from the mid-sagittal structure, and 8.4 mm below the skull surface. A flexible stainless steel wire wrapped around the four stainless steel skull screws served as the current return. The entire assembly was secured to the skull with dental cement.

3.2 Drug

Carrier-free recombinant rat Interleukin-2 (R & D Systems, Canada) was received as a 10 μg filtered solution dissolved in 20 mM of ammonium acetate. Phosphate buffered saline was added to obtain aliquots of 1.0 μg and 0.5 μg which were stored at -60°C. Upon thawing and immediately before use, the potency of IL-2 activity was confirmed following the protocol outlined in Davis et al. (1995), in which the ability to stimulate proliferation of an IL-2 dependent mouse cytotoxic T-cell line was assessed. The ED_{50} for this effect typically ranges from 0.1 to 0.4 ng/mL. Rats were injected intraperitoneally with a volume of 0.5 cc of either a vehicle (ammonium acetate) - Group 1, 0.5 μg IL-2 - Groups 2 and 4, or 1.0 μg IL-2 - Groups 3 and 5, prepared in physiological saline. A sixth group of animals was included in order to monitor normal weight change and food intake over the course of the study; these animals received no surgery or drug injection.

3.3. Apparatus and Self-stimulation

Behavioural tests were carried out in wooden and Plexiglas boxes with dimensions of 28.6 cm deep, 32.6 cm long and 30.6 cm high; a lever was located on the right wall 3 cm above the floor. Each lever depression gave rise to a 500 ms train of monophasic rectangular, cathodal pulses of 0.1 ms in duration. The stimulation trains were separated by a fixed interval of 500 ms. Stimulation was provided by constant-current amplifiers (Mundl, 1980) and double-pulse generators built in-house. Once the minimum current to support maximum rates of responding was established in individual animals, it was held

constant for the duration of the experiment; across animals, the current ranged from 200 to 600 μA. During tests, stimulation parameters were continuously monitored on an oscilloscope by reading the voltage drop across a precision 1KΩ resistor in series with the rat.

Following one week recovery from surgery, conventional shaping procedures were used to determine if the electrodes supported bar-pressing for brain stimulation. Animals showing neutral or aversive responses were excluded from further BSR tests but retained for the metabolic part of the study (Groups 4 and 5, see below).

The criterion for inclusion in the study was consistent responding of 30 or more lever presses responses per minute for stimulation from at least one of the bilateral pair of electrodes. Animals meeting this condition were then trained in the threshold procedure. This consisted of the delivery of a series of trials in which the frequency was reduced in steps of 0.1 $\log_{10}$ units starting with a value that produced maximum responding (in other words the highest number of bar presses that the animal was capable of producing in a minute trial) and ending with one that gave rise to less than 10% of the maximum rate (an example of a frequency sequence would be 50, 40, 32, and 25 Hz). The frequency threshold, an index of the rewarding value of the stimulation, was determined by interpolating each rate-frequency curve at 50 % of the maximum rate. Usually four rate-frequency curves were collected, of which the first one, a "warm-up" curve, was discarded.

Rats were considered stable when the thresholds did not vary by more than 0.05 $\log_{10}$ units for three consecutive days. Once achieved, an intraperitoneal injection of 0.5 cc of either the vehicle, 0.5 μg IL-2, or 1.0 μg IL-2 was administered. Fifteen minutes later,

three frequency thresholds were determined and thereafter every hour for a period of 6 hours (short-term phase). During the subsequent long-term phase, frequency thresholds were collected every second day for 28 consecutive days. Body weight and food intake were monitored in all animals on alternate days. Rats that did not meet the inclusion criterion described above (consistent high rates) within a few training sessions were retained as a separate control group; they received an injection of either 0.5 or 1.0 μg of IL-2 in order to observe the effects of the cytokine, unconfounded by stimulation, on ingestion and rate of weight gain (Groups identified as 4 and 5).

3.4. Histology

Following completion of the experiment, rats were anesthetized with an overdose of sodium pentobarbital (Somnotol) and perfused intracardially with physiological saline followed by a 10% formalin solution containing 10 % sucrose. Brains were removed immediately and submerged in the formalin-sucrose solution. Frozen 30 μm sections were collected in and around the implantation site and then stained with thionin for verification of the electrode tips.

3.5 Statistical Analyses

Weight gain as well as short- and long-term drug effects on frequency thresholds and on maximum rates for Groups 1, 2, and 3 were assessed using a repeated measures analysis of covariance (ANCOVA), with group (independent) and time (repeated) as factors (Statistica, 1998). Alpha level was set at a probability of 0.05. The Greenhouse-Geisser correction procedure was applied for violations to the assumption of sphericity (Howell, 2002) where appropriate. In addition, the analysis of thresholds included a covariate which was the maximum response rate associated with each rate-frequency function from which the threshold was determined. This was done in order to take into account not only drug effects on the rewarding value of the stimulation (indexed by thresholds), but also the potential impact of the drug on performance variables, in this case, maximum response rates. Significant interactions were further assessed via Tukey LSD pair-wise post-hoc tests (probablility set at 0.01). The correlation between the magnitude of post-injection threshold effect and the side of electrode placement in all three groups of rats was assessed using Pearson's Product Moment test.

To standardize weight gain as a function of food ingested, the ratio of the two measures, usually referred to as % efficiency of food utilization, was calculated weekly (Bernadis & Bellinger, 1978; Boyle et al., 1978; Stenger et al., 1991), and an ANOVA carried out on these data. Note that all statistical tests were performed on the raw data while for clarity of presentation, we show percent change from baseline on all measures.

4. RESULTS

Seven animals displayed reliable self-stimulation thresholds, according to our criteria, from both VTA electrodes and fourteen from one of the two VTA electrodes. Thus, the effects of drug administration on post-injection frequency thresholds were evaluated on the basis of 28 electrode placements, divided into three groups as follows: Group 1 - vehicle injection, n=6 placements; Group 2 - 0.5 μg of IL-2, n=10 placements; Group 3 - 1.0 μg of IL-2, n=12 placements. Rats were assigned randomly to one of these three groups. Two other groups comprised animals that did not meet the criterion for stability in baseline frequency thresholds during training sessions, but were nonetheless retained to assess the metabolic effects of IL-2. Group 4 (n = 5) received the lower 0.5 μg dose, and Group 5 (n = 6) the 1.0 μg dose. No stimulation tests were conducted in these animals after drug injection.

No significant differences in baseline frequency thresholds were found across groups. Values varied from 20-55 Hz for Group 1, 12-42 Hz for Group 2, and 20-52 Hz for Group 3. The range per group for the baseline maximum rates was 39-87 bar presses/minute for Group 1, 59-205 for Group 2, and 40-96 for Group 3. In this case, group baseline differences were significant - $F(2, 25) = 4.99, p = 0.015$ due to higher maximum rates associated with Group 2 relative to the other two groups. For this reason, maximum rate was assigned as the running covariate in the analysis of post-injection thresholds.

Figure 1 shows the location of VTA placements, arranged in anteroposterior order according to group designation with the left column showing the position of electrodes

associated with Group 1 rats (vehicle injection), the middle column, Group 2 rats (0.5 μg dose of IL-2), and the right column, Group 3 rats (1.0 μg dose of IL-2). Although we observed no particular differences between groups with respect to electrode placement, rats in Group 2 had a higher concentration of left side placements. However, no correlations were found between the size of the post-injection effect and the side of the electrode placement. In the three groups of animals, all placements fell within the medial forebrain bundle, consistently in or around the region of the VTA, scattered within an anteroposterior distance of almost 2 mm.

Figure 2 depicts the percent change from baseline in frequency thresholds in the animals with a functional bilateral implant. In the majority of cases, there was no correspondence in the size of the post-injection threshold shift between bilateral placements. For example, in rat 1453, the left VTA yielded an increase in threshold over time of nearly 120% while there was no deviation from baseline values in the threshold associated with the right VTA, suggesting a site-specific effect.

The top half of Figure 3 shows the percent change from baseline maximum rates during the short-term (left side) and long-term (right side) phases of the study. The bottom half of the figure shows the threshold data arranged in the same manner. Recall that in the short-term phase, self-stimulation tests began 30 minutes following the injection and thereafter every hour for six hours. In the long-term phase, animals were tested two days following the injection and thereafter every second day for a total of 28 days. Analyses performed on data gathered during the short-term phase - both maximum rate and threshold values - revealed no significant differences over time or between groups; however, an interaction between these variables was obtained in the

case of thresholds - $F(12,138) = 2.67, p = 0.03$ with the lower dose of IL-2 (0.5 μg) giving rise to increased thresholds at every time point evaluated following injection on the first day of tests relative to the data associated with the vehicle or higher dose conditions. Tukey LSD post-hoc tests indicated that significant differences occurred at each time point except for the fourth and fifth hour post-injection. In contrast, the thresholds obtained with the higher dose (1.0 μg) were significantly greater than that of the vehicle group only towards the latter portion of the first day - the fifth and sixth hours post-injection. A similar pattern was obtained for the results associated with the long-term portion of the study, that is, a significant interaction between groups and time - $F(26, 299) = 2.82, p = 0.01$, due to elevated thresholds in both the 0.5 and 1.0 μg but not vehicle conditions. However, post-hoc tests revealed consistent significant differences between the vehicle and low dose groups only, evident at every time point but two over the month long phase of this portion of the study.

The rate of weight gain in Groups 1-5 over time is presented in Figure 4, expressed as weight change from baseline values. Both significant time $F(13,351) = 261.28, p = 1X10^{-5}$ and interaction effects (time x group - $F(52, 299) = 3.63, p = 1X10^{-7}$) were found (top half of Figure 4) with groups receiving stimulation maintaining a consistent higher rate of weight gain throughout the study. No significant effects of the data pertaining to % efficiency of food utilization were obtained.

To insure that the weight loss recorded in all groups at the end of the short-term phase was not drug induced, body weight was monitored in an additional ten animals (Group 6) each morning and evening for one week. While each animal gained weight

over the week, body weight was always lighter (9 g on average with a range across animals of 7-13 g) in the morning compared to evening values.

5. DISCUSSION

In the present study, post-injection changes in the rewarding value of the stimulation differed considerably from one bilateral site to the other in the same animals, with side-to-side variations as great as 100%. Histological analysis confirmed that electrode sites were scattered in a region encompassing about 2 mm within the vicinity of the VTA. Our data suggest that IL-2 does not alter reward processes within this locus in an indiscriminate manner. Rather, it would appear that this cytokine acts in a site-specific way on VTA reward neurons, indicating potential disparities in IL-2 and IL-2-like receptor densities within this region, and possibly in a more widespread area of the medial forebrain bundle. Although to our knowledge, there are no data that touch specifically on this question, such variations have been reported in other contexts. For example, studies of the rat brain using *in situ* hybridization have found that the distribution of endogenous IL-2-like immunoreactive material differs greatly from one site to another. The highest density appears to be located in the hippocampus, but moderate density is also present in neurons of the caudate nucleus, locus coeruleus, and most relevant to this study, hypothalamus (a major component of the medial forebrain bundle) (Araujo et al., 1989; Lapchak et al., 1991, Nistico, 1993; Otero et al., 1995).

Recent electrophysiological evidence supports the contention that the medial forebrain bundle contains neurons whose activity is susceptible to modification by IL-2.

Of particular interest is the VTA, not only due to its robust rewarding properties (see Stellar & Stellar, 1985), but also to the fact that this structure provides the source of the mesolimbic projection (Lindvall, 1974, 1977). Using the whole-cell patch clamp technique, Ye et al. (2001) recently showed that IL-2 modifies the firing capacity of VTA dopaminergic neurons, and that this modulation appears to involve interactions with the *N*-methyl-D-aspartate receptor/channel. In another study, *in vivo* microdialysis in freely moving rats was used to measure the effects of IL-2 on a diverse range of monoamines in the nucleus accumbens, a locus which receives major dopaminergic projections from the VTA (Lindvall, 1974, 1977). It was found that systemic IL-2 administration significantly inhibited not only dopamine efflux in the nucleus accumbens (Anisman et al., 1996; Song et al., 1999), but also and concurrently, the rewarding value of lateral hypothalamic stimulation (Anisman et al., 1996).

The idea that reward mechanisms within the medial forebrain bundle are susceptible to change as a result of IL-2 administration is supported by the results of a series of investigations carried out by Anisman and his group (Anisman et al., 1996, 1998; Hebb et al., 1998). In one study (Hebb et al., 1998), the effects of an intraventricular administration of IL-2 on BSR were assessed from different subregions of the VTA in mice. It was found that in some, but not all VTA loci, the cytokine significantly elevated the frequency threshold for up to a week following the injection, indicating an enduring reduction in the stimulation reward value. Similar to our case, not all VTA sites were equally affected by the cytokine challenge.

In two other closely related studies, (Anisman, 1996, 1998), peripheral administration of IL-2 was found to significantly decrease the rewarding value of BSR

elicited from the lateral hypothalamus as indexed by an elevation in current thresholds. Although their target stimulation site differed from the one used in the present investigation, it is known that both sites, the lateral hypothalamus and VTA, have common reward elements (Bielajew & Shizgal, 1986).

Because dopamine activity has been closely linked with reward mechanisms, especially at the level of the medial forebrain bundle (Fibiger et al., 1987), one possible interpretation for the threshold increases reported here and in Anisman's investigations is that IL-2 administration inhibited reward-relevant dopaminergic neurons within the VTA. However, this idea requires careful scrutiny since varying doses of IL-2 have been shown to induce different profiles of dopaminergic responses within the medial forebrain bundle and its adjacent brain structures. In mesencephalic cell cultures, for instance, dopamine was found to be released in response to moderate but not to high doses of IL-2 (Alonso et al., 1993; Lapchak, 1992). Additionally, peak immune responses associated with IL-2 activation were found to be associated with low levels of dopamine release in the nucleus accumbens but with high levels in the prefrontal cortex (Shanks et al., 1994).

Another element to address is that any interpretation of these data and of our own is mitigated by the fact that IL-2 is a pleiotropic cytokine, so that any modification in its availability within an organism necessarily has a variety of unanticipated effects. Because in normal conditions, IL-2 does not freely circulate, but rather is synthesized for local use, cells throughout the body that express its receptor are activated indiscriminately (Kaplan, 1994), causing a series of systemic consequences which may influence the hedonic experience of the electrical stimulation.

Also important to consider are other features shared by our and Anisman et al.'s (1996, 1998) studies, namely the onset and dose level at which IL-2 altered thresholds. The medial forebrain bundle sites that exhibited post-injection decreases in the rewarding value of the stimulation did so immediately in the short-term phase of testing; threshold changes became progressively more pronounced during the first week of testing and finally stabilized during the remainder of the long-term phase.

Our long-term data, based on four weeks of observation, suggest that the cytokine can alter hedonic processes in a long-term manner, but conditional on a continuous excitation of medial forebrain bundle reward neurons. Thresholds in animals that did not receive regular stimulation tests returned to baseline values in Anisman et al.'s (1998) study. Moreover, this group (Anisman et al., 1996, 1998) observed that in order for IL-2 to induce sustained performance effects, it was crucial that animals be tested soon after the cytokine administration. The importance of timing in drug administration with regards to BSR modulation has already been observed with other neuroactive drugs. Lin, Koob and Markou (2000), for instance, obtained different patterns of alterations for BSR elicited from the lateral hypothalamus that depended on the time interval separating amphetamine injections. In the present case, although the long-term effects of IL-2 on thresholds are somewhat surprising given the short half-life of the cytokine, the above data taken together with our own suggest that the cytokine has the capacity of altering hedonic processes in a long-term manner. The mechanism underlying this effect appears to be a single, albeit complex one, which involves a cascade of events beginning almost immediately after its administration, and spanning a relatively long period of time.

Notwithstanding the similarities between our data and that reported by others (Anisman et al., 1996, 1998; Hebb et al., 1998), there are many methodological differences that should be considered, an important one being the route of administration of IL-2: peripheral in this case and central in Hebb et al.'s (1998) study, for instance. One possibility to account for our data is that the cytokine or some of its active metabolites diffuse across the blood-brain barrier and reach target neurons in the VTA to assert their effects. Indeed, IL-2 has been found to cross the blood-brain barrier in limited quantities (Woodroofe, 1995) and its receptors are distributed in the rat brain (Araujo et al., 1989; Lapchak et al., 1991, Nistico, 1993; Otero et al., 1995). However, because their complete molecular structure is not yet known, it is unclear whether they are of cerebral or peripheral origin (Hanisch & Quirion, 1996).

This quandary could suggest a second interpretation of our data - the possibility that a systemic administration of the cytokine may induce a *de novo* synthesis of brain-derived IL-2. Such a mechanism has been found to occur in other cytokines. When interleukin-1 is released by activated immune cells, for instance, it appears that the vagus nerve is stimulated and sends a signal to the brain, which then locally synthesizes its own interleukin-1 (Ek et al., 1998).

These two interpretations do not exclude the possibility that other factors may have contributed to the observed anhedonic effects of IL-2. One of the goals of the present study was to investigate whether the use of rat recombinant IL-2 would have a different impact on BSR than that observed previously by others (Anisman et al., 1996, 1998; Hebb et al., 1998) using human recombinant IL-2. Although the unique arrangement of amino acid sequences such as the ones seen in rat recombinant IL-2 differ only slightly

from human recombinant IL-2 (Petitto et al., 1998), such minute dissimilarities have been shown to play an essential role in the mounting of an immune response (Campbell & Mathieu, 1995; Petitto et al., 1998). Recent studies show that the greater the similarity of the intruder to the organism, the lesser the resulting immune response (Iho et al., 1999; Lohnas et al., 1998; Men et al., 1999), so that rat IL-2 in itself may be less immunogenic than the human strain of the cytokine. Thus, although IL-2 administration is used in our and other studies with the goal of mimicking endogenous increases in the cytokine, it appears prudent to acknowledge the fact that an activation of the immune response and subsequent cascade of events elicited by its introduction in itself could be a factor in modifying the rewarding value of the stimulation. In this case, the observed reductions in the rewarding value of the stimulation could potentially be - at least partially - due to the mobilization of chemical mediators other than IL-2 and subsequent intercommunication between these substances and the brain.

This consideration is particularly important in light of the dose and source-dependent results obtained by Anisman and his collaborators (1998). They observed that the same dose of IL-2 obtained from two different suppliers had distinctive effects on current thresholds for BSR, despite a verified optimal activity for all the cytokines used in the experiment. It may be that slight variations in the amino acid sequences of human IL-2 obtained from different suppliers elicited different levels of immunogenicity in the rats which in turn, influenced the hedonic experience of brain stimulation. In the case of our investigation, a typical inverted u-shaped dose-function curve was obtained with the 0.5 μg dose being most efficient in producing sustained and long-lasting reductions in the rewarding value of the stimulation. Another element to consider in the

interpretation of our data is based on the behavioural and cognitive changes resulting from IL-2 immunotherapy in humans (see introduction) that have also been observed in experimental rodent models (reviewed in Anisman et al., 2002). Administration of IL-2 directly in the locus coeruleus, for instance, induces strong soporific effects (Nistico & De Sarro, 1991), and spatial learning in a Morris water-maze task is severely disrupted following chronic intracerebroventricular infusions of IL-2 (Hanisch & Quirion, 1996). These data point towards the possibility that threshold changes in BSR may be confounded by the presence of such types of cognitive and behavioural alterations. This interpretation, however, is not consistent with our finding that changes in BSR elicited from the VTA appear to be dependent on the precise stimulation locus, thus reflecting site-specific differences in the susceptibility to IL-2 with regards to anhedonia rather than general cognitive modifications.

A final point is our finding that animals receiving either dose of IL-2 gained slightly less weight over the four-week testing period than rats injected with the vehicle, suggesting at first glance a mild anorectic effect of the cytokine. Interestingly, BSR appeared to have a protective effect in that respect; in comparison with the non-stimulated rats, the profile of weight gain for those animals receiving stimulation resembled more closely that of the vehicle-treated rats. This phenomenon of BSR as having counteracting properties against drug-induced (paroxetine) anorectic effects has been previously observed in our laboratory (Konkle & Bielajew, 1999). In spite of this finding, the interpretation of our metabolic data requires some qualification. Although group differences did emerge statistically with respect to weight gain, no statistical pattern was observed when the measure was standardized as a function of food ingested

(% efficiency of food utilization) which fluctuated over time in all groups. Because this index takes into account not only weight gain but also food consumption, it is considered to be a much more reliable indicator of metabolic activity (Bernadis & Bellinger, 1978; Boyle et al., 1978; Stenger et al., 1991).

In summary, this investigation suggests that systemically administered IL-2 does not significantly affect metabolic function as indexed by percent efficiency of food utilization. However, peripheral IL-2 does alter hedonic processes arising from medial forebrain bundle stimulation in a significant and long-term manner. Furthermore, these alterations appear to be highly site-specific, suggesting that IL-2 receptors or its metabolites may be unevenly distributed inside this region.

Study 1

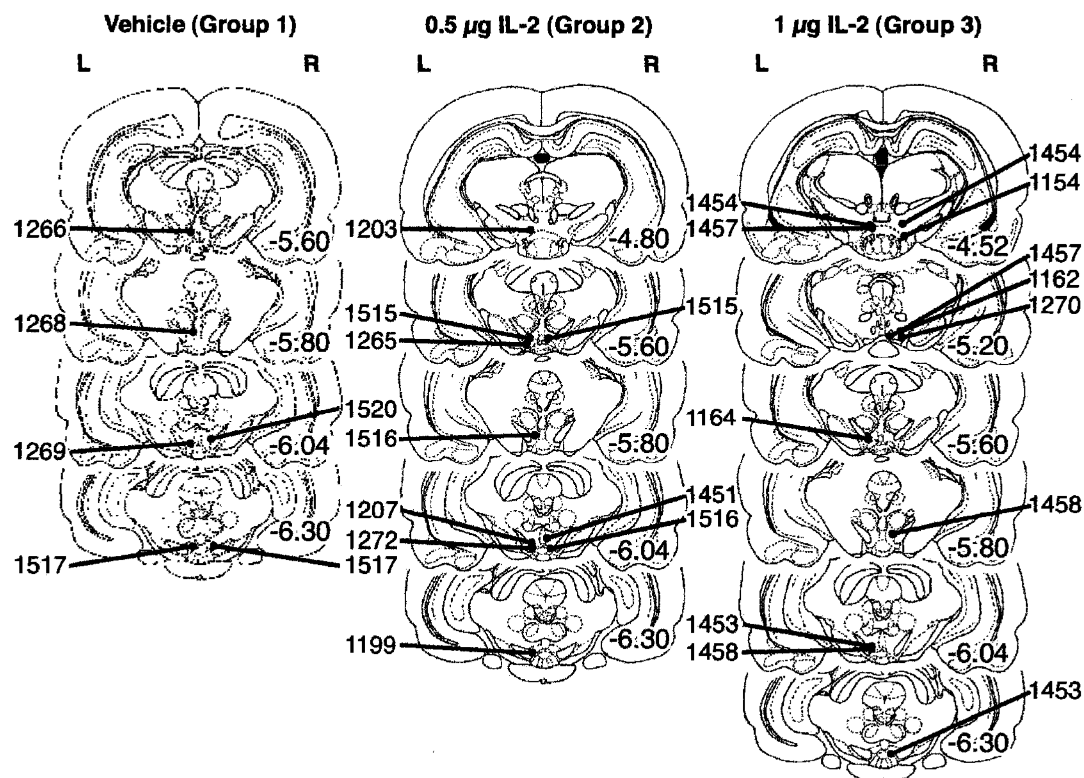
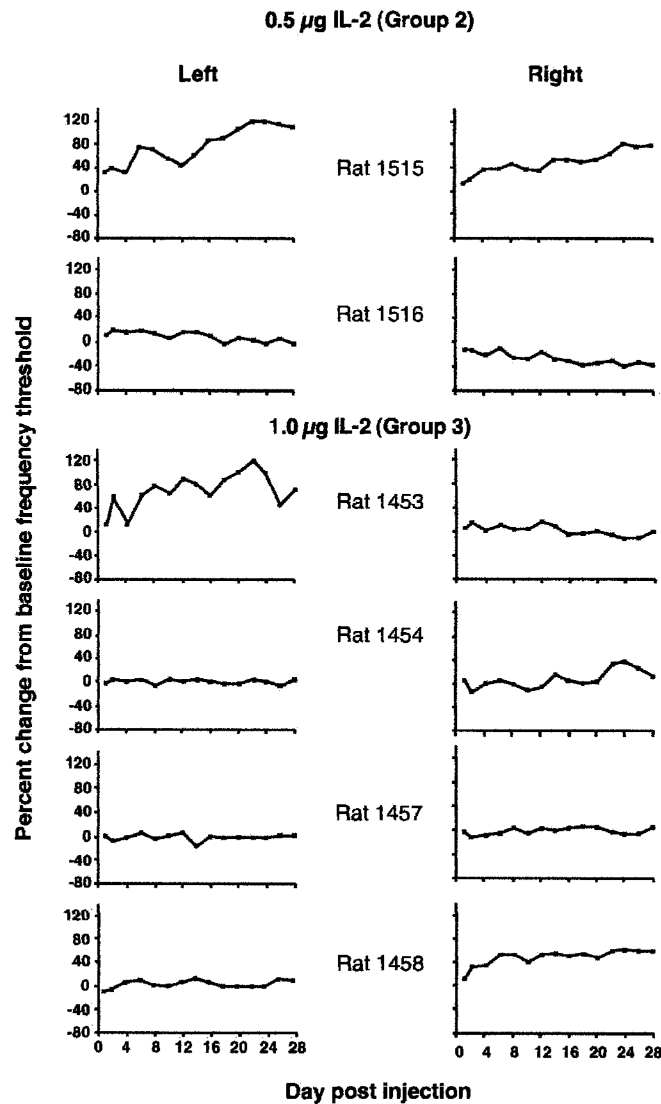


FIG. 1. Tracings of anatomical figures adapted from Paxinos and Watson (1998), showing the location of electrode tips (filled circles) for rats in Group 1 (vehicle): left-hand column, Group 2 (0.5 μg IL-2): middle column, and Group 3 (1.0 μg IL-2): right-hand column. For each group, the tracings are arranged from top to bottom, in anteroposterior order. The rat identifier is shown at the side, and the plate's location (in millimeters relative to bregma) is shown at the bottom right of each tracing.

Study 1

FIG. 2. This figure depicts the shifts in frequency threshold, expressed as a percent change from baseline threshold over time for individual bilateral rats. Upward shifts (> 0) indicate an increase in frequency threshold, which is interpreted as a reduction in the rewarding effect. Downward shifts indicate the reverse: a decrease in the frequency threshold and, therefore, greater reward.



Study 1

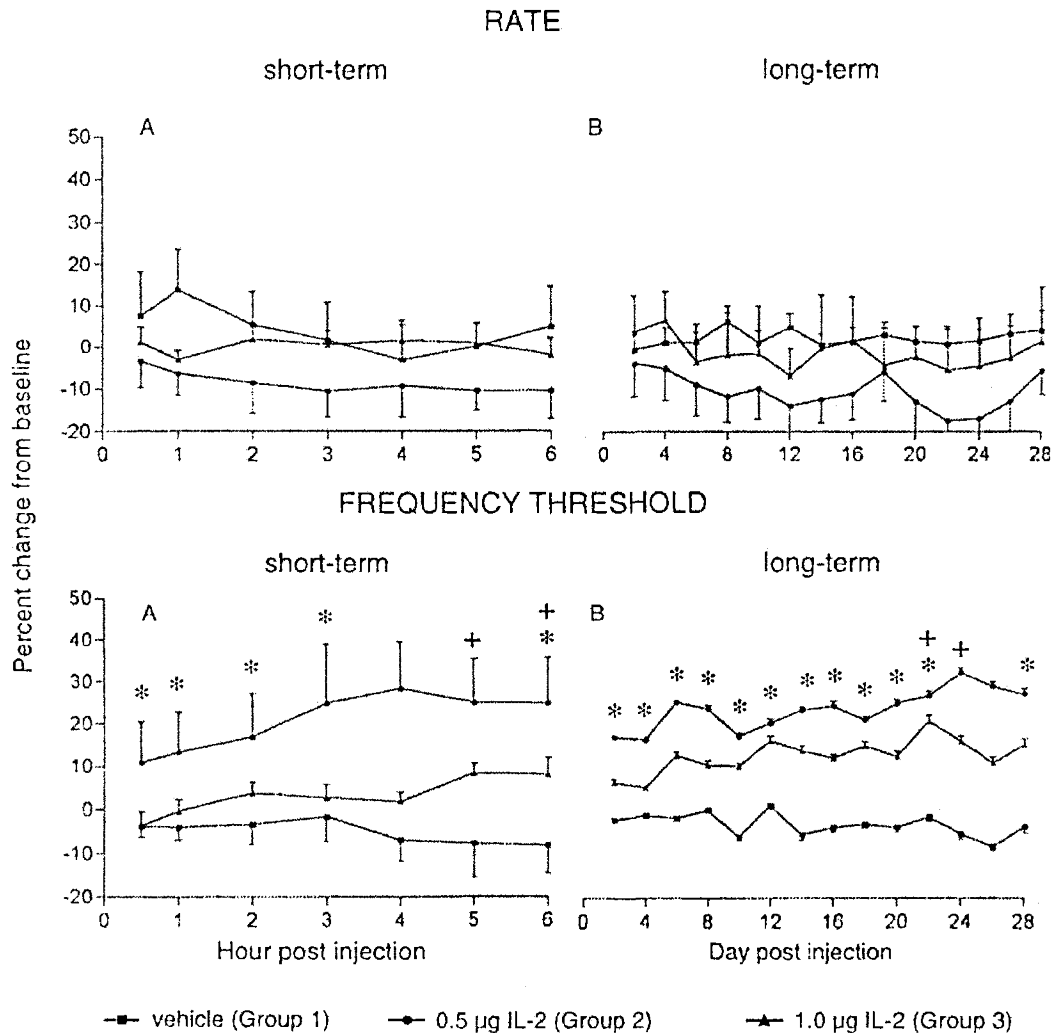
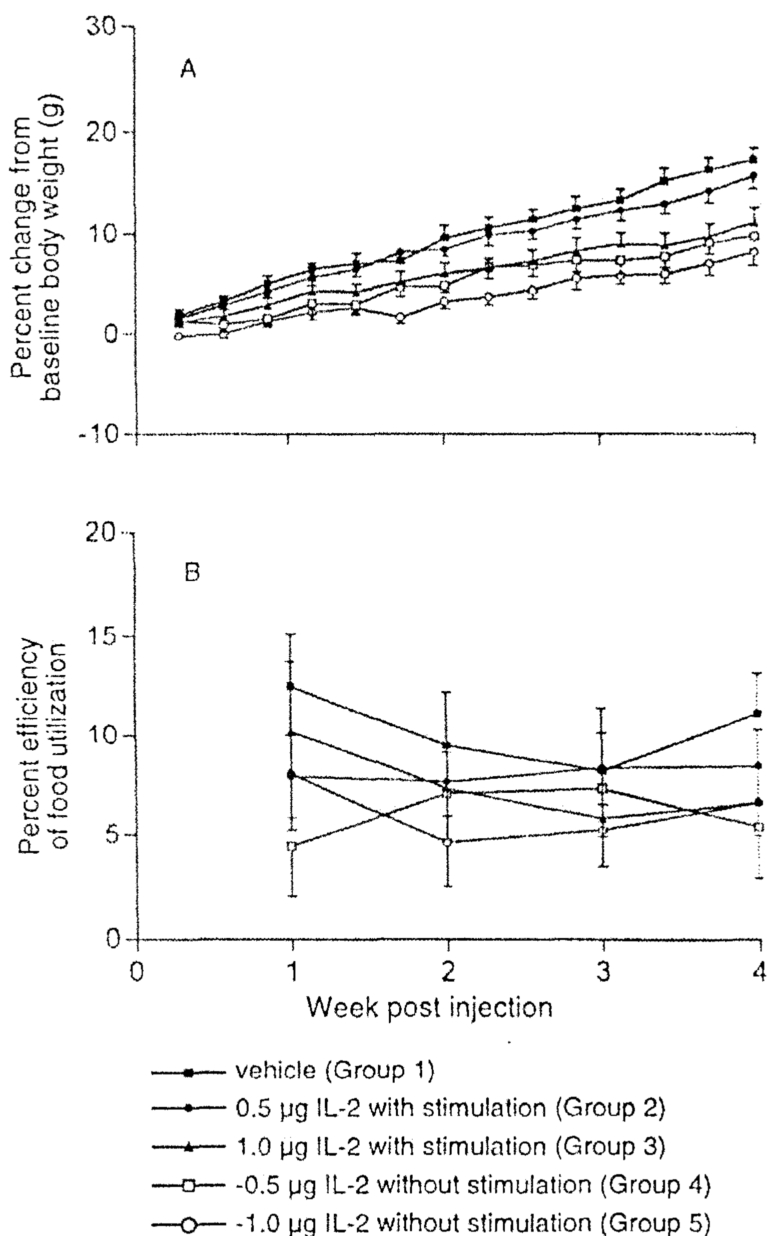


FIG. 3. Percent change values from baseline in response rates per minute (top panel) and in frequency threshold (bottom panel) are presented along with their corresponding standard error of the mean. The data are divided into three groups as follows: Group 1: vehicle injection, Group 2: 0.5 µg IL-2, Group 3: 1.0 µg IL-2. The left-hand side of the graphs depicts data collected during the short-term phase of the study, whereas the right-hand panel shows data from the long-term phase. Significant results (≤.01) obtained from Tukey LSD post-hoc comparisons between individual time points in Groups 1 and 2 are indicated with asterisks, and between Groups 1 and 3 with crosses.

Study 1

FIG. 4. This figure shows the percent change from baseline body weight (top panel) and the percent efficiency of food utilization (bottom panel) along with their corresponding standard error of the mean for each of the five groups during the long-term phase of testing : Group 1: vehicle injection, Group 2: 0.5 μg IL-2 with self-stimulation, Group 3: 1.0 μg IL-2 with self-stimulation, Group 4: 0.5 μg IL-2 without self-stimulation, and Group 5: 1.0 μg IL-2 without self-stimulation.



Experiment 2

Published as: **Kentner, A.C.,** Miguelez, M., James, J.S., Bielajew, C. Behavioural and physiological effects of a single injection of rat interferon-alpha on male Sprague-Dawley rats: a long-term evaluation. **Brain Research,** 2006; 1095:96-106.

1. Abstract

Interferon-α (IFN-α) is a cytokine used as a first line of defense against diseases such as cancer and hepatitis C. However, reports indicate that its effectiveness as a treatment is countered by central nervous system (CNS) disruptions in patients. Our work explored the possibility that it may also cause long-term behavioural disruptions by chronicling the behavioural and physiological disturbances associated with a single injection of vehicle, 10, 100, or 1000 units of IFN-α in male Sprague-Dawley rats (n = 5/ dose). Following one day of locomotor baseline collection, we monitored sickness behaviours (ptosis, piloerection, lethargy, and sleep), food and water intake, body weight, temperature, and motor activity. Observations were recorded four days prior to and four days following the IFN-α injection. Temperature and sickness behaviours were recorded three times daily at 9:00, 15:00 and 21:00 hrs, and all other indices, once daily. On the injection day, temperature values were highest in the animals receiving the 10-unit IFN-α dose 15 minutes, and 13 hours post-injection. In the case of sickness behaviours, a significant increase was observed in piloerection in all IFN-α groups at each time point measured, while the scores of the rats in the vehicle condition remained unchanged between pre- and post-injection days. Analyses of overall sickness behaviours during morning and night observation periods indicated increased scores in all IFN-α groups following injection. Cumulatively, these data suggest that a single IFN-α exposure may elicit long-term behavioural disruptions and that its consequences should be thoroughly investigated for its use in clinical populations.

2. Introduction

Patients administered cytokine therapy as a treatment for cancer, hepatitis C, and other ailments often experience a plethora of adverse symptoms (Capuron et al., 2004; Davis et al., 1994; Iorio et al., 1997). Termed "sickness behaviours" (Kent et al., 1992), they refer to a vast array of generalized non-specific side effects, such as disrupted thermoregulation, lethargy, hypersomnia, suppressed appetite, decreased activity, and lowered mood (Hart, 1988; Kent et al., 1992), and they often lead patients to discontinue their treatment regimens (Musselman et al., 2001). These symptoms are a consequence of specific interactions between the immune and central nervous systems that are thought to be related to the organism's recovery from infection (see Hart, 1988).

In animals, sickness behaviours may be induced by cytokines such as interleukin-1 (IL1), and tumor necrosis factor (TNF), as well as others (reviewed in Dantzer, 2001; see also Anisman et al., 2003; Brebner et al., 2000). However, there has not been a well-developed animal model to study the sickness-inducing effects of interferon-α (IFN-α).

Briefly, IFN-α is a cytokine synthesized by leukocytes to aid in the defense against viral infection (Pestka et al., 1987). It is approved as an immunological therapy for the treatment of, among others, hairy cell leukemia, malignant melanoma, follicular lymphoma, condylomata acuminata, AIDS-related Kaposi's sarcoma, and chronic hepatitis B and C (Nguyen, 2003; Schering-Plough, 2004). In addition, IFN-α is occasionally prescribed for conditions such as multiple sclerosis (Arnaso et al., 1994).

However, the benefits of this drug in chronic illnesses are tempered by the influenza-like symptoms that it induces.

The current hypothesis is that IFN-α exerts its behavioural effects by gaining access to the brain via areas not protected by the blood brain barrier (Smith et al., 1986) and acting on brain sites such as the pons and hypothalamus (Smith et al., 1985; Smith et al., 1986). Indeed, most of the binding sites for IFN-α have been localized to the hypothalamus (Janicki, 1992). Frequent side effects of IFN-α in clinical settings include depression, fatigue, fever, chills, appetite loss, and other influenza-like symptoms (Iorio et al., 1997).

In the animal literature, similar behavioural effects have been documented in response to TNF and interleukin-2 (IL-2) (Hayley et al., 2002, 1999; Sudom et al., 2004), but to our knowledge, only one study has reported such symptoms in rats administered IFN-α (Reyes-Vazquez et al., 1994). Indeed, the adverse effects of IFN-α administration on physiological measures, such as locomotor activity, food intake, and temperature, are not consistently observed in the animal literature. For example, some animals administered IFN-α display reduced motor activity (Crnic and Segall, 1992; Dunn and Crnic, 1993; Kumai et al., 2000; Segall and Crnic, 1990) and food intake (Crnic and Segall, 1992; Reyes-Vazquez et al., 1994; Segall and Crnic, 1990), whereas others fail to demonstrate any such changes in motor abilities (Bethus et al., 2003; De La Garza et al., 2005, 2003; Makino et al., 2000) and food intake (Sammut et al., 2001; Segall and Crnic, 1990). Temperature changes have also been observed as a result of IFN-α administration, with some investigators reporting elevated temperatures (Blatteis et al., 1991; Dinarello et al., 1984), and others, slightly decreased temperatures

(Sammut et al., 2001). Unfortunately, the methodological differences between studies, including the type of IFN-α administered, route of administration, and dose, make it difficult to attribute these discrepancies to anything beyond procedural variances.

One conundrum is whether to use the human or rat form of the cytokine. It has been established that human IFN-α binds in the rat brain (Janicki, 1992), and is the most frequently employed version of the cytokine in animal work. Differences in behavioural effects between the human and rat forms have been demonstrated (Makino et al., 2000; Saphier et al., 1994), causing concern over the appropriateness of the human source (De La Garza et al., 2005).

Because differing administration routes and IFN-α doses between studies may account for the lack of consistency in behavioural profiles, we opted to use the findings of Sammut and colleagues (2001) and Saphier et al. (1993, 1994) to guide our choice of administration route and dosages for the current work, and to establish a consistent framework within the literature using the rat source of the cytokine. The range of these rat IFN-α doses (10, 100, and 1000 units) was considered comparable to that used in clinical settings. For example, 66 units of the rat source of the cytokine have been shown to be equivalent to the human dose currently administered to patients with viral hepatitis (Shinozaki et al., 2005).

To address some of the above issues, we chose to systematically chronicle the consequences of a single intraperitoneal injection of rat IFN-α. Sickness behaviours (piloerection, ptosis, lethargy, and sleep) were tracked during the day and night. We also monitored locomotor activity, food and water intake, temperature, and weight in male Sprague-Dawley rats.

We were interested in evaluating the long-term consequences of a single injection of the IFN-α before embarking on studies employing repeated administration of the compound. While the drug schedule in clinical settings is chronic in nature, establishing an animal model of cytokine challenge requires a thorough investigation of its immediate and enduring effects from a single to multiple exposures.

3. Materials and Methods

3.1. Subjects

Twenty Sprague-Dawley male rats (Charles River Canada, St-Constant, Québec, Canada) weighing between 350-475 g, upon arrival to the laboratory, were singly housed on a 12-hour light/12-hour dark cycle (lights on at 7:00 hrs) with free access to Purina rat chow and water. The animals underwent a one-week acclimatization period before data collection began. All procedures were completed in accordance with the guidelines of the Canadian Council on Animal Care.

3.2. Materials

3.2.1. Drug

Rat recombinant IFN-α was purchased from Sigma-Aldrich (Missouri, U.S.). The concentration of the drug, once reconstituted with 1 ml sterile distilled water, was 1 x 10^5 units/ml. This was aliquoted in 60 μl stock vials and stored at -80°C until use. On injection day, the stock solution was used to prepare a working solution of 10, 100, and

1000 units per ml. These doses, as well as the vehicle, were injected intraperitoneally at a concentration of 1 ml/kg. A vehicle solution was prepared according to the protocol provided by Sigma-Aldrich.

3.3. Procedure

3.3.1. Sequence of Events

Because of the number of phases and measures in this study, a chart of the sequence of procedures is provided in Table 1.

3.3.2. Baseline Tests

Five days before injection (see day 8 in Table 1), sickness behaviour and locomotor scores were collected simultaneously for a one-hour period in the morning and again in the evening. During both occasions, sickness behaviour scores were recorded every 15 minutes, based on an adaptation of the methods of Hayley et al. (2002, 1999) and Sudom et al. (2004). Each animal was examined for the presence of piloerection, ptosis, lethargy, and sleep. Animals remained undisturbed in the locomotor test cage (on day 8, as well as on injection days 13 and 45) during collection of sickness scores. Each sickness behaviour (piloerection, ptosis, lethargy, and sleep) was determined separately. If no piloerection, lethargy, or ptosis was present, the animal was assigned a score of '0'. If the appearance was mild in nature, the animal was assigned a '1'. If the appearance was severe, then a score of '2' was assigned.

If piloerection was only present in the area of the haunches, no score was attributed, because this can be typical of healthy animals. 'Mild' piloerection was scored when a considerable amount of fur was erect, and was distributed around the animal's body (rather than being localized to, for example, the haunches). Piloerection was considered severe if a considerable amount of the fur was obviously erect (like a hedgehog's quills). 'Mild' ptosis was scored if the animal's eyes were a little droopy (between 1/3 and 1/2 closed) and 'severe' if the eye opening appeared as a slit. Sleep was either present '1', or not '0'. If the animal was sleeping, no score for ptosis was attributed.

With respect to lethargy, if the animal was moving, engaging in active behaviours (e.g., grooming), or standing/rearing, a score of '0' was given; if inactive, a score of '1'; and if curled up or sleeping, a score of '2'. Cohen's kappa (Howell, 2002) was used to determine the level of agreement between two independent raters.

Locomotor activity was measured in a standard laboratory Plexiglas box identical in design to the rats' home cage. A fresh cage was provided for each locomotor test in order to stimulate motor activity. Each test box was divided into six equal areas, and every section was equipped with an infrared photoelectric beam positioned above the area. The beam was interrupted by passage of the rat from one area to another. The distance traveled and the number of location changes (locomotor rotations) were recorded every ten minutes via software designed in-house.

3.3.3. Pre- and Post-injection Measures

Following the initial tests outlined above, sickness behaviours and physiological indices were evaluated for four consecutive days prior to (days 9-12 in Table 1) and following drug injection (days 14-17 in Table 1). Physiological measures, consisting of body weight and food and water intake, were recorded once daily at 09:00 hrs. In addition, rectal temperature readings (Harvard Apparatus digital thermometer) and sickness behaviour scores were collected three times daily (09:00, 15:00, 21:00 hrs) during the pre- and post-injection periods. Animals were evaluated for sickness behaviours in their home cage, prior to the physiological evaluations.

On injection day (day 13 in Table 1), rats were randomly divided into four groups corresponding to the dose of interferon-α (vehicle, 10 units, 100 units, and 1000 units, n = 5 rats per dose). Rectal temperature was recorded immediately prior to drug administration, which occurred between 8:00 a.m. and 10:00 a.m. Temperature was also recorded at 15 min, 75 min, 7 hrs, and 13 hrs post-injection.

Approximately 15 minutes after injection, locomotor activity was assessed for one hour to observe any change from the pre-injection values attained on day eight (see Table 1). Sickness behaviours were again noted every 15 minutes during each one-hour locomotor test. All morning tests were recorded between 8:00 and 11:00 hrs, and evening tests between 21:00 and 24:00 hrs on both the pre-injection and injection collection days.

3.3.4. Sensitization Period

One month (day 45 in Table 1) following the single IFN-α injection, all rats were re-administered the same dose in order to determine the presence of sensitization effects. Data were collected on all the measures described above according to the schedule employed in the initial pre- and post-injection phases (see days 41-49 in Table 1).

3.3.5. Statistical Analyses

Sickness behaviour scores were collected four times over a one-hour period during the locomotor tests at baseline (day 8) and on injection day (day 13), to assess the immediate impact of IFN-α dose on sickness behaviours. In a few cases, only three scores could be obtained, thus the top three scores observed during each one-hour period were summed for each behaviour. Difference scores were then determined between the baseline and injection phases, and a Kruskal-Wallis non-parametric test (Howell, 2002) employed with dose (vehicle, 10, 100, and 1000 units) as the grouping variable was conducted for each sickness behaviour. If found to be significant, then post-hoc tests were employed using the Wilcoxon Signed Ranks Test (Howell, 2002).

The same procedure was applied to the analysis of difference scores obtained from pre-injection and post-injection sickness behaviour scores. However, in this case, note that scores were recorded at three different time points on four consecutive days pre- and post-injection (days 9-12 and 14-17).

We also compiled a total chronic sickness score, computed by adding the summed scores for each behaviour during the pre- and post-injection phases; these data were analyzed as above.

The temperature, food and water intake, and body weight data collected during the four day pre- and four day post-injection phases were analyzed via a mixed analysis of variance (ANOVA) design, with group as the independent factor with four levels (vehicle, 10, 100, or 1000 IFN-α units) and time as the repeated factor with eight levels (SPSS, 2004).

The temperature scores recorded on the first injection day (day 13) and the sensitization injection day (day 44) were likewise examined via mixed ANOVA procedures, using five intervals of time (before, 15 min, 75 min, 7 hrs, and 13 hrs after injection).

Day and night-time locomotor rotations and distance data were analyzed in a similar manner except that the repeated factor, time, had 12 intervals. Alpha level was set at a probability of 0.05 for all analyses and the Huynh-Feldt correction procedure was applied for violations to the assumption of sphericity (Howell, 2002). Significant interactions were further assessed via simple main effects with alpha set at 0.01 (Bonferroni correction).

4. Results

4.1. Sickness Behaviours

There were no significant effects of dose on any of the sickness behaviours during the one-hour locomotor test conducted on injection day (day 13) relative to baseline values (day 8). Cohen's kappa test (Howell, 2002) was used to determine the level of agreement between two independent raters. The kappa coefficients were found to be

significant for all sickness behaviours (sleep, $\kappa = .90$, $T = 5.692$; $p = .0001$, ptosis $\kappa = .91$, $T = 5.612$; $p = .0001$, piloerection $\kappa = .85$, $T = 5.303$; $p = .0001$, and lethargy $\kappa = .92$, $T = 5.317$; $p = .0001$), indicating very high inter-rater agreement.

Figure 1 shows the summed scores for piloerection data recorded during the pre- and post-injection phases at the three daily time points. During this chronic monitoring period, a significant dose effect for this behaviour was found for morning ($\chi^2(3) = 12.065$; $p = .007$), afternoon ($\chi^2(3) = 10.843$; $p = .013$), and night ($\chi^2(3) = 10.858$; $p = .013$). The follow-up tests indicated that these scores (except for the vehicle data) were consistently higher after, than before, drug injection for all monitoring periods (morning: $Z = -3.504$; $p = .0001$, afternoon: $Z = -3.528$; $p = .0001$, and night: $Z = -3.599$; $p =.0001$). The scores associated with the vehicle group remained stable from pre- to post-injection. The other three sickness behaviours were not significantly affected by any dose of IFN-α at any time point on any day.

Figure 2 presents the total chronic sickness scores for all behaviours (ptosis, piloerection, and lethargy), including sleep (morning scores appear on the top half and night scores on the bottom half of the figure). The pattern suggests that all scores were increased following drug injection, except for those corresponding to the vehicle condition. A Kruskal Wallis test confirmed the presence of dose effects for both morning ($\chi^2(3) = 9.241$; $p = .026$), and night ($\chi^2(3) = 10.779$; $p = .013$) on the total chronic sickness measure. A Wilcoxon Signed Ranks Test revealed a significant increase in total chronic sickness scores between pre- and post-injection phases for morning ($Z = -2.238$; $p = .025$), and night ($Z = -3.437$; $p = .001$), respectively.

Table 2 lists the contribution of the individual sickness behaviours to the composite or total score for both the pre- and post-injection chronic observation time points.

The sensitization period failed to show any sickness behaviours in response to IFN-α.

4.2. Locomotor Activity

Figure 3 illustrates the average total number of locomotor rotations (left graphs) and distance traveled (right graphs) over the one-hour test for each IFN-α dose, for both morning (top graphs), and night (bottom graphs). The analysis revealed a significant interaction of dose and time for both the morning ($F(33,176) = 1.654$; $p = .021$), and night ($F(21.34, 113.811) = 1.923$; $p = .015$) locomotor periods. Follow-up tests using simple main effects confirmed significant differences at 20 ($F(3,16) = 7.082$; $p = .003$), and 30 minutes ($F(3,16) = 5.411$; $p = .009$), in the morning. The pair-wise Tukey test revealed that the vehicle group had the lowest activity. The simple main effects conducted on the night-time data were not significant.

Further analysis of locomotor activity revealed that distance traveled during the morning locomotor test was dose- ($F(3,16) = 5.839$; $p = .007$), and time-dependent ($F(11, 147.01) = 61.88$; $p = .0001$), with animals receiving the 10-unit IFN-α dose being more active than vehicle animals. While a significant dose and time interaction ($F(21.38, 113.698) = 1.886$; $p = .018$) was obtained at night for this measure, follow-up tests did not identify the source of the interaction.

No changes in locomotor activity due to IFN-α were observed during the sensitization period.

4.3. Food, Water, and Body Weight Data

There were no changes in food and water intake, or body weight, attributable to IFN-α administration. All animals maintained the normal increase in body weight expected over the time course of the study, $F(1.737, 27.794) = 108.729$; $p = .0001$.

4.4. Temperature Analyses

The administration of IFN-α induced significant temperature changes on both the first and the sensitization injection days. Figure 4 presents the temperature values obtained after the first (top graph) and second or sensitization injection (bottom graph). On the first injection day, the data revealed a significant interaction of dose and time, $F(12,64) = 3.565$; $p = .0001$. Simple main effect analyses showed the greatest difference to be at 15 minutes ($F(3,16) = 6.594$; $p = .002$) and 13 hours ($F(3,16) = 4.284$; $p = .021$) post injection. These differences (Tukey test) were apparent between the vehicle and 10-unit IFN-α dose groups at 15 minutes post injection ($p = .002$); this pattern just missed significance at 13 hours post-injection ($p = .016$) based on a Bonferroni correction. Overall, it appears the animals receiving the 10-unit IFN-α dose exhibited the highest temperatures over time.

A similar pattern was found after the sensitization injection with a significant interaction of time and dose, $F(12,64) = 3.335$; $p = .001$. This was confirmed by the simple main effect analysis at the 13-hour time point ($F(3,16) = 11.229$; $p = .0001$), and was due to the difference between the control and 10-unit IFN-α dose groups, with the former eliciting higher temperatures ($p = .0001$).

Evaluation of the temperature time points collected during the four days before, and after IFN-α injection revealed a significant interaction of dose and time for the data collected at night ($F(16.347, 87.186) = 1.931$; $p = .027$). These data are not shown.

5. Discussion

The goal of this study was to evaluate the effect of a single intraperitoneal injection of rat IFN-α on sickness behaviours and a variety of physiological consequences in male Sprague-Dawley rats. These included sickness behaviours, locomotor activity, food and water intake, and body weight. All, with the exception of locomotor activity, were tracked four days prior to and following IFN-α administration. This is one of the first evaluations of chronic sickness behaviour since most investigations have focused on the acute or anhedonic effects of IFN-α. Generally, we demonstrate a consistent increase in piloerection following IFN-α administration, in addition to temperature and locomotor changes.

5.1. Sickness Behaviours

The sickness behaviours induced by IFN-α immunotherapy have been validated in the clinical setting via the Sickness Impact Profile, which assesses health-related quality of life (Bergner et al., 1976; Iorio et al., 1997). To our knowledge, no study has systematically documented sickness behaviours in animal models in response to IFN-α administration. The qualitative observations reported by Reyes-Vazquez and colleagues (1994) following repeated administration of IFN-α are consistent with the

data described in this study with respect to increased piloerection. Our study was guided by the quantitative procedures used by Hayley et al. (1999, 2002) and Sudom et al. (2004) to evaluate the acute sickness behaviours elicited by TNF-α and IL-2. In our case, a single IFN-α injection failed to induce sickness behaviours in the short term; however, the chronic profile indicated mild to moderate symptoms of sickness behaviours. There were, nonetheless, individual differences in the magnitude and number of sickness behaviours expressed.

In the group analyses, only piloerection was significantly elevated between the four-day pre- and four-day post-injection periods as a function of IFN-α dose, consistent with the observations reported by others (Reyes-Vazquez et al., 1994). Piloerection has been elicited by IL-1, TNF-α, and lipopolysaccharide (Hayley et al., 1999, 2002; Lacosta et al., 1999; Linthorst et al., 1994). This sickness behaviour is thought to play a role in heat production and its maintenance in response to the fever induced by an infectious state (see Dantzer, 2001; Maier and Watkins, 1998). Because IL-1, TNF, and lipopolysaccharide also induce febrile responses (Kettelhut and Goldberg, 1988; Konsman et al., 2000; Linthorst et al., 1994), the mechanism underlying piloerection may be similar across immune challenges that are associated with fever.

Indeed, several cytokine actions in the brain, including IFN-α's role in the fever response, are mediated through prostaglandin E2 activation in the hypothalamus (Dinarello et al., 1984; see also Konsman et al., 2002). Interestingly, previous work investigating the thermoregulatory responses of the rat reported shivering and other 'heat-gain' behaviours such as piloerection as being induced by an inhibitory tonic

mechanism in the pons (Amini-Sereshki and Zarrindast, 1984). Although the main pyrogenic area of the brain is localized to the pre-optic anterior hypothalamus (Myers et al., 1994), electrical stimulation of the pons has been shown to affect the activity of the former, and reciprocal communication between the two structures is thought to occur (Eisenman, 1974). Since IFN-α is presumed to cross into the brain through more permeable areas of the blood brain barrier, allowing for access to the pons and the hypothalamus (Smith et al., 1985; Smith et al., 1986), it is conceivable that the cytokine exerts an influence on these two structures, eliciting a disruption in temperature, and resulting in piloerection and other heat-maintenance behaviours.

In our hands, the lowest (10 units) and highest (1000 units) IFN-α doses had the strongest impact on piloerection, with the 10-unit dose being the most potent. The vehicle group remained stable across both pre- and post-injection periods, for all recorded time points, while all IFN-α groups showed increased piloerection behaviour. Previous work has also pointed to the efficacy of lower cytokine doses. For example, our group examined the effect of a single injection of rat IL-2 on the thresholds for brain stimulation reward. In that study, the lowest dose (0.5 μg) produced the greatest increase in the thresholds for rewarding brain stimulation relative to baseline values, indicating an anhedonic consequence of the cytokine. These effects appeared shortly post-injection, reached maximum values several hours later, and persisted over the month-long test period (Miguelez et al., 2004). In another study, Montkowski and colleagues (1997) noted that in the elevated plus maze, the dose response profile resembled an inverted U-shaped curve, or a non-linear relationship between IL-1 dose and performance. Saphier's group (1994) observed a quadratic dose-response profile

using rat IFN-α in that only the 1- and 10-unit dose inhibited plasma corticosterone levels, while lower and higher doses did not. Together, these suggest an effective dose window for cytokine administration that needs further delineation.

Lethargy, ptosis, and sleep are important sickness behaviours associated with the adaptive recovery of an organism in response to illness. These behaviours have all been induced as a consequence of cytokine treatment, with a specific focus on sleep because of the somnogenic properties of some cytokines (see Larson and Dunn, 2001); maintaining this quiet state aids in the recovery process. Such behaviours reduce activity and increase energy stores, which are utilized for fever production, and conservation of heat (see Hart, 1988; also refer to Maier and Watkins, 1998).

In animal models of IFN-α immunotherapy, such somnolent-related (lethargy, ptosis, and sleep) behaviours have been well documented. There is empirical evidence to show that intracerebral ventricular injection of recombinant human and rabbit sources of IFN-α result in non-rapid-eye-movement sleep and fever in rabbits. Of interest, the potency of rabbit IFN-α has been reported to be higher than that of the human source (Kimura et al., 1994). In addition, injection of both rat and human recombinant IFN-α into the third cerebral ventricle results in sleep, as does IFN-α administration to the locus coeruleus (DeSarro et al., 1990). In this study, we failed to observe any somnolent-related behaviours following the single systemic injection of IFN-α. One explanation may be that there are genetic differences with respect to cytokine actions. For example, one study investigating the effects of IFN inducers on sleep patterns demonstrated a strain effect with C57BL/6 mice showing increased slow-wave sleep levels, whereas BALB/c mice did not exhibit such effects. In addition,

neither strain displayed elevated slow-wave sleep time following administration of IFN-α, or IFN-α/β (Toth, 1996). In this study, the absence of customary sickness behaviours, such as those categorized as somnolent-related, may indicate that the dose range was inadequate or that these behaviours are not acutely elicited. Note that sickness behaviours were not evaluated in the groups of investigators who used the same dose range to explore the effects of IFN-α (Sammut et al., 2001; Saphier et al, 1993, 1994).

One aspect of the sickness behaviour profile that was not evaluated in this study was anhedonia, an underlying feature of depression. Anhedonia is characterized as a loss of interest or enjoyment in stimuli once found to be pleasurable (Willner, 1987). Clinically, IFN-α has been reported to induce this symptom of depression (see Loftis et al., 2004; Musselman et al., 2001), although recent animal studies that have attempted to model this effect report inconsistent results (De La Garza et al., 2005; Makino et al., 2000, 1998; Sammut et al., 2002, 2001). These findings underscore the importance of developing more sensitive behavioural measures to the anhedonic consequences of cytokine and other challenges.

5.2. Temperature

Fever is an adaptive physiological response that occurs as a consequence of a pathogen; the accompanying increase in body temperature stimulates the proliferation of immune cells, and stalls the growth and spread of pathogens (see Dantzer, 2001). Both the endogenous and the exogenous release of cytokines elevate set body

temperature in order to induce a perception of cold, and the ensuing compensation of increased heat production (Dantzer, 2001; Maier and Watkins, 1998).

We documented significant increases in body temperature 15 minutes following IFN-α injection (day time); animals that received the 10-unit IFN-α dose exhibited the highest temperatures, and vehicle animals the lowest; a similar pattern was observed 13 hours post-injection (night time). Our findings are consistent with the classic elevation in temperature reported in the literature in response to IFN-α treatment (Blatteis et al., 1991; Dinarello et al., 1984).

In the chronic phase of the study, a mild increase in temperature was observed in the animals that received 10 units of IFN-α. This effect was only evident during the night period, suggesting circadian influences. Indeed, the immune response of rats, when confronted with a pathogen, has been shown to be most effective in the evening, which corresponds to the animals' active phase (Ucar et al., 1983).

Pan and associates (1997) have demonstrated that approximately 25% of ^{125}I labelled IFN-α was detectable in whole brain 20 minutes after vascular exposure and that most of the cytokine entered into the parenchyma, as opposed to being sequestered within the vascular space. The detectable levels of IFN-α 20 minutes after cytokine administration coincide with our reported increase in temperature 15 minutes following injection. However, the increased temperature profile observed 13 hours, and four days following IFN-α may be difficult to reconcile. Nonetheless, it has been reported that despite unquantifiable serum amounts of LPS, or other cytokines such as IL-1β, the physiological consequences of cytokine challenge, for example, fever, can be induced (Kluger, 1991; Long et al., 1990; see also Watkins et al., 1995). Watkins and

colleagues (1995) propose that these effects may be elicited via the vagus nerve through paraganglia expressing cytokine receptors, or through other neural non-vagal routes (reviewed in Watkins et al., 1995). Indeed, TNF-α, IL-1β, and LPS have CNS effects via these pathways (Watkins et al., 1995). Current examination of the literature reveals a paucity of research investigating the role of IFN-α and its receptors in relation to the vagus nerve or paraganglia.

In this work, the acute temperature profile following the second IFN-α injection, or sensitization test, 31 days later, was similar to that obtained in response to the first injection, and not the expected heightened reactivity according to the model of sensitization (see Wichers and Maes, 2002). Segall and Crnic (1990) have also noted a lack of sensitization effects from IFN-α administration on a variety of motor behaviours. However, based on physiological (Opree and Kress, 2000) and biochemical indices (Hayley et al., 1999), the classic sensitization profile in response to other proinflammatory cytokines has been described.

5.3. Locomotor Activity

The current perspective in the animal literature is that IFN-α administration results in decreased motor activity as assessed by most forced swim, open field, and locomotor tests (Crnic and Segall, 1992; Dunn and Crnic, 1993; Kumai et al., 2000; Makino et al., 2000; Segall and Crnic, 1990). Relative to the vehicle group, we observed a different profile: an increase in locomotor activity in response to IFN-α treatment (see Figure 3). This inconsistency in locomotor behaviour has also been reported following administration of other cytokines. For example, while Zalcman and colleagues (1998)

show an increased level of ambulatory behaviours in mice following injection of interleukin-6 (IL-6), Dunn and Swiergiel (1998) reported no change.

One problem is that most studies exploit the human source of IFN-α, even though the murine form is believed to be more potent (Crnic and Segall, 1992; Saphier et al., 1994). Despite this difference, both are considered comparable in eliciting behavioural effects (De Sarro et al., 1990). However, our examination of the literature suggests that, at least with respect to locomotor behaviours, this does not appear to be the case. Dunn and Crnic (1993) reported that BALB/c mice administered a recombinant human hybrid of IFN-α (1600 U) for five days exhibited a decline in float time (one of their measures of motor activity) in a swim test. On the other hand, Wistar rats administered either a single or repeated intraventricular injection of human IFN-α significantly increased immobility time in the forced swim test; however, the rat form of the cytokine failed to induce any changes in activity level (Makino et al., 2000). The story is complicated further by suggestions that repeated intraperitoneal injections of recombinant human IFN-α do not alter immobility time in the forced swim test (De La Garza et al., 2003, 2005). Likewise, this pattern has been reported using the locomotor measure. Sprague-Dawley rats given daily injections of recombinant human IFN-α (10^4 U/kg) for 24 days did not display changes in locomotor activity, either acutely or chronically, compared to their vehicle counterparts (Bethus et al., 2003).

In the sensitization aspect of our study, we failed to observe any effects in locomotor activity 31 days after the first IFN-α exposure. Segall and Crnic (1990), employing a different dose schedule, found no evidence of sensitization effects. However, they did observe decreased motor activity, as measured by head pokes into a

food chamber, as well as reduced horizontal activity in mice administered an intraperitoneal injection of mouse IFN-α. In our work, the presence of any sensitization effects cannot be discredited until further studies investigating additional IFN- α doses and intervals between injection and observation time points are examined.

It is difficult to reconcile the differences in motor activity given the lack of consistency across studies in terms of administration schedule, dose, and source of IFN-α employed. The compounded effects of a single versus repeated injections of IFN-α from a human versus murine source needs particular attention before the disruptive potential of this cytokine is better understood.

5.4. Food, Water, and Body Weight

Interferon-α administration did not result in any decreases in food or water intake, or weight loss in this experiment. All animals gained weight at a steady rate, irrespective of IFN-α dose. While some studies do report reductions in food-related behaviour (Crnic and Segall, 1992; Reyes-Vazquez et al., 1994; Segall and Crnic, 1990), and body weight (Reyes-Vazquez et al., 1994) as a consequence of IFN-α treatment, others fail to observe changes in weight gain despite a decline in food intake (Crnic and Segall, 1992), suggesting a more efficient metabolic response to IFN-α challenge (Boyle et al., 1978). In this study, rats received only a single injection of IFN-α, whereas typically, reported reductions in food consumption and weight take place over a course of several repeated drug administrations. Although repeated injections of IFN-α are typical in clinical settings, in establishing an animal model, it is imperative to understand the primary effects of a single exposure.

6. General Conclusions

Overall, a single injection of rat IFN-α induces mild to moderate sickness behaviours in a chronic manner, as demonstrated by increased piloerection and temperature. In addition, the present study underlines the importance of the circadian cycle on immune functioning, given the persistence in night-time temperature and locomotor disruptions following rat IFN-α injection.

The effects observed here were of a lesser magnitude than that reported in response to the human source of IFN-α, despite the alleged higher potency of the rat product (Crnic and Segall, 1992; Saphier et al., 1994). Since cytokines are glycoproteins with an endocrine-signalling potential (Turrin and Plata-Salamán, 2000) to elicit pleiotropic effects dependent on species, as well as individual differences (see Walker et al., 1999), disparity in the actions between the human and rat form of IFN-α are not surprising. Indeed, it may be that behavioural and physiological responses to the human source of IFN-α are the consequence of an antigenic reaction, a suggestion cited in a personal communication between De La Garza and A.H. Miller (2005).

Study 2.

Table 1. *Sequence of Procedures.*

Day	Phase	Procedure	Details
1 –7	Acclimatization	N/A	N/A
8	Baseline	Sickness behaviors	Tracked every 15 minutes during 1 hr locomotor tests.
		Locomotor Activity	Recorded 1 hour in morning, and 1 hour in evening.
9-12	Pre-injection	Sickness behaviors	Recorded daily (09:00, 15:00, 21:00 hrs).
		Rectal temperature	As above.
		Food & water intake, body weight	Daily at 9:00am
13	Injection	Sickness behavior	Tracked every 15 minutes during 1 hr locomotor tests.
		Locomotor Activity	Recorded 1 hour in morning, and 1 hour in evening.
		Rectal temperature	Before, 15 min, 75 min, 7 hrs, and 13 hrs after injectior
14-17	Post-injection	Sickness behaviors	Recorded daily (09:00, 15:00, 21:00 hrs).
		Rectal temperature	As above.
		Food & water intake, body weight	Daily at 9:00am
41-44	Sensitization	Sickness behaviors	Recorded daily (09:00, 15:00, 21:00 hrs).
		Rectal temperature	As above.
	Pre-injection	Food & water intake, body weight.	Daily at 9:00am
45	Sensitization	Locomotor Activity	Recorded 1 hour in morning, and 1 hour in evening.
		Sickness behavior	Tracked every 15 minutes during 1 hr locomotor tests.
	Injection Day	Rectal temperature	Prior to, 15 min, 75 min, 7 hrs, and 13 hrs after injectio
46-49	Sensitization	Sickness behaviors	Recorded daily (09:00, 15:00, 21:00 hrs).
		Rectal temperature	As above
	Post-injection	Food & water intake, body weight.	Daily at 9:00am.

Study 2

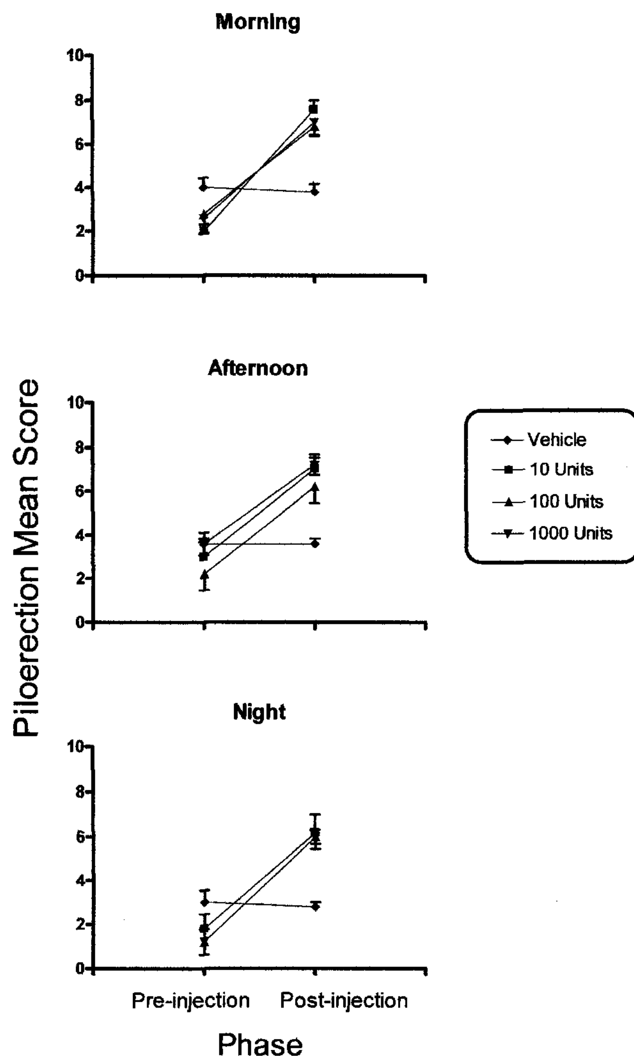


FIG. 1. Displayed are the average (± S.E.M.) total piloerection scores associated with morning (top graph), afternoon (middle graph), and night (bottom graph) time periods for each dose (vehicle, 10, 100, and 1000 units) of IFN-α. The two time points (pre- and post-injection) represent the summation of the top three piloerection scores obtained from each rat four days before, and four days following IFN-α injection and vehicle injection. The summation data were then averaged across animals. Please note that in the night-time graph, the 10- and 100-unit doses display identical patterns. Note that because the analyses were based on repeated designs, the error associated with each mean would be less than shown because the contribution due to subject differences was removed from the overall error term.

Study 2

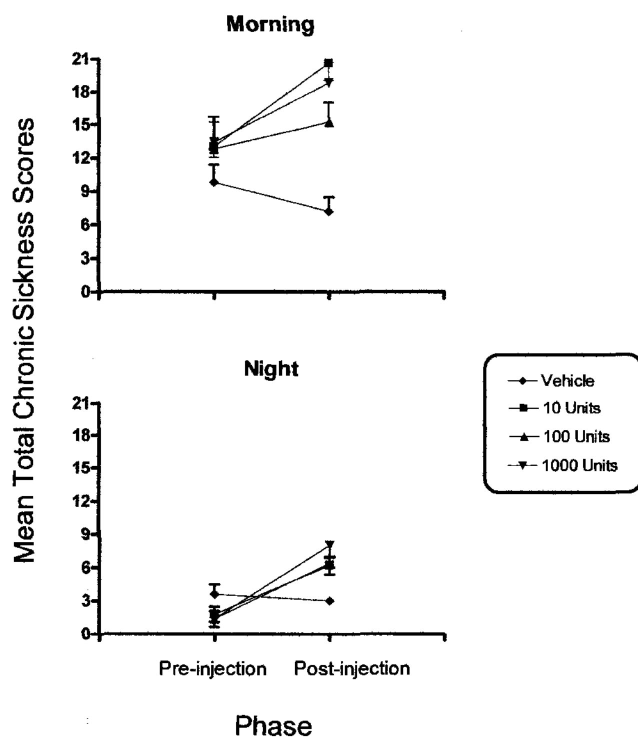


FIG. 2. The figure shows the total chronic sickness scores for all sickness behaviours evaluated (piloerection, ptosis, lethargy, and sleep) pre- and post-injection. Data are based on the average (± S.E.M.) summation of the top three scores across animals. The top graph represents the morning values and the bottom graph, the night-time values for each group (vehicle, 10, 100, or 1000 units of IFN-α). Note that because the analyses were based on repeated designs, the error associated with each mean would be less than shown because the contribution due to subject differences was removed from the overall error term.

Study 2

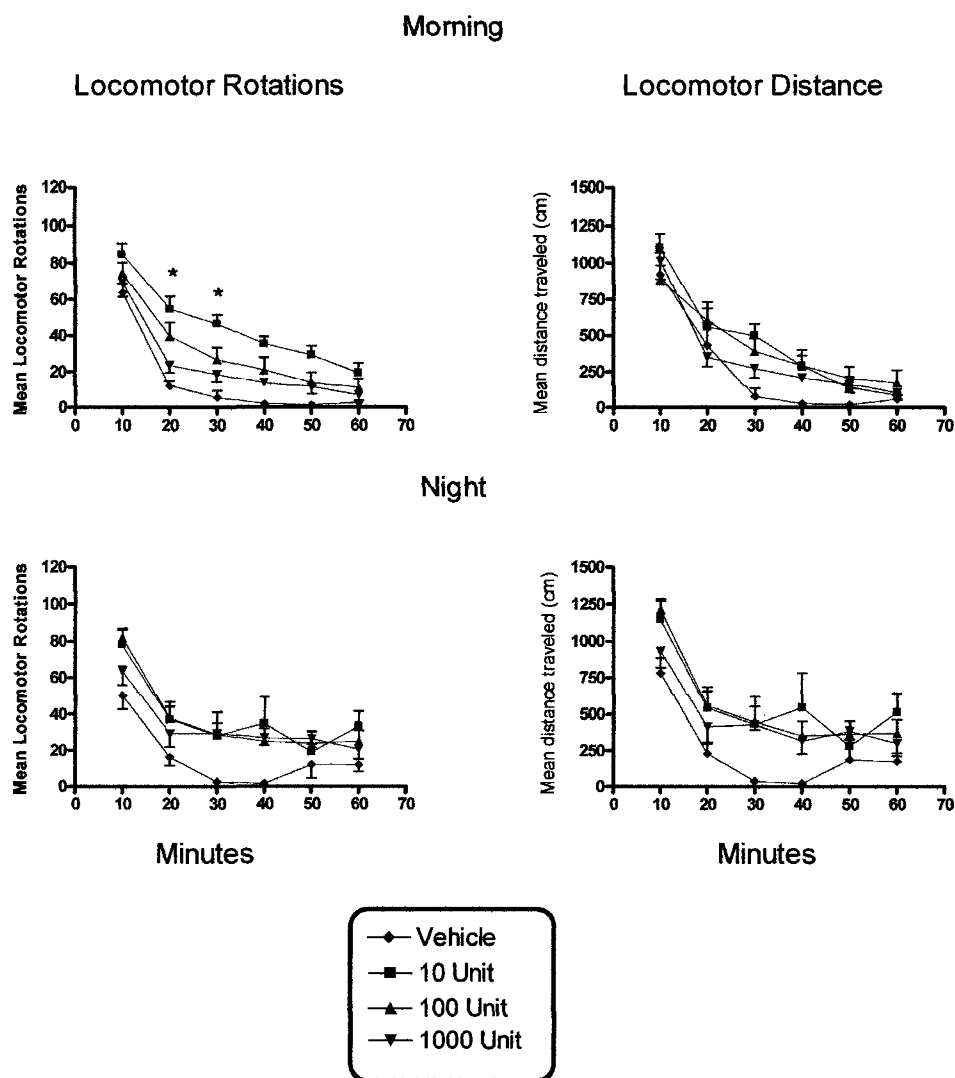


FIG. 3. This figure shows the total number of locomotor rotations (left) and distance traveled in cm (right) recorded for both morning (top), and night (bottom) time periods over a one-hour duration for each IFN-α dose (vehicle and 10, 100, or 1000 units). Data are expressed as means (± S.E.M.) with each of the six time points separated into 10-minute intervals. Note that each rat was placed into the locomotor box for observation immediately following the first temperature reading after drug administration. The * represents a significant simple main effect ($p<.01$). Note that because the analyses were based on repeated designs, the error associated with each mean would be less than shown because the contribution due to subject differences was removed from the overall error term.

Study 2

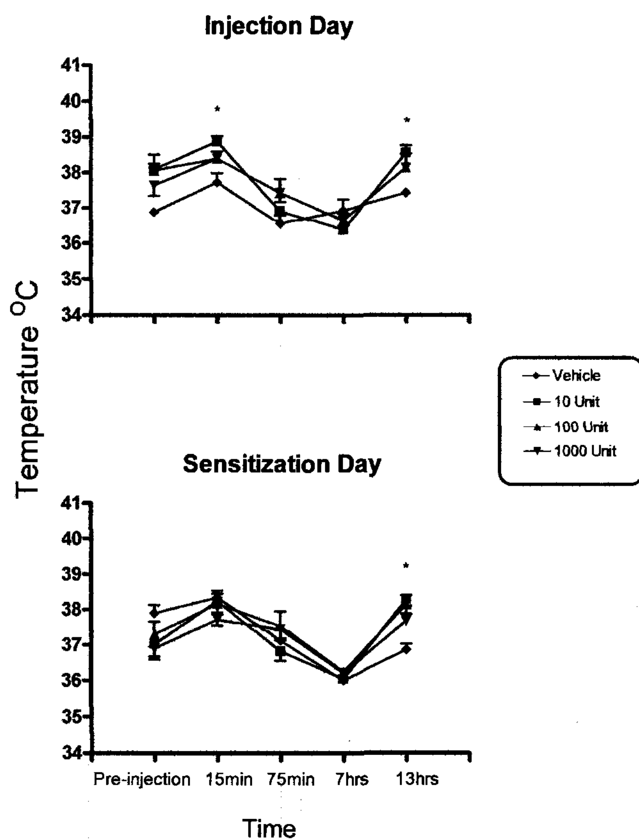


FIG. 4. Illustrated are the single injection day (top) and sensitization injection day (bottom) temperature graphs. Shown are mean (± S.E.M.) temperature (°C) values for baseline, 15 min, 75 min, 7 hrs, and 13 hrs post-IFN-α injection. The * represents significant simple main effect ($p<.01$). Note that because the analyses were based on repeated designs, the error associated with each mean would be less than shown because the contribution due to subject differences was removed from the overall error term.

Table 1. *Sequence of Procedures.*

Day	Phase	Procedure	Details
1 –7	Acclimatization	N/A	N/A
8	Baseline	Sickness behaviors	Tracked every 15 minutes during 1 hr locomotor tests.
		Locomotor Activity	Recorded 1 hour in morning, and 1 hour in evening.
9-12	Pre-injection	Sickness behaviors	Recorded daily (09:00, 15:00, 21:00 hrs).
		Rectal temperature	As above.
		Food & water intake, body weight	Daily at 9:00am
13	Injection	Sickness behavior	Tracked every 15 minutes during 1 hr locomotor tests.
		Locomotor Activity	Recorded 1 hour in morning, and 1 hour in evening.
		Rectal temperature	Before, 15 min, 75 min, 7 hrs, and 13 hrs after injectior
14-17	Post-injection	Sickness behaviors	Recorded daily (09:00, 15:00, 21:00 hrs).
		Rectal temperature	As above.
		Food & water intake, body weight	Daily at 9:00am
41-44	Sensitization	Sickness behaviors	Recorded daily (09:00, 15:00, 21:00 hrs).
		Rectal temperature	As above.
	Pre-injection	Food & water intake, body weight.	Daily at 9:00am
45	Sensitization	Locomotor Activity	Recorded 1 hour in morning, and 1 hour in evening.
		Sickness behavior	Tracked every 15 minutes during 1 hr locomotor tests.
	Injection Day	Rectal temperature	Prior to, 15 min, 75 min, 7 hrs, and 13 hrs after injectio
46-49	Sensitization	Sickness behaviors	Recorded daily (09:00, 15:00, 21:00 hrs).
		Rectal temperature	As above
	Post-injection	Food & water intake, body weight.	Daily at 9:00am.

Table 2. *Means and standard errors for pre- and post-injection sickness scores for ptosis, lethargy and sleep for morning, afternoon, and night for each IFN-alpha dose. Please note: all values are non-significant.*

Behavior		Pre-injection		Post-injection	
Morning		**Mean**	**SEM**	**Mean**	**SEM**
Ptosis	*Vehicle*	2.400	0.748	0.600	0.245
Lethargy		3.200	0.374	2.200	0.735
Sleep		0.200	0.200	0.600	0.400
Ptosis	*10 units*	2.000	0.447	4.400	1.077
Lethargy		6.800	0.800	7.200	0.374
Sleep		2.200	0.200	1.400	0.400
Ptosis	*100 units*	2.000	0.632	1.600	0.510
Lethargy		5.800	0.970	6.000	0.894
Sleep		2.200	0.800	0.800	0.583
Ptosis	*1000 units*	2.000	1.095	3.800	0.860
Lethargy		6.600	0.872	7.000	0.775
Sleep		2.200	0.583	1.000	0.447
Afternoon					
Ptosis	*Vehicle*	2.600	1.435	2.400	0.678
Lethargy		3.800	0.970	3.200	0.200
Sleep		0.200	0.200	0.000	0.000
Ptosis	*10 units*	3.200	0.374	3.200	0.860
Lethargy		7.000	0.775	6.600	0.510
Sleep		2.000	0.707	1.200	0.374
Ptosis	*100 units*	2.000	1.140	2.400	0.872
Lethargy		5.400	1.249	6.200	0.663
Sleep		1.400	0.678	1.200	0.374
Ptosis	*1000 units*	3.200	1.158	4.800	0.970
Lethargy		7.000	0.775	5.600	0.812
Sleep		2.600	0.678	0.800	0.374
Night					
Ptosis	*Vehicle*	0.200	0.200	0.000	0.000
Lethargy		0.400	0.245	0.200	0.200
Sleep		0.000	0.000	0.000	0.000
Ptosis	*10 units*	0.000	0.000	0.000	0.000
Lethargy		0.000	0.000	0.000	0.000
Sleep		0.000	0.000	0.000	0.000
Ptosis	*100 units*	0.000	0.000	0.200	0.200

Experiment 3

Published as: Kentner, A.C., James, J.S., Miguelez, M., Bielajew, C. Investigating the hedonic effects of Interferon-α on female rats using brain-stimulation reward. **Behavioural Brain Research**, 2007; 177:90-99.

1. Abstract

Interferon-α (IFN-α) is used as a front-line treatment for cancer and other diseases. Reports of depression as a consequence of IFN-α therapy scatter the literature, generating interest in the CNS disruptions elicited by this cytokine. In the present work, we investigated the short- and long-term effects of a single systemic injection of vehicle, 10, or 1000 units of IFN-α on temperature, body weight, food intake, sickness behaviours, locomotor activity, and brain stimulation reward (BSR) thresholds elicited from the ventral tegmental area in female Long Evans rats. Pioneered for studying motivational processes, BSR has been exploited as a tool for tracking hedonic status in animal models of depression. In this study, the main findings were that IFN-α did not induce anhedonia as defined by no increase in frequency thresholds. However, the analyses of sickness behaviours unveiled a significant increase in piloerection in all sham control animals that received an IFN-α injection while the BSR animal scores remained relatively unchanged between pre- and post-injection days. This pattern was also evident in the overall total sickness behaviour scores. Our data suggest that a single exposure to IFN-alpha treatment in female rats elicits long-term somatic effects, without altering hedonic status.

2. Introduction

The immune system is regulated through bidirectional interactions between the central and peripheral nervous systems (Blalock, 1989). Specifically, the peripheral route is modulated by the brain through both the endocrine and autonomic systems, and the complex method by which this communication takes place is mediated by cytokines. Cytokines are substances released by cells that have activational (pro-inflammatory) and inhibitory (anti-inflammatory) effects on other cells that influence behaviour (see Larson & Dunn, 2001). For example, the administration of cytokines has been shown to disrupt feeding (see Plata-Salamàn, 1998), induce cognitive deficits (Oitzl et al., 1993), alter activity levels (Zalcman et al., 1998), and change motivational state (Miguelez et al., 2004; Anisman et al., 1996, 1998). Such disturbances have been reported following the administration of a number of cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-2 (IL-2), and tumor necrosis factor- α (TNF- α). In this context the anti-inflammatory cytokine, interferon- α (IFN-α), has been generating renewed interest, given its widespread use in clinical populations for the treatment of hairy cell leukaemia, malignant melanoma, follicular lymphoma, Condylomata Acuminata, AIDS-related Kaposi's sarcoma, and chronic hepatitis C (Schering Corporation, 2002).

Interferon-α is an anti-inflammatory cytokine synthesized by leukocytes to ward against viral infection (Pestka et al., 1987). Its administration elicits several central nervous system disruptions that are comparable to those induced by pro-inflammatory cytokines, although the adverse effects of IFN-α have not been consistently demonstrated (De La Garza et al, 2005a; De La Garza et al., 2005b; Kentner et al.,

2006). In the animal literature, contradictory findings have emerged between laboratories with regards to IFN-α's effects on activity level (Kentner et al., 2006; Bethus et al., 2003; Kumai et al., 2000; Makino et al., 2000), food intake (Crnic et al., 1992; Segall et al., 1990), and core temperature (Sammut et al., 2001; Blatteis et al., 1991; Dinarello et al., 1984). Differences in motivational states have also been reported as a result of IFN-α exposure with some animal investigators observing depressive symptoms (Sammut et al., 2002, 2001; Makino et al., 2000, 1998) and others, no evidence of hedonic change (De La Garza et al., 2005a; De La Garza et al., 2005b). This is similar to the pattern reported in the clinical literature (see Loftis et al., 2004; Musselman et al., 2001). The appearance of depressive symptoms has recently been attributed to heightened baseline immune activation in patients that go on to develop major depressive disorder in the context of IFN-α treatment, at least for hepatitis C patients (Wichers et al., 2006).

Anhedonia was first coined in 1896 by Théodule Ribot, a French psychologist, and is referred to as the loss of interest in normally rewarding stimuli.While there has been some debate concerning the inclusion of anhedonia as one of the core features of depression (Auriacombe et al., 1997), animal models of cytokine and drug exposure have been developed with this as an underlying feature.

Typically, anhedonia is interpreted from decreases in sucrose intake and preference and increases in the thresholds for brain stimulation reward that are elicited following exposure to a series of stressors (Katz, 1982; Willner, 1997). This effect is reversible following antidepressant treatment (Moreau et al., 1993; Moreau et al., 1994; Moreau et al., 1996). Similar findings have been reported following the administration of cytokine

drugs, using sucrose consumption as an indicator of reward sensitivity (Brebner et al., 2000; Sammut et al., 2002, 2001). This behavioural consequence of cytokine challenge appears to be related to the activation of the HPA axis, thus eliciting the stress response (Anisman et al., 1993; Dunn, 1990).

It is generally believed that sex differences underlie the difference in prevalence of neuropathological disorders in males and females (Palanza, 2001). Clinical depression is purported to occur more frequently in females between adolescence and menopause before stabilizing to rates comparable to males (Gutiérrez-Lobos et al., 2002; Jorm, 1987). These observations lead one to speculate on the relatedness of hormonal and biological sex differences in the pathogenesis of depression. Recent investigations of this idea with respect to the chronic mild stress (CMS) paradigm have demonstrated that female rats display reduced sucrose intake or preference following stressors (Baker et al., 2006; Dalla et al., 2005; Konkle et al., 2003; Duncko et al., 2001), interpreted as anhedonia. Thus, in our study, we have chosen to employ female Long Evans rats, in order to assess the impact of IFN-α cytokine challenge on hedonic status and estrous cyclicity. Previously we have shown that in the Long-Evans strain, the estrous cycle is more susceptible to stress (Konkle et al., 2003).

Brain stimulation reward (BSR), long employed for studying motivational processes, has been exploited as a tool for tracking hedonic status in animal models of depression. In the context of cytokine drug administration, the thresholds for BSR are increased in male rats, interpreted as evidence for anhedonia (Miguelez et al., 2004; Anisman et al., 1998, 1996; Hebb et al., 1998). In our own work, we have found that one systemic injection of IL-2 was sufficient to elicit increased thresholds for BSR

from the ventral tegmental area. These effects were observed on injection day and persisted for a period of four weeks, the duration of the study (Miguelez et al., 2004). It was our intention, in the present work, to determine if a single intraperitoneal injection of IFN-α might elicit similar results. To our knowledge, this is the first experiment to evaluate the effects of IFN-α exposure on thresholds for BSR; the current literature has focused primarily on sensitivity to sucrose rewards in this context (De La Garza et al., 2005b; Sammut et al., 2002, 2001).

In addition, we tracked other behavioural and physiological changes induced by rat IFN-α two days prior and two days following drug administration. Specifically, we assessed sickness indices including piloerection, ptosis, lethargy and sleep, as well as locomotor activity, food and water intake, temperature, and weight.

3. Materials and Methods

3.1. Animals and Surgery

Twenty-eight female Long Evans rats (Charles River Canada, St-Constant, Québec, Canada), weighing between 242 - 329 g at time of surgery, were implanted with a bilateral pair of electrodes aimed at the ventral tegmental area (VTA) using standard stereotaxic techniques. Each animal received continuous administration of the inhalant anaesthetic, halothane, throughout the surgery. The VTA coordinates were 4.8 mm posterior to bregma, 0.7 mm lateral to the midsagittal suture, and 8.0 mm below the skull surface (Paxinos & Watson, 1998). The electrodes (Plastics One, Inc., Roanoke, VA) were constructed from stainless steel wire, 250 μm in diameter, and insulated with

polyimide to the tip. The current return consisted of a pliable stainless steel wire wrapped around three stainless steel skull screws. The entire assembly was secured to the skull by dental acrylic.

Animals were singly housed and maintained on a 12 h light/12 h dark cycle with Purina food and water available ad lib. Vaginal swabs were collected each weekday morning in order to track the estrous cycle. All animals had a black polyethelene tube, 9.5 cm round by 12.5 cm long and a nestlet (Ancare) available in their home cage at all times. Procedures were approved by the institutional protocol review board according to the guidelines of the Canadian Council on Animal Care.

3.2. Drug

Rat recombinant IFN-α was procured from Sigma-Aldrich (Missouri, USA). The concentration of the drug, once reconstituted with 1 ml sterile distilled water, was 1 x 10^5 units/ml. This was aliquoted into 60 μl stock vials and stored at -80°C until use. On the injection day, the stock solution was used to prepare working solutions of 10 and 1000 units per ml. These and the vehicle dose were injected intraperitoneally at a concentration of 1 ml/kg. The vehicle was prepared according to the protocol supplied by Sigma-Aldrich. We determined our cytokine administration regimen (10 and 1000 IFN-α units) based on the findings of Saphier and colleagues (1993, 1994), Kentner et al. (2006), and the anhedonic observations reported by Sammut and associates (2001). Previous assertions that 66 units of the rat source of the IFN-α is equivalent to the human dose currently administered to patients with viral hepatitis (Shinozaki et al., 2005) suggests that our choices are appropriate and comparable to that employed in the

clinical environment. All injections were administered between the hours of 8:00 and 10:00 am.

3.3. Sequence of Procedures

Figure 1 provides a flow chart of the sequence of procedures in this study outlined below.

3.4. Brain Stimulation Reward

Following recovery from surgery, screening for BSR was conducted in a wood and Plexiglas box with dimensions 27 cm deep by 37 cm wide by 51 cm high. Animals that demonstrated neutral or aversive responses to the stimulation were immediately placed into the sham control group. The remaining animals were trained to press a lever for brain stimulation using conventional shaping procedures. Animals that pressed the bar 30 times or more per minute without apparent motoric artefacts were retained in the BSR group (see Figure 1, box 1a and box 1b).

The stimulation consisted of 300 or 500 ms trains of square-wave monophasic cathodal pulses, 0.1 ms in duration. Stimulation was provided by a constant-current amplifier and a pulse generator (Stimtek Systems, S1202 ICSS, Acton, MA, USA). The 300 ms train was used in some animals in order to reduce stimulation-induced motor artefacts. The lowest current and frequency of pulses to elicit reliable responding were determined for each rat.

Following training, stabilization of the frequency thresholds began (see Figure 1, box 2a). In this procedure (method of limits), the current was held constant and

stimulation frequencies were delivered in a descending sequence. This began with a value that elicited maximum responding, and was reduced in equal $logarithmic_{10}$ steps (0.1 log_{10} between adjacent values) during each 60 sec trial, until a value that yielded little or no responding (example of a typical sequence - 40 Hz, 32 Hz, 25 Hz, 20 Hz, 16 Hz). The beginning of the first available frequency (40 Hz trial, for example) was signalled by three trains of "priming" stimulation, having the same parameters as that available during the trial. Priming stimulation was not delivered during the subsequent descending frequency values. The frequency threshold was interpolated from the rate-frequency function and defined as the value that supported half the maximum response rate. Four rate-frequency curves were collected during each session (using RF_CURVE software from Stimtek, Acton, MA, USA); the first was considered a warm-up, and discarded. The average threshold per session was based on the three remaining rate-frequency functions. Across rats, the current ranged from 250 to 630 μA. Animals were considered stable when the average frequency threshold did not vary more than 0.1 log_{10} units over three consecutive test days.

After stabilization, the rats in the BSR group received an intraperitoneal injection of the vehicle, 10 units, or 1000 units of IFN-α. Fifteen minutes later, four frequency thresholds were determined, and continued to be tracked every hour for five hours (see Figure 1, box 4a). Following this short-term phase, frequency thresholds were collected twice weekly for four weeks (see Figure 1, box 6a).

3.5. Locomotor Activity

The sham control animals were evaluated for locomotor activity in response to IFN-α administration. Three days prior to dug injection, sickness and locomotor scores were determined over a one hour period in the morning. After being placed in the locomotor apparatus, baseline sickness behaviour scores were collected from each rat, every 15 minutes, for the duration of the locomotor test (see Figure 1, box 2b). Our sickness behaviour measures were adapted from those employed by Hayley and collegues (2002, 1999) and Sudom and et al (2004) and included ptosis (droopy eyelids), piloerection, lethargy, and sleep. Each behaviour was scored on a three point scale (none = 0, mild = 1, or severe = 2), except for sleep which was scored on a two point scale as either 0 (absent) or 1 (present), as previously described (Kentner et al., 2006). We modified these previous methods in order to discriminate between the behaviours induced by a single IFN-α administration, and to evaluate the contribution of individual sickness behaviours to the overall sickness total, as opposed to creating a composite score based on the individual behaviours. Cohen's kappa (Howell, 2002) was employed in order to measure inter-rater agreement between two observers for each behaviour.

Locomotor activity was assessed in standard laboratory boxes identical to the animals' home cages. Each box was divided into six equal areas, each section fitted with an infrared photoelectric beam positioned above the area. Interruption of the beam by the rat's movement from one area into another was scored as a crossing. The distance traveled and the number of location changes were recorded every 15 minutes via software created in-house.

Following this baseline phase, animals were divided into three groups based on dose of interferon-α (vehicle, 10 units, and 1000 units).

Fifteen minutes following drug administration (see Figure 1, box 4b), rats were placed in a fresh locomotor cage for one hour in order to analyze any change in locomotor activity from pre-injection values. Sickness behaviours were again observed every 15 minutes during the one hour locomotor test. The tests were conducted between 8:30 and 11:00 hrs on both the pre- and post injection days.

3.6. Physiological and Behavioural Measures

After the frequency thresholds had been stabilized and the baseline locomotor test conducted, a series of physiological indices were collected from each rat over a two day period both before and after the IFN-cytokine challenge (see Figure 1, box 3). Body weight and food and water intake were documented once daily at 09:00 hrs. Rectal temperature (Harvard digital thermometer) and sickness behaviours were recorded three times daily (09:00, 15:00, 21:00 hrs) during this period. The sickness behaviour measures were identical to those described above.

On injection day, a baseline rectal temperature was recorded immediately prior to drug administration. Temperature was also documented at 15 min, 75 min, 7 hrs, and 13 hrs post injection (see Figure 1, box 5).

3.7. Histology

At the completion of the experiment, rats were anesthetized with an overdose of sodium pentobarbital (Somnotol). Animals in the BSR group were perfused

intracardially with physiological saline followed by a 10% formalin solution containing 10 % sucrose. Brains were removed immediately and stored in a formalin-sucrose solution. Frozen 40 μm sections were collected and subsequently stained with thionin for verification of the electrode tips.

3.8. Statistical Analyses

For the analysis of the sickness behaviour scores collected during the locomotor test (applies to sham control group only), the top three values of the four recorded during the one-hour test were summed for each behaviour (ptosis, piloerection, lethargy, and sleep). We used this method because in a few cases, only three scores could be obtained, due to animal position (crouching, for example), making it difficult to score the behaviour of interest. Difference scores (pre-injection minus post-injection) were computed and a Kruskal-Wallis non-parametric test (Howell, 2002) was used to evaluate any dose effects. If significant, pair-wise post-hoc tests were applied using the Wilcoxon Signed Ranks Test (Howell, 2002).

The same procedure was applied to the analysis of difference scores associated with each sickness behaviour assessed in all animals two days before and after injection. In addition, a composite score was compiled, determined by adding the summed scores for each sickness behaviour during the pre- and post-injection phases; these data were analyzed as above.

The temperature, food and water intake, and body weight data were each analyzed via a mixed ANOVA design, with two levels (control and BSR) of the independent factor group, three levels (vehicle, 10, or 1000 IFN-α units) of the independent factor

dose, and four levels of the repeated factor time (SPSS, 2004). Follow-up trend analyses were applied to the difference scores associated with the temperature data. These were based on the difference between the average of the two days pre-injection and two days post-injection. The temperature scores recorded on the injection day were similarly treated, except in this case there were five levels of time (before, 15 min, 75 min, 7 hrs, and 13 hrs after injection).

Locomotor rotations and distance data were likewise analyzed except that the repeated factor, time, had eight intervals. The BSR thresholds collected on the injection day and during the chronic phase were also evaluated using mixed ANOVA procedures.

Alpha level was set at a probability of 0.05 for all analyses and the Huynh-Feldt correction procedure was applied for violations to the assumption of sphericity (Howell, 2002).

4. Results

4.1. Sickness Behaviours

During the one hour locomotor tests, there were no significant effects of dose or group on any of the sickness behaviours (difference between baseline and injection days). A kappa test (Howell, 2002) was used to determine the level of agreement between two evaluators and was found to be significant for each of the sickness behaviours (sleep, $\kappa = 1.00$, $T = 6.481$; $p = .0001$, ptosis $\kappa = .905$, $T = 5.864$; $p = .0001$,

piloerection κ = .714, T = 4.629; p = .0001, and lethargy κ = 1.00, T = 6.481; p = .0001), indicative of high inter-rater agreement.

Figure 2 shows the summed scores for the piloerection data recorded during the pre- and post injection phases at the three daily time points for both the sham control (left panel) and BSR (right panel) groups (refer to Figure 1, box 3 and box 5). During this chronic monitoring period, a significant effect of group was found for morning ($\chi^2(1)$ = 4.637; p = .031) but not for the afternoon ($\chi^2(1)$ = 3.077; p = .079) or night ($\chi^2(1)$ = 1.554; p =.213) group data. Pre- and post-injection scores were assessed using Wilcoxon pair-wise tests. Significant differences were observed in the 1000 unit dose only for morning (Z = -2.2323; p = .026) and afternoon (Z = -2.070; p = .038) in sham control rats. The follow-up tests revealed no significant dose effects for either the sham control or BSR groups with respect to the vehicle or 10 unit doses. Although all of the piloerection scores increased in the sham control animals following injection, this trend was not seen in the BSR group.

Thus, the general pattern observed with respect to piloerection was that a 1000 unit dose of IFN-α did not produce long-term effects of piloerection that were observed in the sham control group. In this latter group, the effect of piloerection was observed in the morning, and afternoon, but extinguished at night.

Note that the analyses of the ptosis, lethargy, and sleep data did not give rise to any significant effects.

Figure 3 presents the composite scores of all sickness behaviours for each group (sham control – left panel and BSR – right panel) and time point. A Kruskal Wallis test conducted on the afternoon scores associated with the sham control group indicate a

significant dose effect of IFN-α ($\chi^2(2) = 6.286$; $p = .043$). The pre- and post-injection scores were then analyzed using Wilcoxon pair-wise tests. Significant differences were seen in the 1000 unit dose ($Z = 2.032$, $p = .031$) while the 10 unit dose approached significance ($Z = 1.826$, $p = .063$). The pattern observed suggests that the total sickness scores were elevated in the afternoon following both doses of IFN-α, an effect not seen in the comparable BSR group.

4.2. Locomotor Activity

Figure 4 illustrates the average total number of locomotor rotations (top graph) and distance traveled (bottom graph) during the one hour test following the IFN-α injection, in the case of the sham control rats only (see Figure 1 box 4b). The interaction effect (dose by time) associated with locomotor rotations ($F(14,77) = 1.792$; $p = .055$) and distance traveled ($F(5.025, 27.639) = 1.845$; $p = .136$) were non-significant. In general, the activity level associated with both IFN-α doses appeared to be lower compared to vehicle-treated animals. As expected, the main effect of time was significant in both analyses – locomotor rotations ($F(7,77) = 73.385$; $p = .0001$), and distance traveled ($F(7,77) = 31.833$; $p = .001$); the main effect of group was not.

4.3. Thresholds for Brain Stimulation Reward

In our animals, histological examinations confirmed that electrode placements were scattered within the ventral tegmental area. Figure 5 shows the average frequency threshold and maximum rate collected in each dose group over the long-term course of the study. Neither frequency thresholds ($F(12.038, 102.322) = .929$; $p = .522$) nor rates

(F(12.043 90.322) = .622; p = .819) were altered by any of the drug doses. The threshold measure remained impressively stable during the acute and subsequent four weeks of data collection. This pattern was confirmed by the results of the analyses. There were no group differences in baseline frequency thresholds which ranged from about 32 to 45 Hz.

Some variability was observed in the average maximum rate over time, most noticeably in the groups receiving the cytokine challenge. However, these appeared to be proportional to the baseline maximum rate, which was higher in this group.

4.4. Physiological Indices

A single injection of IFN-α was not associated with any changes in food and water intake and body weight, nor did it disrupt estrous cyclicity throughout the monitoring period. A chi-square test of independence between phase of estrous cycle and dose was non-significant. All animals maintained the normal increase in body weight expected over the time course of the study (F(4.175, 91.843) = 32.222; p = .0001).

4.5. Temperature Analyses

Figure 6 displays the temperature difference scores obtained during the morning collection period with the data for sham control animals on the left and BSR animals on the right. Recall that temperature was recorded two days before and two days post IFN-α injection (see Figure 1 box 3 and box 5). The plotted scores represent the difference between the average of the two days before and two days after injection. The statistical analyses performed on the original scores revealed a significant main effect of group

(F(1,22) = 8.628, p = .008). Given the group by dose pattern obtained on examination of the plotted difference scores, we performed trend analyses and found a near significant quadratic trend associated only with the sham control data ($F(1,12) = 4.302$, $p = .06$) versus the BSR data ($F(1,10) = 1.210$; $p = .297$). No other obvious trends were revealed.

5. Discussion

In the present study, it was our intention to investigate the potential anhedonic consequence of a single systemic injection of rat IFN-α, in addition to other behavioural and physiological measures in female Long Evans rats. In healthy human volunteers, administration of a single dose (1.5 MIU//m^2) of IFN-α interferes with cognitive performance on a stimulus tracking task while a low dose (0.1 MIU/m^2) causes disruptions in alertness (Smith et al.,1988). In cancer patients, depression has been reported in a subset of individuals undergoing treatment with IFN-α (Collier and Chapman, 2001). It is these cognitive and affective symptoms elicited by IFN-α and other cytokines that have captured the attention of many investigators, contributing to the development of animal models of cytokine-induced sickness behaviours.

Stemming from findings of prolonged symptom duration as a result of IFN-α challenge (Smith et al., 1988), we chose to explore the consequences of a single administration before determining the effects of repeated injection schedules. In addition, we employed the rat form of the cytokine as opposed to the human subtype. Since human IFN-α binds in the rat brain (Janicki, 1992), it is justifiably the most

widely used form of the cytokine in animal studies. However, differential behavioural effects have been demonstrated between the human and rat form of IFN-α (Makino et al., 2000; Saphier et al., 1994), and concern over the appropriateness of the human source in animal studies has been expressed (Kentner et al., 2006; De La Garza et al., 2005b; Miguelez et al., 2004). Other methodological differences, such as drug dose and administration route, may also explain some of the inconsistencies in behavioural outcomes between studies.

In response to a single systemic injection of rat IFN- α, we observed slight temperature increases, a decline in locomotor activity, and the manifestation of sickness behaviours as gauged by physical appearance measures, but no changes in hedonic status, estrous cyclicity, or food-related behaviours.

5.1. Sickness Behaviours

Observed physiological disruptions were in the form of elevated long-term morning temperature which was accompanied, not surprisingly, by increased piloerection scores. In general, during the two day pre- versus post-injection morning and afternoon time periods, piloerection was significantly higher following the IFN-α injection in sham control rats, particularly in those receiving the 1000 unit dose. Although we do concede to non-significantly higher piloerection levels in our vehicle-treated control animals, comparatively, our BSR rats administered either the vehicle, or rat IFN-α displayed attenuated levels of piloerection. Persistent levels of piloerection have also been reported as a general observation by Reyes-Vazquez and colleagues (1994). These behavioural responses are likely energy conservation mechanisms related to the febrile

disruption caused by IFN-α challenge. However, female rats undergoing BSR tests failed to demonstrate any significant elevations in piloerection levels. Interestingly, male rats challenged with IFN-α demonstrate persistent long-term temperature scores alongside increased piloerection, primarily in the evening observation periods, as well as during the evening of injection day (Kentner et al., 2006) which follows the premise that immune activation is most effective at night corresponding to the rat's more active phase (Ucar et al., 1983).

Depression is one of many in a long list of generalized non-specific side-effects of cytokine treatment that includes disrupted thermoregulation, lethargy, hypersomnia, suppressed appetite, decreased activity, and lowered mood (Hart, 1988; Kent et al., 1992). In many investigations of animal models of depression, reductions in sucrose intake and preference are used to determine the presence of anhedonia following cytokine challenge with IL-1β, TNF-α, and IFN-α (Sammut et al., 2002; Sammut et al., 2001; Brebner et al., 2000; Hayley et al., 1999). In Sammut et al's studies (2002; 2001)intake of a 1% sucrose solution has been shown to be dramatically reduced in male Lister Hooded rats administered repeated intraperitoneal injections of both human and rat IFN-α (Sammut et al., 2001). Subsequent work confirmed that human IFN-α (1000 units) inhibited intake of a 1% sucrose solution in a reversible manner when rats were administered antidepressants (Sammut et al., 2002). Unfortunately most tests of palatable-intake are preceded by food and water restriction which in itself adds a stressful component to the procedure. However, it has been shown that latency to consume a sucrose pellet is reliable in mice without any deprivation protocols (Merali et al., 2003). Using tests dependent on consumption in order to assess cytokine-induced

sickness behaviours introduces the difficulty of interpreting the suppression of fluid or food intake due to anhedonia from that as a consequence of anorexia. As a result, there has been increased reliance on tasks that do not require edible substances as primary motivators.

Brain stimulation reward has been used to assess the anhedonic effects of cytokine challenge and is the paradigm that we chose to employ in the current study. Our frequency threshold data suggest that the rewarding properties underlying this site are not disrupted by a single systemic injection of IFN-α. Interestingly, our group has previously observed an anhedonic effect induced by a solitary administration of a low dose of rat IL-2 on responding for ventral tegmental BSR. In that study, increased frequency thresholds evolved shortly post-injection, progressed to maximal values several hours later, and were sustained until the end of the month-long monitoring period (Miguelez et al., 2004). Similar anhedonic effects of IL-2 were reported by Anisman and colleagues (1998) as well as Hebb et al. (1998). These findings nicely corroborate the clinical literature in showing that a single administration of IL-2 produces immediate changes in affective states (Maes et al., 2001; Capuron et al., 2000), whereas comparatively, symptoms of major depressive disorder are observable within the first month of treatment in some patients receiving repeated administration of IFN-α therapy (Capuron et al., 2000).

5.2. Locomotor Activity

Ambulatory behaviours are often used as an index of the overall physical health of an animal with sick animals typically displaying reduced activity compared to healthy

ones (Hart, 1988). Although we did observe a general decline in locomotor activity, we, like others have failed to show any significant changes in locomotor activity following IFN-α administration (Bethus et al., 2003; Makino et al., 2000;Crinc and Segall, 1992).

The general decline in motor activity observed following cytokine challenge did not appear to be gender dependent in one study investigating IL-1 in rats (Yirmiya et al., 1995). However, other work by the same laboratory suggested that administration of IL-1 decreased open field activity in male rats but in female rats only during their estrus phase (Avitsur et al., 1995). Note that activity levels in female rats tend to be generally higher during estrus (Avitsur et al., 1995). In the present study, estrous cycle was not an indicator of activity level, nor was estrous cyclicity disrupted by a single IFN-α administration

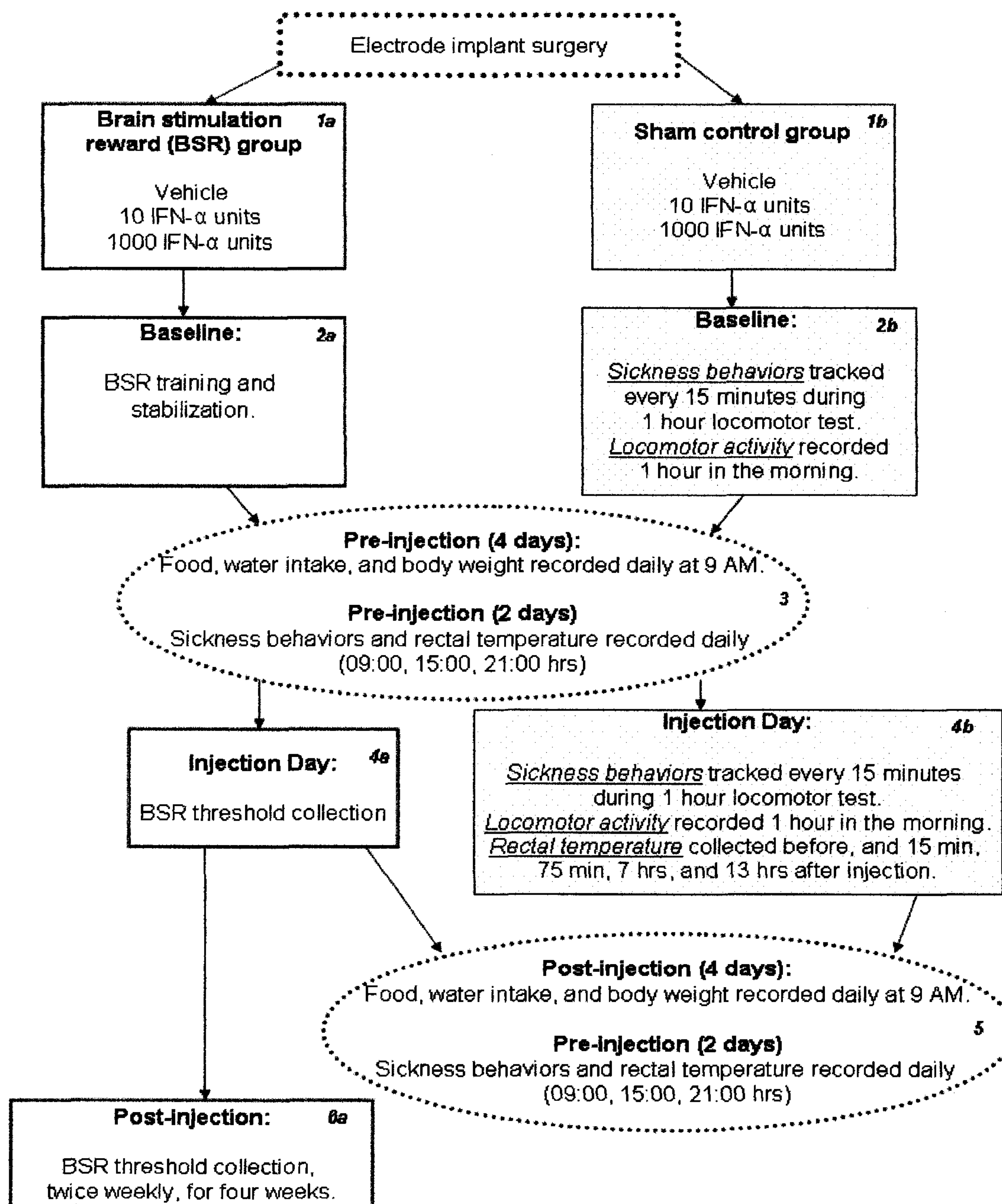
5.3. Conclusions

In this study we did not demonstrate disruptions in hedonic status in female rats following IFN-α challenge, contributing to a growing animal literature that suggests that females may be less vulnerable to stressful stimuli than once assumed (Dalla et al., 2005; Drossopoulou et al., 2004; Konkle et al., 2003; Bielajew et al., 2003; Bowman et al., 2003; Karandrea et al., 2002). Despite clinical reports that females outnumber males in the incidence of depression at rates of more than 2:1 (Hankin & Abramson, 1999; Kessler et al., 1993), hormonal and biological factors have yet to be established as the primary determinants underlying this assertion (Kornstein, 1997). Indeed, some clinical studies report that marital and child bearing status are correlated with depression rates. For example, females without children, married or not, have rates of depression that are

lower than that associated with females with children, regardless of marital status (Lucht et al., 2003; Garter et al., 1989). This is worth noting particularly since depression rates are reported to steadily decline in males, but less rapidly in females after the age of 25 (childbearing years) until 50 years of age when depression rates become comparable between the sexes (Gutiérrez-Lobos et al., 2002). It may be that the combination of bearing and raising children with hormonal changes that accompany childbirth contributes to an increased susceptibility for depression in females. Of course, additional factors such as differences in symptom recognition (Cotton et al., 2006), help-seeking behaviour, and differences in the willingness to report depressive symptoms between males and females (Andrews et al., 2001; Barsky et al., 2001; Verbugge, 1980) undoubtedly also contribute to the preponderance of clinical depression reported in females.

Study 3

FIG.1. Flow chart showing the different conditions and phases of the study.



Study 3

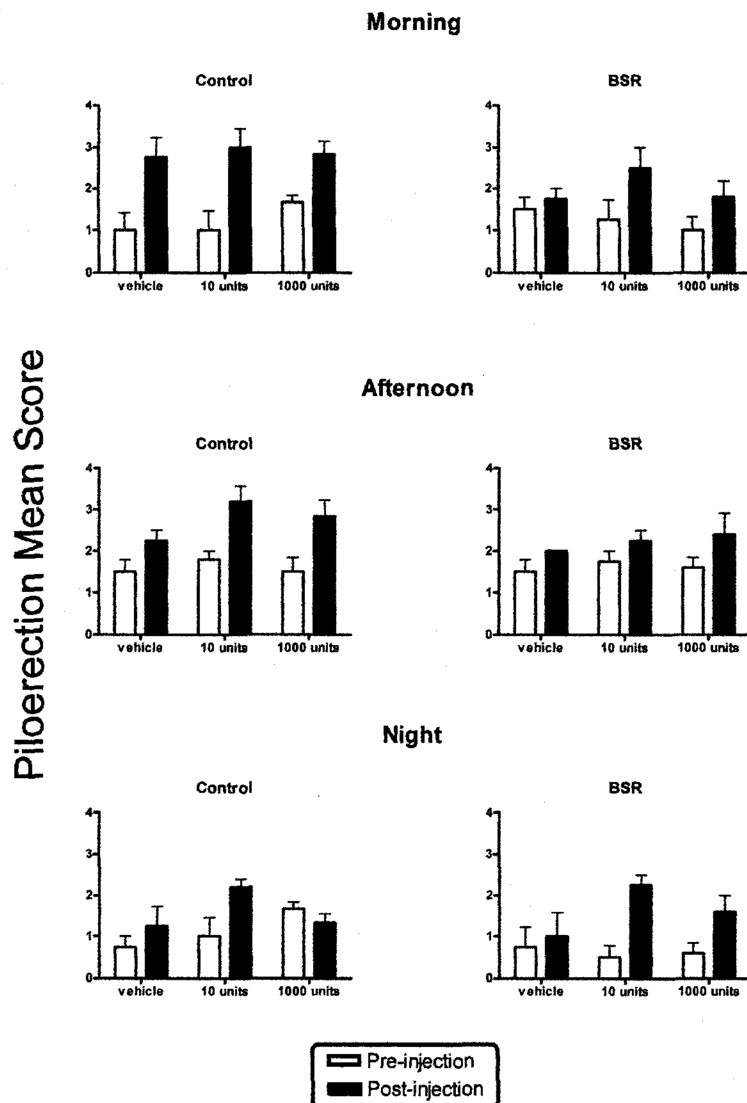


FIG.2. The average total piloerection scores associated with morning (top graph), afternoon (middle graph), and night (bottom graph) time periods for each dose (vehicle, 10, and 1000 units of IFN-α) and group (sham control on the left and BSR on the right) are displayed. The two bars (pre- and post-injection) represent the summation of the top three piloerection scores observed in each rat.

Study 3

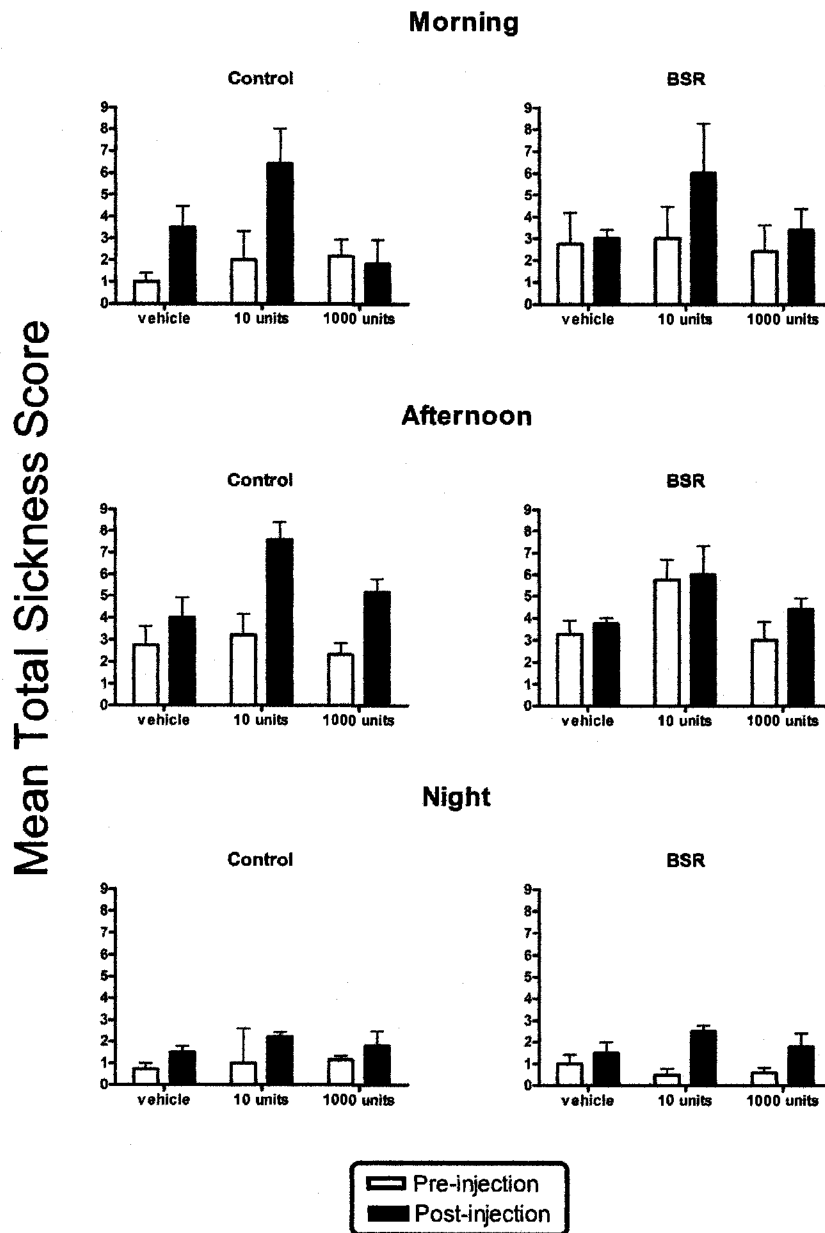


FIG.3. The figure shows the composite score based on all sickness behaviours (piloerection, ptosis, lethargy, and sleep) pre- and post-injection. Data are based on the summation of the top three scores observed in each animal and are expressed as means. The top graph represents the morning values and the bottom graph the night time values for each dose (vehicle 10, and 1000 units of IFN-α) and group (sham control on the left and BSR on the right).

Study 3

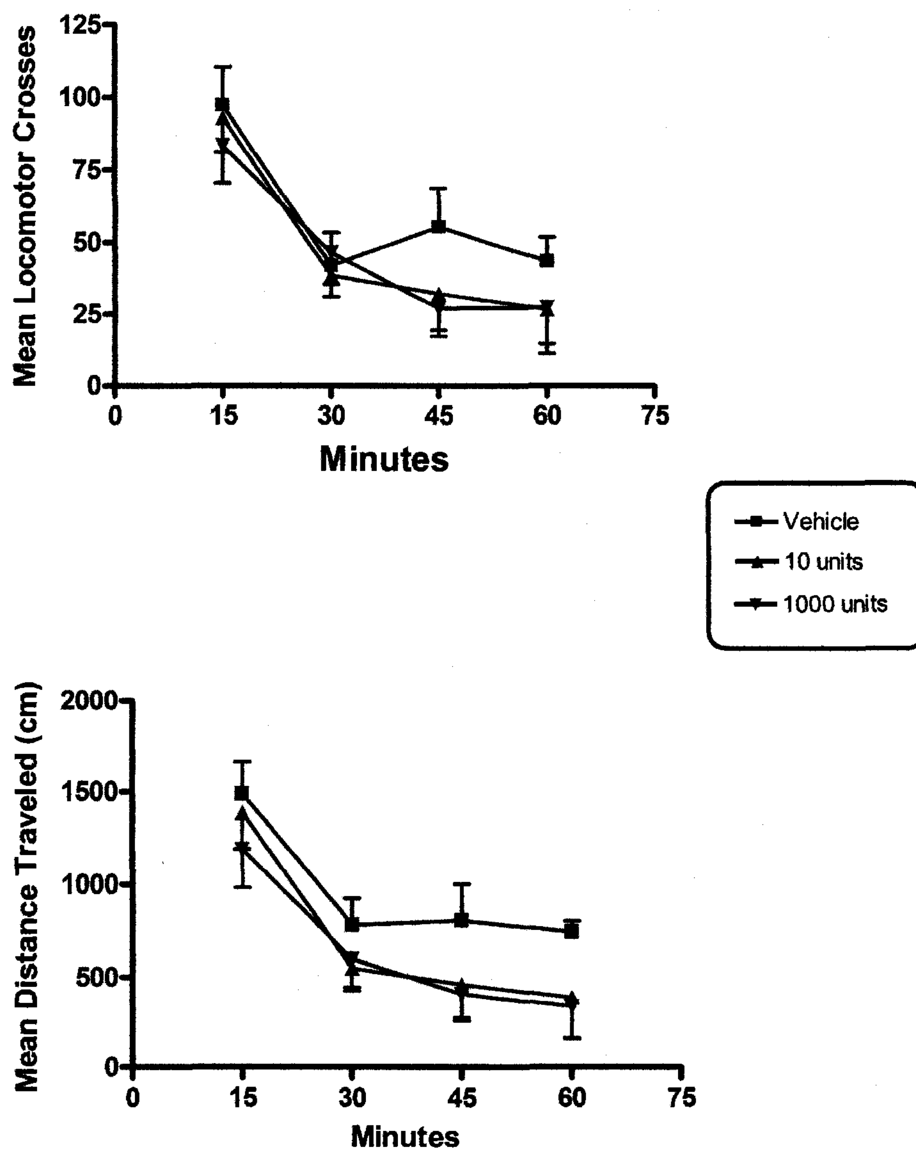


FIG.4. This figure shows the total number of locomotor rotations (top) and distance traveled in cm (bottom) recorded during the one-hour test for each IFN-α dose (vehicle, 10, and 1000 units). Data are expressed as means with each of the four time points representing a 15-minute interval. Note that each rat was placed in the locomotor box for observation immediately following the first temperature reading after drug administration.

Study 3

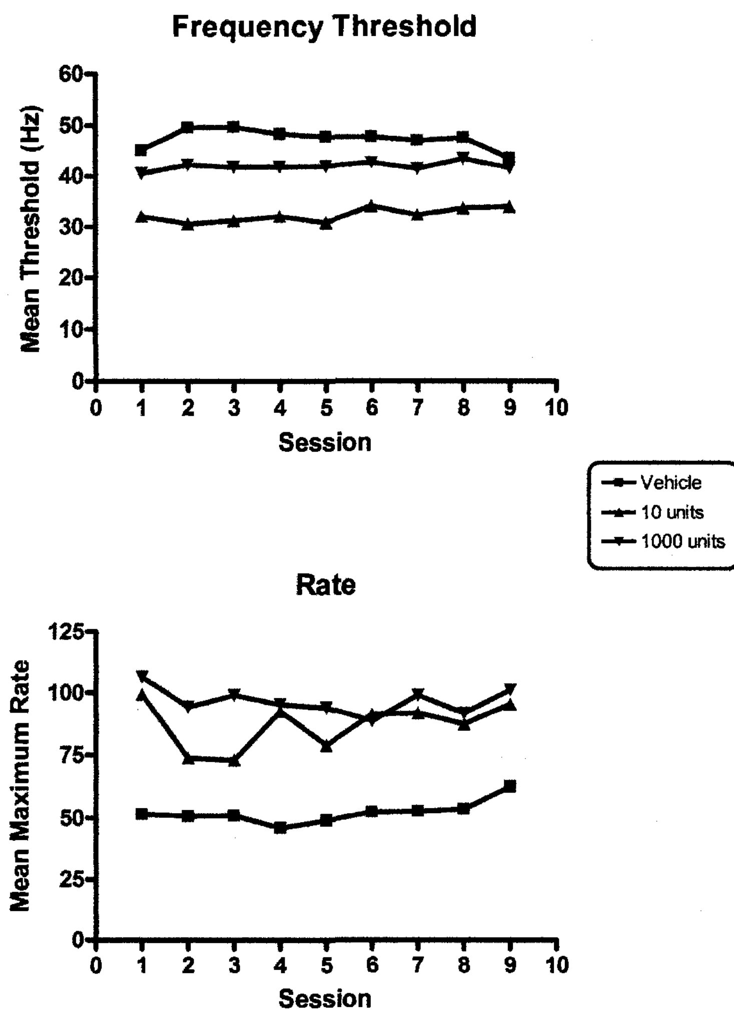


FIG.5. This figure shows the frequency threshold (top) and rate (bottom) during the long-term BSR tests that occurred twice weekly over four weeks for each IFN-α dose (vehicle, 10, and 1000 units). Data are expressed as means with the first point representing pre-injection and each additional point thereafter representing post-injection.

Study 3

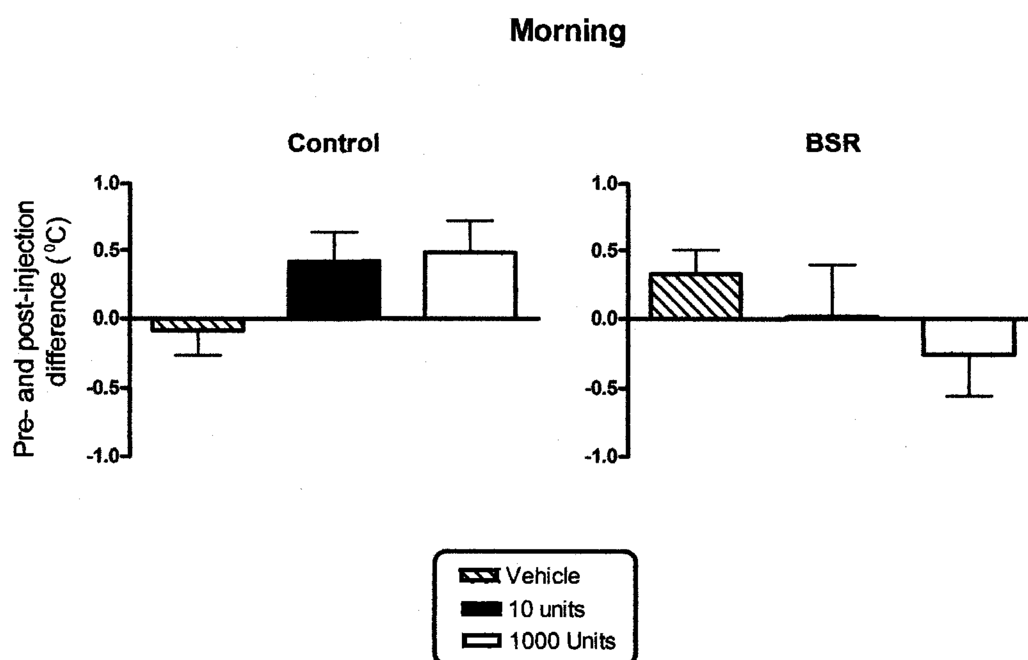


FIG.6. The plot illustrates the mean difference in temperature (°C) scores obtained during the morning period that were collected two days before and two days after IFN-α injection in the sham control (left) and BSR (right) groups, according to IFN-α dose (vehicle, 10, and 1000 units).

Experiment 4

Submitted to Brain Behaviour and Immunity as: Kentner, A.C., Takeuchi, A., James, J.S. Miki, T., Seino, S., Hayley, S., Bielajew, C. The effect of 'reward' on lipopolysaccharide induced sickness behaviours and cytokine expression in the female Sprague-Dawley rat.

There is at the bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes.
-George Bernard Shaw
The Doctor's Dilemma, Act I, 1906.

Abstract

Responding for rewarding brain stimulation has been used to track hedonic status in animals. In addition to neurochemical alterations, stimulation of the lateral hypothalamus or ventral tegmentum has been shown to influence immunological processes, including elevation of peripheral natural killer cell activity. In the present study, we examined whether ventral tegmental area stimulation or environmental enrichment altered the severity of lipopolysaccharide (LPS)-induced sickness behaviours and the provocation of cytokine expression induced by the endotoxin. Accordingly, rats received either trials of brain stimulation reward or exposure to an enriched environment and subsequently challenged with a 150 ug/kg i.p. of LPS. Groups receiving LPS and saline injections without further manipulation were also included. Using the real-time RT-PCR and a multiplex bead assay, mRNA and protein levels for several cytokines and their receptors were determined to evaluate how these may vary as a consequence of reward. Both brain stimulation and environmental enrichment similarly diminished sickness behaviours associated with the endotoxin. Receptor gene levels were generally stable across groups. Differences in peripheral cytokines were found in IFN-γ (elevated in enriched animals) and reduced IL-10 in the BSR group. Brain IL-6 levels were increased in the group receiving LPS challenge alone. Taken together, these data suggest that rewarding hypothalamic stimulation and environmental enrichment may buffer against malaise provoked by inflammatory stimuli and influence immunological status through changes in specific cytokine subsets.

I. Introduction

One goal of brain stimulation reward (BSR) studies is to understand how the activation of a central motivational state has biological consequences. Our specific interest is related to its impact on immunological and endocrine function. The rewarding element of brain stimulation is elicited most robustly from sites within the medial forebrain bundle (reviewed in Gallistel, 1973) including the lateral hypothalamus (LH) and the ventral tegmental area (VTA), the latter being a main source of dopamine pathways (Robbins and Everitt, 2003). Fessel and Forsyth were the first to observe a modulatory effect of brain stimulation reward on immune functioning, as indicated by increased levels of rat immunoglobulin-γ (see Vlajković et al., 1993).

Subsequently, several investigators demonstrated elevated levels of peripheral lymphocytes and enhanced mitogen-induced humoral and cellular immune responses as a consequence of BSR (Wrona et al., 2004; Wrona and Trojniar, 2003; Wenner et al., 2000; Wenner et al., 1996; Vlajković et al., 1993; Šakić and Vlajković, 1990; Janković et al., 1988).

For example, rats that passively received stimulation of the LH had increased splenic natural killer cell (NK) activity; in the same study, stimulation of brain regions that do not generally support BSR (frontal cortex) had no such effect. The results suggested that it was the rewarding property of the stimulation specifically that altered immune activity (Wenner et al., 1996). Although there was no verification that the electrode sites supported reward, this is an important consideration, as not all LH

electrodes elicit rewarding responses and some frontal cortex sites do (Bielajew and Trzcinska, 1998).

Others have reported that non-contingent stimulation of the LH and VTA, but not the thalamus, had marked immunomodulatory effects in conscious rats, including elevations of NK cytotoxicity (Wrona et al., 2004; Wrona and Trojniar, 2003) and large granular lymphocytes, the latter following LH stimulation only (Wrona and Trojniar, 2003). It is noteworthy that non-contingent stimulation, even to rewarding brain areas, have been shown to produce aversive responses (escape) (see Steiner et al., 1969; Sudakov et al., 1989; Cantor et al., 1971), and that aversive stimulation itself may affect immunocompetence (Wrona and Trojniar, 2005; Demetrikopoulos et al., 1994). In this regard, stimulation applied to brain sites thought to induce aversive behavioural responses suppressed several immune factors (Wrona and Trojniar, 2005; Demetrikopoulos et al., 1994), and it is possible that the variations among these immune factors is tied more to site specificity rather than hedonic mechanisms. Nevertheless, it appears that brain stimulation does influence the immune system; however the rewarding contribution of such stimulation has not been properly validated.

The immunomodulatory effects of BSR may have important functional implications applicable to several central conditions, including depression and other stressor-related disorders. In this respect, non-contingent LH stimulation was found to moderate the impact of a number of stressors, such as tail shock, in which case the stimulation reduced the size and number of stressor-induced gastric lesions and stomach ulcers (McCutcheon et al., 1986; Freimark, 1973; Marshall and McCutcheon, 1976).

Moreover, BSR has been shown to have protective effects against the anorectic consequences of drugs such as interleukin-2 (IL-2) (Miguelez et al., 2004) and paroxetine (Konkle and Bielajew, 1999), compared to non-stimulated rats receiving the same treatment.

Similar protective (or immuno-enhancing) effects have also been observed as a consequence of environmental enrichment; however these distinctions are usually reported in indices related to learning and memory (Jones, et al., 1996; Escorihuela et al., 1995; Mohammed et al., 1993; Rosenzweig, 1966) such as neuronal development (Johansson and Belichenko, 2002; Globus, et al., 1973; Diamond, 1967; Diamond et al., 1964).

There is a paucity of studies investigating the effects that environmental enrichment has on biochemical/immune responses, and the findings have not been consistent. For example, recent experimental evidence suggests that plasma corticosterone levels in enriched animals increase *prior* to stressor application (Monceck et al., 2004) while others have found slight decreases (Belz et al., 2003), or no effect on resting plasma concentrations from exposure to an enriched environment (Morely-Fletcher et al., 2003; Schrijver, et al., 2002). Recently, one study reported higher levels of NK activity in the spleens of environmentally enriched mice, compared to standard housed controls, indicating increased host resistance to disease (Benaroya-Milshtein et al., 2004). However, other work has reported lower interferon (IFN)-γ/IL-2 and IL-2/IL-10 levels in environmentally enriched mice which may indicate vulnerability to viral challenge (Marashi et al., 2003). Whether these differences are due to increased or decreased

immunocompetence in response to environmental enrichment, or are merely a result of redistribution of immune markers and their responses is unknown.

There have also been reports regarding the functional nature of environmental enrichment. For example, Döbrössy and Dunnett (2004) used quinolinic acid to unilaterally lesion the dorsal striatum of rats pre-trained on various motor tasks. Of the animals given neuronal grafts, those housed in the enriched condition outperformed grafted animals kept in standard housing when tested on a skilled staircase task. Another study in pigs evaluated the role of rewarded behaviour on wound healing following a biopsy punch. Animals trained to respond to a tone in order to receive food as a reward had significantly smaller wounds five days following biopsy than control pigs fed under regular conditions (Ernst et al., 2006).

It is this background that prompted us to investigate the possibility that brain stimulation, and reward in general, may have protective consequences against immune challenges. As yet, there is a scarcity of data regarding the role that reward may play in immunity, specifically in the regulation of cytokines.

Cytokines are glycoproteins synthesized by immune cells and have pro- and anti-inflammatory effects on other cells (Abbas & Lichtman, 2003; Janeway et al., 2001) and act in an autocrine and/or paracrine manner in addition to being endocrine signals (Plata-Salamán, 1998). Cytokines are linked to a series of behavioural disturbances including anorexia, REM sleep changes, lethargy, altered activity levels, fever, and changes in motivational state; these behaviours, as a group, are known as 'sickness behaviours' (Kent et al., 1992). The endotoxin, lipopolysaccharide (LPS) is a potent

stimulator of the release of cytokines which results in some of these behavioural signs of sickness (see Maier and Watkins, 1998).

In this study, we investigated the potential protective role that BSR and another form of reward, environmental enrichment, may have on sickness behaviours associated with LPS challenge. In addition, endogenous levels of a panel of pro- and anti-inflammatory cytokines were assessed following BSR and environmental enrichment in order to delineate if these immunotransmitters are linked to any immunomodulatory consequences of the treatments.

2. Methods

2.1. Animals and surgery

Forty-six female Sprague-Dawley rats were obtained from Charles River Canada (St-Constant, Québec, Canada). They were individually housed in standard cages and kept on a 12 h light/12 h dark cycle with light onset at 0700 hour. Each cage was outfitted with a hiding tube (9.5 cm round by 12.5 cm long) and a nestlet (Ancare Corporation). Food and water were freely available at all times. Body and food weights were collected once weekly throughout the study. Vaginal swabs were taken daily in order to track the estrous cycle. All procedures were followed according to the guidelines of the Canadian Council on Animal Care.

Each rat was implanted with a bilateral pair of electrodes aimed at the VTA using standard stereotaxic techniques. The coordinates were 4.8 mm posterior to bregma, 0.7 mm lateral to the midsagittal suture, and 8.0 mm below the skull surface (Paxinos &

Watson, 1998). Anaesthesia was supplied via continuous administration of either halothane or isoflurane, inhalant anaesthetics (Sigma-Aldrich, Canada).

The electrodes, (HRS Scientific, Montreal, Canada) were made from stainless steel wire, 250 μm in diameter, and insulated with polyimide to the exposed tip. The current return consisted of a pliable stainless steel wire wrapped around three stainless steel skull screws. The entire assembly was secured to the skull using dental acrylic.

2.3. Brain stimulation reward

Following a one week recovery from surgery, rats were screened for BSR using conventional shaping techniques. All simulation tests were conducted in a wood and Plexiglas chamber with dimensions 27 cm deep X 37 cm wide X 51 cm high. Animals that demonstrated neutral or aversive responses were assigned to either a drug control group (n = 12), or an environmentally enriched group (n=12) on the basis of one 20 minute screening session per electrode. Animals that responded at a rate of at least 30 bar-presses/minute with little or no stimulation-induced motor artifacts (n = 12) were retained in the BSR group. An additional group (n =10) served as sham control vehicle animals. Thus, the study comprised four groups of animals - a group that received BSR and drug, a group that received environmental enrichment and drug, a group that received drug and no manipulation, and a group that received vehicle injections and no manipulation. Figure 1 outlines the group designations and procedures for this study via a flow chart.

The BSR animals received 500 ms trains of square-wave monophasic cathodal pulses, 0.1 ms in duration. Stimulation was provided by a constant-current amplifier

and a pulse generator (S1202 ICSS Systems from Stimtek, Acton, MA, USA). The lowest current and frequency of pulses to elicit responding were determined individually for each rat. Across rats, the current ranged from 250 to 800 μA.

After the initial screening for BSR, training and stabilization of the threshold procedure began. Using the method of limits, the current was kept constant and the stimulation frequencies delivered in a descending sequence, beginning with a value that elicited maximum responding, and reduced in equal logarithmic steps (0.1 $\log_{10}$ between adjacent values) each 60 sec trial, until a value that yielded little or no responses per minute (example of a typical sequence - 50 Hz, 40 Hz, 32 Hz, 25 Hz, 20 Hz). The frequency threshold was interpolated from the rate-frequency function and defined as the value that supported half the maximum response rate. Four rate-frequency thresholds were determined each session (RF_CURVE software from Stimtek, Acton, MA, USA), the first always considered a warm-up and discarded. The average threshold per session was based on the three remaining rate-frequency functions.

During the training phase, each rat received nine sessions staggered over a four-day period followed by one session a day for 12 days until the first tail blood sample was collected. Subsequently, rats received one daily BSR session for an additional 12 consecutive days until the second and final tail blood sample was collected.

2.3 Enrichment housing

The environmentally enriched animals had access to a large three-floor cage built with grid sides for climbing (0.73 m height X 0.44 m width X 0.75 m depth). They

were housed in groups of six from 08:00 to 16:00 hrs daily for the duration of the experiment, except for the two days on which tail blood was collected. Each cage was equipped with a climbing rope, hammock, and large playing tubes in addition to small kitten toys. Between enrichment sessions, rats were housed individually in standard cages. The groups receiving no manipulation, and rats that obtained BSR, were similarly housed.

2.4 Sickness behaviour evaluation

Sickness behaviours were evaluated three times during the study - at the baseline phase and two hours after each injection. The behaviours included ptosis (droopy eyelids), piloerection, lethargy, and sleep. Each behaviour was scored on a three point scale (none = 0, mild = 1, or severe = 2), except for sleep which was scored on a two point scale as either 0 (absent) or 1 (present). Additional details of the sickness behaviour procedure have been described recently in Kentner et al., 2006.

2.5. LPS injections

Following the baseline phase, all groups received an intraperitoneal injection of 150 μg/kg of lipopolysaccharide (LPS) (Sigma-Aldrich) or an equivalent volume of saline between 0600 and 0730 hrs in order to stimulate an immune response (please refer to Figure 1).

2.6. Tail blood collection

When the scoring of sickness behaviours was complete, each animal was placed under light halothane or isoflurane-induced sedation. Tail blood was drawn under sterile conditions using a 23-gauge butterfly syringe flushed with anticoagulant EDTA inserted into the tail vein. Collection occurred between 8:00 and 10:30 hrs and took approximately five minutes per rat. The samples were spun and the plasma aliquoted and stored at -80 degrees Celsius until analysis via a multiplex bead-based immunoassay. Lysis and homogenization of the remains of each sample occurred immediately and were likewise stored at -80 degree Celsius until RNA isolation for PCR analysis. All animals were then returned to their respective housing conditions for an additional twelve days until the second LPS challenge, at which point the sickness behaviour and tail blood collection procedures were repeated.

2.7. Brain collection and histology

Eight hours following completion of the second LPS challenge, rats were euthanized in a carbon dioxide chamber. Spleens were removed and weighed, and brains extracted, flash frozen, and stored at -80°C for later sectioning and analysis.

Frozen 30 μm sections were collected from animals in the BSR group in and around the implantation site and then stained with thionin for verification of the electrode tips. The remaining brains were placed in a brain blocker and cut into 2 mm coronal sections. Using the Paxinos and Watson (1998) brain atlas as a guide, tissue from the

VTA was collected for further analysis via a multiplex bead-based immunoassay. Samples were obtained by micro-punch using a 20-gauge microdissection needle.

2.8. Real-time reverse transcriptase PCR

Total RNA from both tail blood samples was isolated from rat whole blood leukocytes via the QIAamp RNA Blood Mini Kit (QIAGEN GmbH, Hilden,Germany) as per the manufacturers' protocol. cDNA was synthesized in 20 µl of solution using 0.5 µg of total RNA in the presence of 1.25 pmol/µl random hexamer,1 mM dNTP, 1×RT buffer, 2 u/µl RNase inhibitor (Rnasin®, Promega, Madison, WI), and 0.5 u/µl reverse transcriptase (ReverTra Ace®, TOYOBO, Osaka, Japan). Quantification of NK receptor, interleukin (IL)-6, IL-6 receptor, IFN-α, IL-2 receptor, IL-1β, and IL-1β receptor antagonist transcript levels was completed using the TaqMan real time PCR system as directed by the manufacturer (Perkin-Elmer Applied Biosystems, Foster City, CA). PCR was carried out in 20 µl of solution using cDNAs synthesized from10 ng of total RNA. Standard curves were obtained using cDNA generated from whole blood leukocytes from four Sprague-Dawley female rats challenged with 150µg/kg of LPS in order to achieve measurable amounts of cytokine transcript levels. For each sample, the expression of the transcript level was divided by the expression of phosphoribosyltransferase (HPRT) for normalization.

2.9 Multiplex bead-based immunoassay

Levels of several brain and peripheral cytokines, collected from the second plasma sample, were assessed via a multiplex bead-based immunoassay, described in Anisman

et al., 2007 and Gandhi et al., 2007. The Rat Cytokine/Chemokine Linco*plex* kit (Linco Research, St. Charles Missouri, Cat # RCYTO-80K) was used alongside a Luminex 100 system in order to determine the plasma cytokine quantities of IL-2, IL-6, IL-10, IL-18, IL-1β, IFN-γ, and TNF-α, according to the manufacturer's instructions. The identical procedure was used to assess cytokine levels in the VTA, except for the replacement of IL-18 and IFN-γ with the growth factor, MCP-1. Samples from animals in the BSR group were not included in this analysis. In addition, the lower limit of cytokine detection was 0.1 pg/ml (TNF-α) to an upper limit of 3.2 pg/ml (IL-10).The assays were performed in duplicate and resulted in less than 10% intra- and inter-assay reliability.

2.10. Statistical analyses

All analyses were conducted using SPSS version 15 software (2006). Recall that sickness behaviour scores were collected at three time points: baseline and following each injection. Difference scores (baseline versus injection) were then computed for each injection day and a Kruskal-Wallis non-parametric test (Howell, 2002) employed to assess group differences with respect to each sickness behaviour. Significant results were further evaluated using the Mann-Whitney U test with Bonferonni correction. In addition to the analyses of the individual sickness behaviours, a composite score was also computed by summing the scores associated with ptosis, piloerection, and lethargy at each phase; these data were analyzed as above. Sleep was not included because its occurrence was very rare (3 times in total). Cohen's kappa test (Howell, 2002) was used to determine the inter-rater reliability between two independent scorers.

Body weight data were assessed via a two-way mixed ANOVA design with group as the independent and time as the repeated factor. Spleen weights were analyzed using a one-way ANCOVA on group with body weight on the second injection day as the covariate.

Chronic drug effects on BSR frequency thresholds and maximum response rates were examined using two-way mixed ANOVA procedures (factors group and time).

Cytokine data associated with the real-time PCR and multiplex bead-based immunoassay procedures were each submitted to a one-way ANOVA with group as the independent factor. Occasionally samples were unusable due to extreme variability or detection error.

Significant omnibus tests were followed by Tukey HSD comparisons with Bonferonni correction in order to maintain the family-wise error rate at 0.05.

3. RESULTS

Figure 2 illustrates the difference scores associated with sickness behaviours (piloerection, ptosis, lethargy, and sleep) for both injection days. Significant group differences were found on the second injection day for piloerection (X^2 (3) = 13.772; p = .003), ptosis (X^2 (3) = 11.624; p = .009), and lethargy (X^2 (3) = 10.719; p = .013). Follow-up tests indicated that LPS control animals consistently demonstrated higher sickness scores relative to their BSR counterparts on all sickness variables but sleep (piloerection, z = -3.126, p = .002; ptosis, z =-2.955, p = .006; lethargy, z =-2.713, p = .010). The same pattern was observed between LPS control and environmentally

enriched animals for piloerection (z = -2.673, p = .010). The kappa coefficients (Howell, 2002) were found to be significant for all sickness behaviours (sleep, κ = 1.00, T = 5.657; p = .0001, ptosis, κ = 1.00, T = 5.657; p = .0001, piloerection, κ = .672, T = 3.810; p = .0001, lethargy, κ = .934, T = 5.297; p = .0001), indicating a high level of inter-rater reliability.

The results of the analysis on the composite sickness behaviour scores are shown in Figure 3. A significant group effect was found on the second injection day (X^2 (3) = 14.907; p = .002) due to the control LPS group yielding higher difference scores than animals receiving either BSR (z = 16.00, p = .001) or environmental enrichment (z = 25.500, p = .006).

None of the analyses of sickness behaviour on the first injection day gave rise to any significant effects. Saline-treated control animals generally had low scores on all sickness measures on both injection days. Furthermore, these scores were comparable to the ones associated with the BSR and environment enrichment groups.

There was also notable variation in the degree and number of sickness behaviours expressed between the groups. For example, all but one of the control LPS rats displayed moderate to severe sickness. One BSR rat demonstrated moderate sickness while a few others had mild signs of piloerection only. In the environmentally enriched group, roughly half of the animals showed moderate to severe sickness signs while the remainder displayed mild or no behavioural indications of illness.

There were no group differences in body weight gain over the duration of the study; only the rate of weight gain was significant (F (2.5, 105.1) = 49.86; p < 0.001; η^2 =

.478) with no interaction between group and time. Baseline weight values were similar across groups (F (3,42) = 0.86; p = 0.469 $\eta^2 = .058$).

All groups that received the LPS injection showed a reduced food intake during the week following the first LPS injection ($F(3,42) = 4.124$; $p = 0.012$; $\eta^2 = .228$) relative to the saline control group (data not shown).

The spleen weights for each group are shown in Figure 4. A group difference in wet weight was found using body weight on the second injection day as a covariate. The analysis indicated that there was a significant group effect of wet spleen weight ($F(3,41) = 6.991$; $p < 0.001$, $\eta^2 = .338$). In general, animals challenged with LPS had higher spleen weights than saline treated rats with significant differences between the control saline group and both LPS control ($p = .0001$) and environmentally enriched animals ($p = .017$).

Histological examination of electrode placements confirmed that all tips were within the VTA, mostly clustered in the rostral division of the structure. Neither frequency thresholds ($F(3.71, 74.82) = 1.725$; p = 0.157; $\eta^2 = .079$) nor maximum response rates ($F(5.13, 102.64) = 1.075$; p = 0.379; $\eta^2 = .051$) were altered by the LPS challenge at any point during the chronic BSR collection phase. There were no group differences in baseline frequency thresholds ($F(1.896, 37.919) = 2.735$; p = 0.080; $\eta^2 = .120$); these values ranged from 23 to 50 Hz across animals.

Figure 5 shows the real-time PCR quantification of plasma transcript levels for each cytokine (IL-6, IFN-α, IL-1β, IL-1β receptor antagonist) and cytokine receptor (NK receptor, IL-6 receptor, IL-2 receptor), derived from LPS challenged rats on the first and second injection days. The results of the analyses are presented in Table 1. In

general, all groups displayed similar levels following each injection. No significant group differences were found. Only a few cases approached significance; see for example IFN-α gene expression on day 1 and NK receptor and IL-1β gene levels on day 2.

Figure 6 displays the results of the blood cytokine levels obtained from plasma on the second injection day, as assessed via a multiplex bead-based immunoassay for each LPS stimulated group. The analysis of circulating levels of IFN-γ elicited a significant group effect ($F(2, 24) = 3.470$; $p = 0.050$; $\eta^2 = .248$), with the environmentally enriched animals demonstrating an elevated expression of this cytokine compared to LPS challenged control animals ($p = .05$). Concentrations of IL-10 also varied across group ($F(2, 28) = 3.921$; $p = 0.033$; $\eta^2 = .239$); in this case, BSR animals yielded significantly lower levels than both LPS treated controls ($p = .05$) and environmentally enriched animals ($p = .06$). There were no group differences in levels of IL-2, IL-6, and TNF-α .

Figure 7 illustrates the brain cytokine levels obtained from the VTA as determined by a multiplex bead-based immunoassay following the second injection. Of the five cytokines assessed, LPS significantly altered IL-6 ($F(2,21) = 5.349$; $p = .013$; $\eta^2 = .337$) levels, which were elevated in LPS control versus saline-treated animals ($p = .013$). In environmentally enriched rats, a trend towards lower levels of this cytokine in the VTA compared to LPS-stimulated control animals ($p = .072$) was observed.

Estrous cycle status was not a relevant variable in any of the above analyses.

4. DISCUSSION

Animal models of cytokine challenge are used to investigate an array of generalized non-specific side effects, typically referred to as 'sickness behaviours' (Kent et al., 1992); examples of these include lethargy, hypersomnia, decreased activity, piloerection, and other febrile related changes, in addition to anhedonic and anorectic effects (see Hart, 1988; Kent et al., 1992). These indicators of sickness are part of an adaptive recovery process that responds to illness. The prototypical endotoxin, LPS, is a constituent of the cell wall of gram-negative bacteria known to elicit some of the above symptoms by stimulating the central and peripheral release of cytokines such as IL-1, IL-6, and TNF-α (see Maier and Watkins, 1998; Larson and Dunn, 2001).

In this study, overall and individual sickness behaviours in BSR and environmentally enriched groups were generally comparable to animals receiving the vehicle injection and reduced relative to animals that received the LPS injection without the accompanying "reward" experience.

Although the groups were relatively stable with respect to sickness behaviours, there was notable individual variation in the sickness response to LPS in the environmentally enriched group, indicating that the enrichment experience was not sufficient to counteract the immune challenge as measured by sickness behaviours. Unlike BSR, in which thresholds indicate the rewarding value of the stimulation, there is no index of enrichment to evaluate individual animal responses to their environment. For animals in subordinate positions, for example, the group experience may have been less satisfying than for animals in more dominant positions, affecting immunocompetence. In one

study, dominant male Wistar rats had higher NK cytotoxicity than their subordinate counterparts following amphetamine administration (Wrona et al., 2005).

Although the attenuating sickness effects of BSR to cytokine challenge, and other stressors, have been reported previously (Kentner et al., 2007; McCutcheon et al., 1986; Marshall and McCutcheon, 1976; Freimark, 1973), to our knowledge, this is the first study to provide endogenous cytokine profiles of a selection of genes and ligands that could potentially underlie BSR and environmental enrichment.

The observation of behavioural sensitization in the control LPS rats was expected given the long interval between injections. Low doses of LPS have been shown to produce a sensitization effect, as measured by increases in corticosterone levels and concomitant sickness behaviours, as early as one day following initial administration, but these markers were not present when assessed 28 days later (Hayley et al., 2001). Typically, the development of tolerance to LPS requires that repeated injections be a few days apart (Ohta et al., 2006).

Although an admittedly gross measure, spleen weights were also determined in order to assess the influence of BSR and enrichment on this organ following LPS challenge. Similar to others, we observed elevated spleen weights in rats following endotoxin exposure (Kumagai et al., 1992). One explanation for the increased organ weight following LPS challenge may lie in the augmentation of iron levels in the spleen, particularly within macrophages of the marginal zone, and ferritin positive cells found within the white pulp of the spleen (Kumagai et al., 1992). In mice given an intravenous administration of LPS, most of the endotoxin, using immunoperoxidase techniques, was confirmed to be taken up by macrophages within the marginal zone,

two hours following injection (Groeneveld and van Rooijen, 1985). Over activation of the hypothalamic pituitary axis has also been linked to increased adrenal organ and spleen weights. Animals undergoing chronic regimes such as cold stress administration (Bhatnagar and Dallman, 1998; Burchfield et al., 1980) and other various stressor schedules (Bielajew, Konkle, Merali, 2002; Herman, et al. 1995; Hu, et al., 2000; Heiderstadt et al., 2000) have been shown to display alterations in organ weights (Burchfield, et al., 1980; Herman et al., 1995; Hu et al., 2000; Heiderstadt et al., 2000). Since immune challenges are also considered stressors (see Maier and Watkins, 1998), it is probable that LPS-induced corticosterone release (Schotanus et al., 1993) contributed to the subsequent elevation of spleen weights, particularly in the LPS-treated control and environmentally enriched rat; this effect was not as evident in the BSR animals.

Endotoxin challenge did not alter the expected rate of weight gain in any LPS group; all animals gained weight at a steady rate, irrespective of their group designation. We observed a short-lived but significant decline in food intake in all LPS groups, without an accompanying weight loss, indicating that LPS challenge briefly decreased metabolic rate (see Boyle et al., 1978). Others have also reported a disruption in food intake following LPS administration (McCarthy et al., 1984; Swiergiel et al., 1997; Kim et al., 2007).

Cytokine Effects

Immediately following the assessment of sickness behaviours, we measured peripheral cytokine gene expression of IL-1β, IL-1β receptor antagonist, IL-6, and IFN-α following each LPS injection (Figure 5). These cytokines were selected based on previous reports of their release in response to LPS (Conti et al., 1991; Flohé et al., 1999; Lindemann, 1989; Schotanus et al., 1993; and others).

Most striking was the finding that the receptor gene levels were impressively stable across injection periods, indicating that sensitization for sickness behaviours was mediated by ligand levels and not by receptor availability. The importance of receptor functioning, and not just ligand level, is now receiving attention for its role in endocrine-immune communication (see Heijnen, 2007 for a review).

Employing a multiplex bead-based immunoassay (Figure 6), we determined peripheral profiles of several cytokine ligands (IFN-γ, TNF-α, IL-1β, IL-10, IL-6, and IL-2) following the second injection (one-time point). Based on the limited profiles of the cytokine ligand and gene data we collected, we did not observe large differences between the groups. However, the trends suggest that environmental enrichment and electrical stimulation may modulate immunity via separate mechanisms. Environmentally enriched rats displayed the lowest and most stable levels of IL-6. Perhaps IL-10 down regulated levels of IL-6 preventing the occurrence of sickness behaviours; when IL-6 is absent, the typical release of proinflammatory cytokines, and concurrent behavioural effects of LPS are disrupted (Sparkman et al., 2006).

Coinciding with the peripheral cytokine data are the results of the VTA analysis with respect to IL-6 (Figure 7). Levels of this cytokine were significantly lower in

saline-treated control animals compared to the LPS control rats that showed severe sickness behaviours following the second LPS injection. Environmentally enriched rats demonstrated a trend for reduced IL-6 levels in this brain region and this group showed attenuated sickness behaviours, comparable to that of saline-treated animals. These observations suggest that the role of the VTA in mediating reward may also be implicated in modulating the organism's behavioural response to cytokine challenge.

Peripheral IL-10 was lower in animals that received BSR compared to LPS control rats. There is some debate regarding the role of IL-10 in sickness behaviour given that this cytokine can also be down regulated during LPS tolerance processes measured *in vitro* (Flach et al., 1997), but it is likely that the contribution of IL-10 to attenuated sickness in our own work is minimal.

It is thought that stimulation of brain sites that mediate reward may cause the release of IFN-γ and IL-2 which in turn activate NK cytotoxicity (see Wrona and Trojniar, 2003; Wenner et al., 1996). In this study, there was a trend for animals that obtained BSR to have higher NK cell receptor gene expression. However, there were no differences between groups with respect to IL-2 activity, nor did the direction or magnitude of IFN-γ release appear to modulate NK receptor gene elevation.

Functional Impact

If the cytokine profiles that we measured do not contribute specifically to the attenuated sickness behaviour following LPS challenge in our rats, what other influences on the organism could BSR have that would account for this behavioural effect? Using microdialysis techniques, electrical stimulation of the LH of the medial

forebrain bundle has been shown to increase hormone levels in a pattern similar to the classical stress response (Terry and Martin, 1978). Specifically, the investigators found that BSR evoked a release of prolactin over the first hour of stimulation, in addition to corticosterone release sustained throughout the session. Suppression of growth hormone was also observed during the test period, with a large rebound immediately following the end of the test (Terry and Martin, 1978). While growth hormone level has been correlated with NK cell cytotoxicity, it is likely mediated through proliferation of IL-2 and IFN-γ from macrophages (see Wrona and Trojniar, 2003) and not by BSR directly.

Recent evidence suggests that changes in GABA neurons within the VTA occur in anticipation of BSR (Lassen et al., 2007) and that this neurotransmitter appears to have neuroprotective abilities (Kuhn et al., 2004). For example, the expression of GABA (B) receptors on microglia in the brain blocks the release of LPS-induced IL-6 and IL-12p40 *in vitro* (Kuhn et al., 2004). Thus, GABA may offer other, unexplored, protective roles against LPS-induced sickness.

The role of HPA activation is another avenue to explore regarding the effects of environmental enrichment and BSR on immunomodulation, since both 'rewards' stimulate the HPA response (Monceck et al., 2004; Marashi et al., 2003; Terry and Martin, 1978). Selye (1980) has argued that the indications of stress not only occur to psychogenic stressors, but to anything registered as a stressor, including physical demands. Selye also differentiated between good stress, or 'eustress', and bad stress, arguing that both elicit the same physiological responses in a non-discriminate fashion (Selye, 1980). However, he acknowledged that because there are different degrees of

stress, the same response is not always produced (Selye, 1980). This might imply that HPA activation and associated neuroendocrine changes may be a function of the relative reward 'experienced' by animals in their respective contexts.

Alternatively, changes in immune functioning could be a result of increased physical activity in the environmentally enriched and BSR animals, and not attributable to reward at all. It is understood that exercise affects immune activity as a function of intensity and duration of activity (reviewed in Gleeson and Bishop, 2005). Immune markers, NK activity for example, tend to be increased immediately following exercise, with a sharp depletion during rest, until a later recovery to baseline levels, mediated in part by corticosterone release (Gleeson and Bishop, 2005). What is less understood is the efficacy of immune parameters within an organism to respond to an immune insult during the baseline and resting phases (see Nieman and Pedersen, 1999).

There have been several assertions in the literature that positive outlook and emotions may improve immunocompetence and survival rates, or at least quality of life, in cancer and AIDS patients (Anderson et al., 2007; Carlson et al., 2007; Spiegel et al., 1998; Fawzey et al., 1993), *in combination with proper medical attention and treatment.* For example, in one case evaluating resistance to the common cold, positive emotional style was not related to the rate of contracting infection, but instead to the clinical expression of illness through visible symptoms as well as subjective reports made by the patient (Cohen et al., 2003). The cold symptoms were speculated to be mediated through proinflammatory mechanisms (which are responsible for signs of illness) (Cohen et al., 2003). An important observation, in that study, was that the effects of positive emotional style were generalizable between two cold types. It is

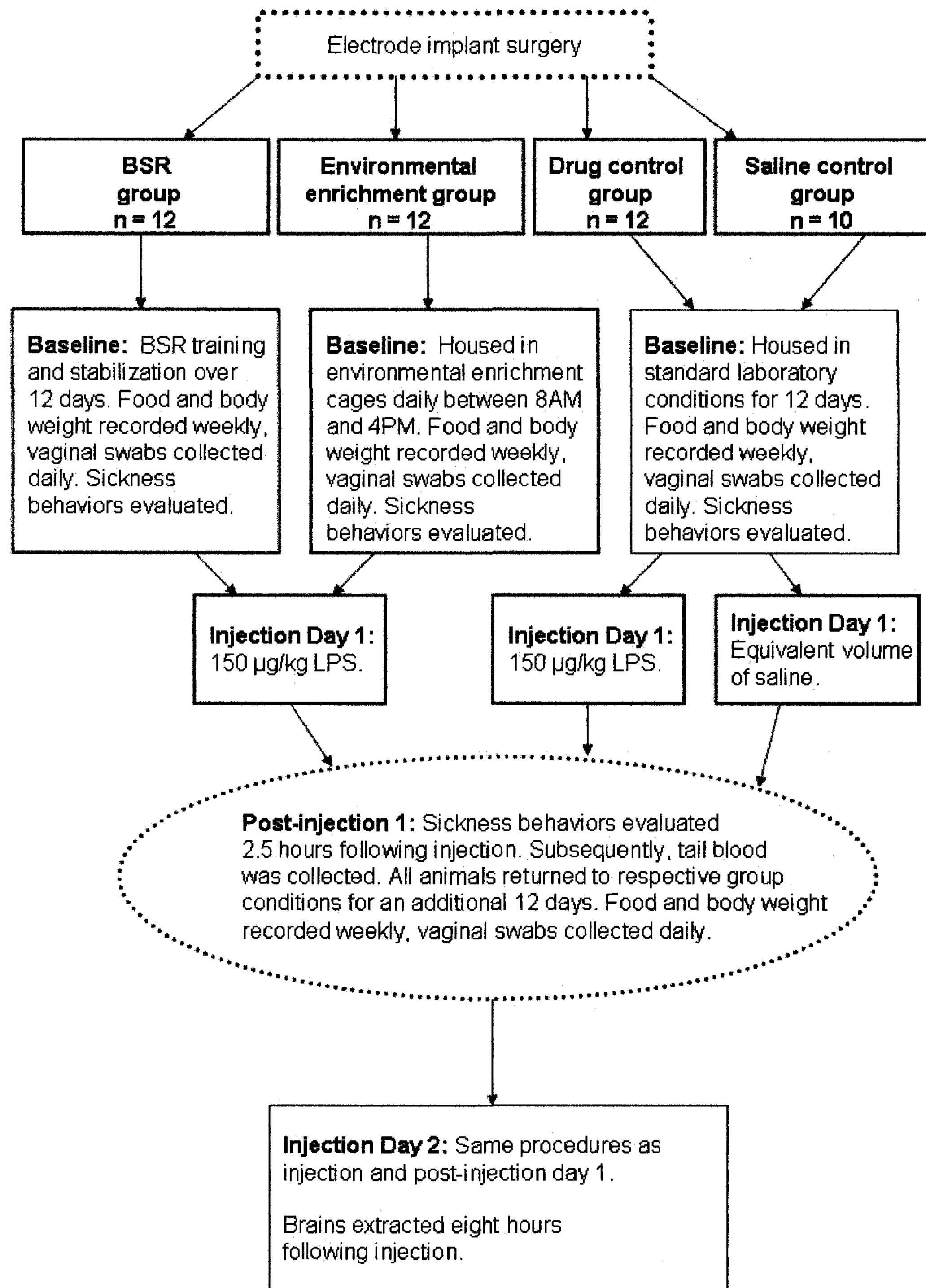
critical to note that different pathogens necessitate different immune responses, and the ability to assess immunocompetence is pathogen dependent (see Adamo, 2004). In our case, all animals were challenged with LPS; however the expression of the sickness indicators differed according to group designation. Determining the prophylactic effects of BSR and environmental enrichment against immune challenges, in order to make a qualitative statement about overall resistance to disease, will require the assessment of several pathogens (bacterial and viral).

Conclusions

In summary, we found that BSR and environmental enrichment were associated with resistance to develop behavioural signs of illness, in response to LPS challenge. However, there were only modest changes in the cytokine profiles following the endotoxin insult. It is possible that the changes in immune parameters that we report may more adequately reflect redistribution of immune mechanisms, as opposed to a change in immunocompetence, per say. As suggested by Adamo (2004), it may be incorrect to characterize changes in a small number of immune markers as either an optimization, or decline, in immune function. If there is instead varying degrees of magnitude and direction of immune markers, as reported here, then it is more appropriate to attribute them to changes in immune resource distributions. Because immune challenges such as LPS are not robust with respect to measures of immune responsiveness, we ought to rely instead on host resistance tests to particular pathogens in order to determine immunocompetence (Adamo, 2004; Nieman and Perderson, 1999).

Study 4

Figure 1 – Flow chart showing the different conditions and phases of the study.



Study 4

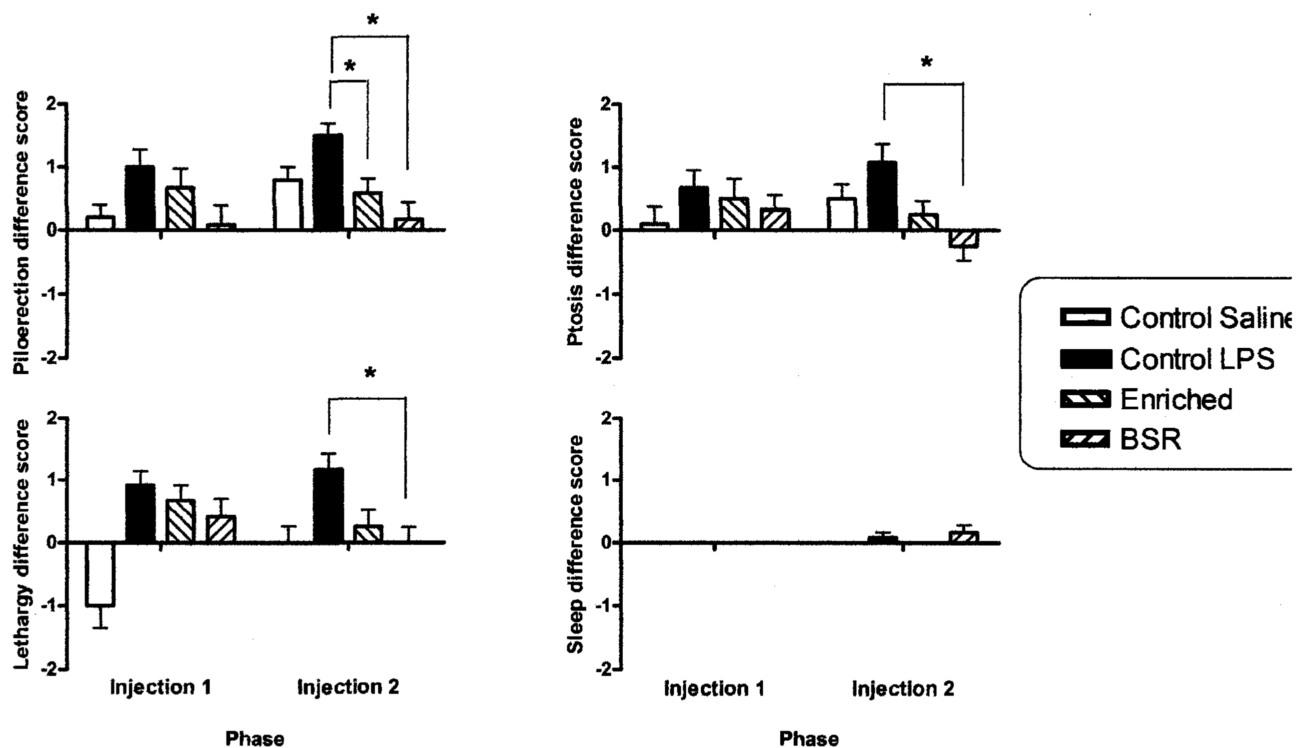


Figure 2 – The mean difference (± SEM) between baseline total sickness scores associated with piloerection (top left panel), ptosis (top right panel), lethargy (bottom left panel), and sleep (bottom right panel) scores collected 2.5 hours following 150 μg/kg i.p. LPS (or vehicle) challenge on injection days 1 and 2. * $p < .05$.

Study 4

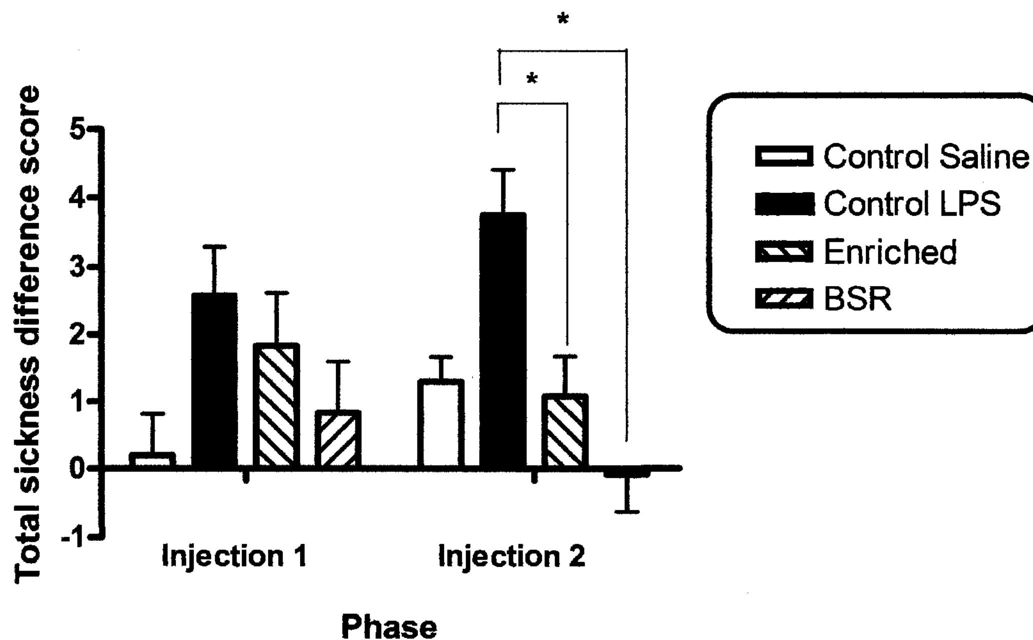


Figure 3 – The average difference (± SEM) from baseline composite scores of all sickness behaviours (piloerection, ptosis, and lethargy), except for sleep (due to its rare occurrence), for each LPS (or vehicle) injection day. The data are based on the summation of the difference scores obtained between baseline and each respective injection day (1 or 2) sickness behaviour collection. * $p < .05$.

Study 4

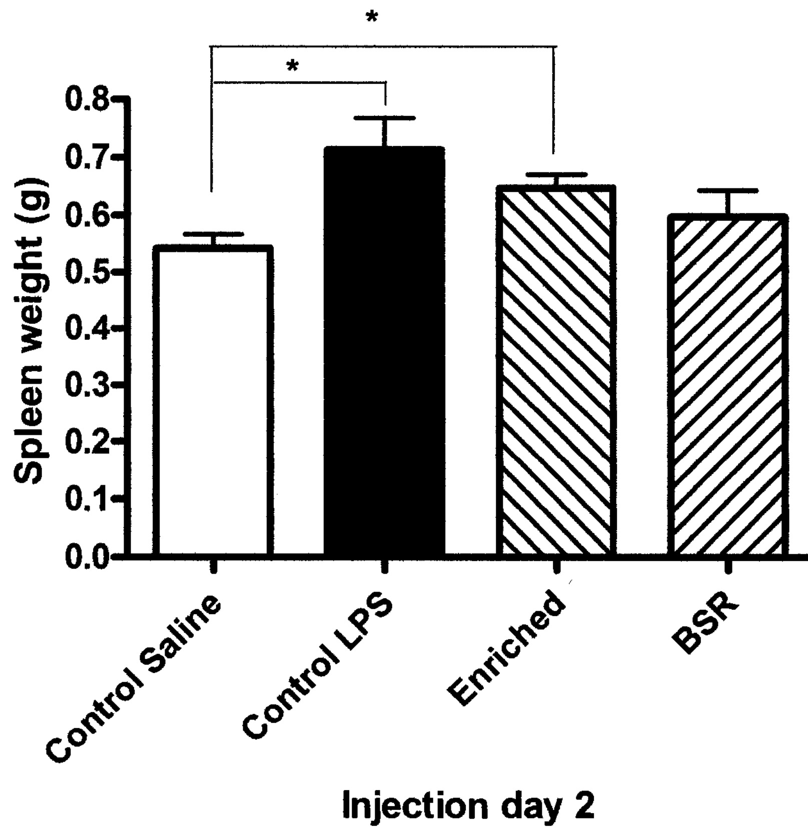


Figure 4 – Displays the raw data of the wet spleen weights (± SEM) in grams for each group (control saline, control LPS, environmentally enriched, and BSR). * $p < .05$.

Study 4

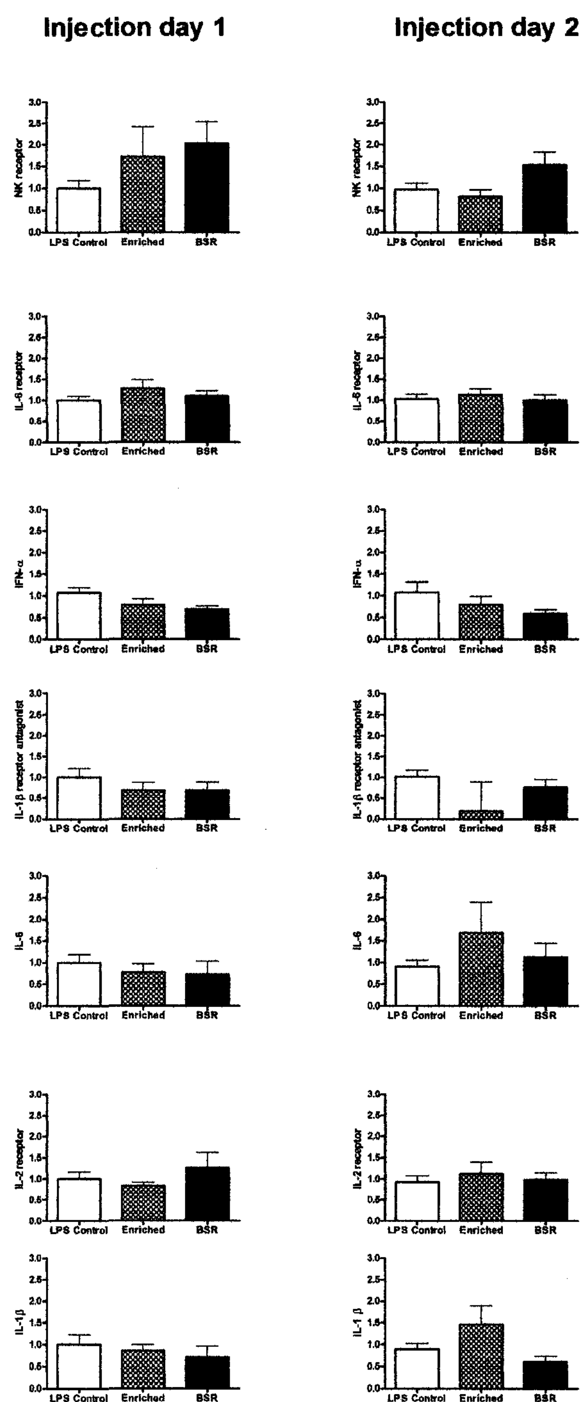


Figure 5 – Profile of the real-time PCR quantification of plasma transcript levels (± SEM) for each cytokine (IL-6, IFN-α, IL-1β, IL-1β receptor antagonist), and cytokine receptor (NK receptor, IL-6 receptor, IL-2 receptor), 2.5 hours after LPS challenge (150 μg/kg i.p.) on injection days 1 (left panel) and 2 (right panel). All data have been normalized to control LPS rats. Animals had either been in the control LPS, environmental enrichment, or BSR condition. * $p < .05$.

Study 4

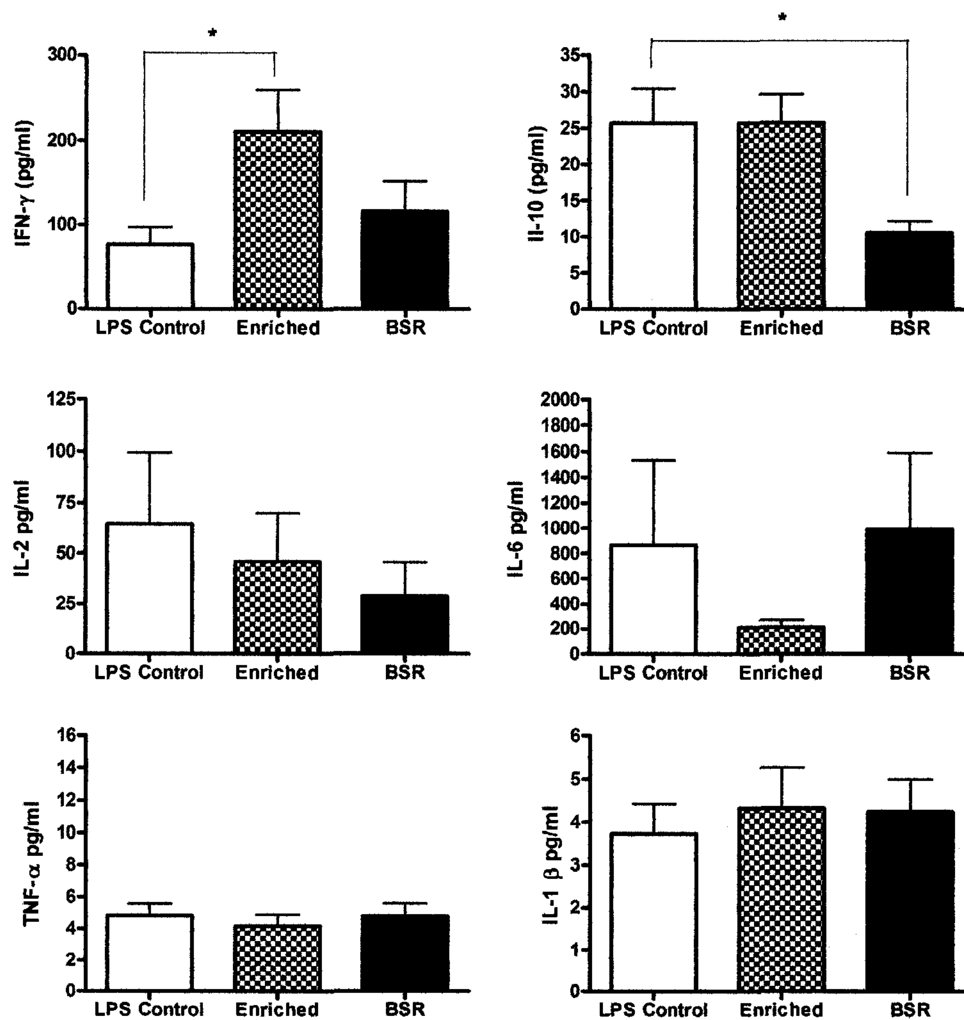
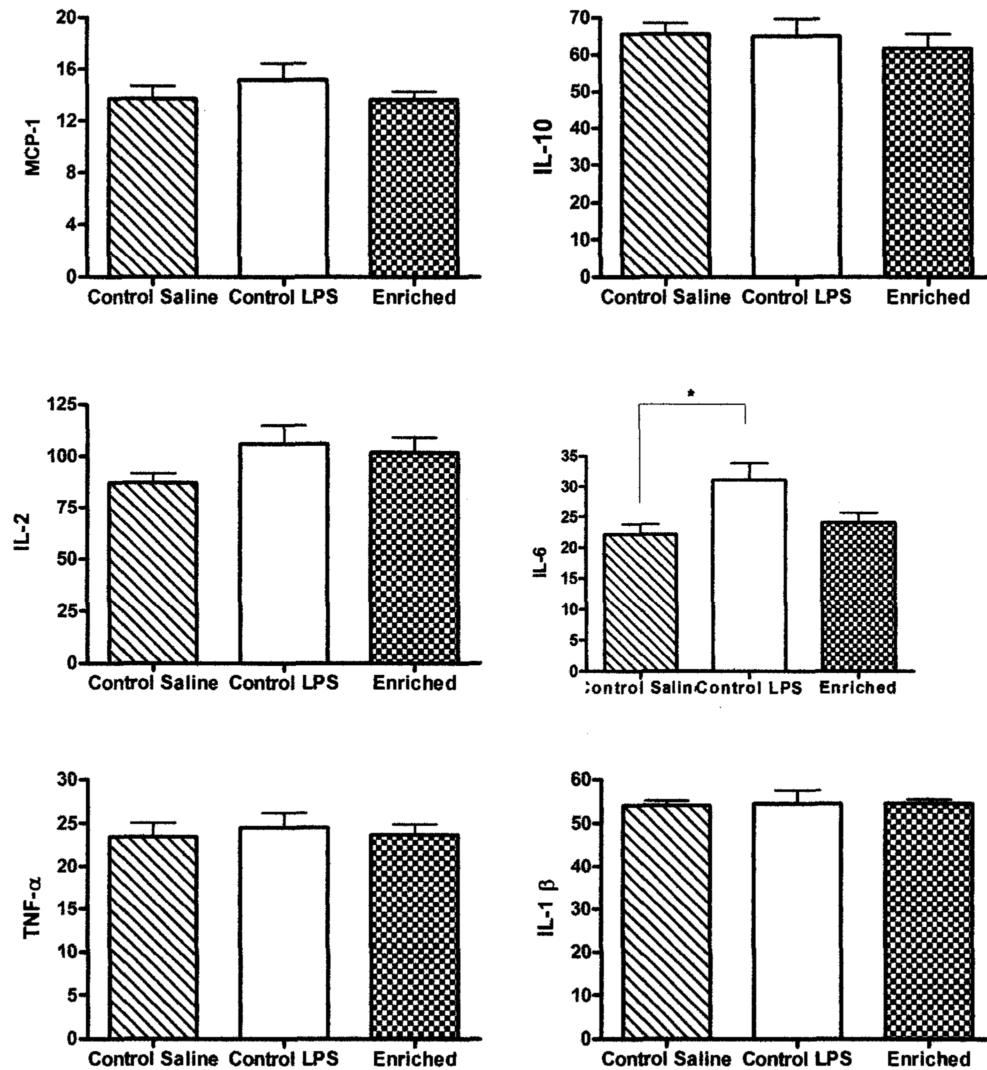


Figure 6 – Plasma cytokine levels (± SEM) 2.5 hours following LPS challenge (150 μg/kg i.p.) for injection day 2. Animals had either been in the LPS control, environmental enrichment, or BSR condition. * $p < .05$.

Study 4



Injection Day 2

Figure 7 – Brain cytokine levels (± SEM) collected from the VTA, 8 hours following the second LPS challenge (150 μg/kg i.p.). Samples were collected from animals in the control saline, control LPS, and environmental enrichment groups. * $p < .05$.

General Discussion

There were two aims of the studies presented in this thesis. The first was to establish an animal model of cytokine-induced sickness behaviour following challenge with interleukin (IL)-2 or interferon (IFN)-α. Although the behavioural effects of pro-inflammatory cytokines such as IL-1 and TNF-α have been thoroughly evaluated (see Danztzer et al., 2001; Anisman et al, 2003; Brebner et al., 2000), these cytokines are not used clinically. Thus, we put our efforts into evaluating the anti-inflammatory cytokines, IL-2 and IFN-α, which are readily administered to patients in order to treat cancer and hepatitis C. This is timely considering that the anhedonic potential of these cytokines has not been validated despite emerging reports of depression when administered in clinical settings.

The second aim was to evaluate the model based on the responses of female animals, an aspect typically ignored in research endeavors. Although clinical depression is more prevalent in females, it is the responses of males that are predominately evaluated in animal models.

The Motivational Path

In the first study of the thesis, the effects of a single systemic injection of IL-2 on thresholds for rewarding VTA stimulation were documented in male Sprague-Dawley rats (Miguelez, et al., 2004). Following IL-2 challenge, frequency thresholds were elevated during the acute portion of the study, and progressively increased throughout the remainder of the four week test period. This result was most prominent in animals that

received the lower IL-2 dose (0.5 μg) compared to the higher dose (1.0 μg), while vehicle treated animals continued to display stable thresholds throughout the duration of the experiment. Animals that had bilateral VTA implants showed differences in threshold alteration as a function of electrode placement. One concern is that threshold changes between the bilateral electrodes were due to interconnections between the two VTA regions. This is likley not the case since other studies employing control bilateral animals failed to show threshold changes following VTA stimulation (see Kentner et al., 2007). Finally, the altered hedonic effect elicited by IL-2 administration did not occur in all animals, indicating that IL-2 receptors, or their metabolites, are not uniformly distributed within the medial forebrain bundle. Taken together, these results suggest that the behavioural effects of IL-2 administration are site specific.

The findings of this first study led us to extend our investigations to an additional immunotheraputic drug, IFN-α, and its effects in male Sprague-Dawley rats (Kentner et al., 2006). Reviews of the literature indicate that the behavioural symptomology and proper dosages of this drug have not been sufficiently identified in rat models of disease. Thus, the first step involved the evaluation of sickness appearance, including ptosis (droopy eyes), piloerection, lethargy, and sleep using IFN-α dose ranges that had been employed in other studies to evaluate anhedonia (see Sammut et al., 2001; 2002). Beforehand, no study had systematically assessed the sickness appearance of IFN-α challenge. In addition, locomotor activity and body temperature were monitored before and after a single exposure of the chosen 10, 100, or 1000 units of rat recombinant IFN-α, or vehicle.

Overall, our findings indicate a significant difference between baseline and post-

injection values of piloerection. All animals, with exception to the vehicle-treated rats, appeared more "sick" during both the acute day, and chronic follow-up period. This result was consistent with previous qualitative reports (Reyes-Vazquez et al., 1994). Moreover, the results are comparable to the IL-2 study described above, with the lowest dose (10 U) giving rise to the most significant behavioural changes. These data suggest that an isolated exposure to IFN-α has the potential to elicit long-term behavioural disruptions and that it is important to assess these consequences in both animal and clinical models.

The third study in the thesis was the assessment of the anhedonic potential of IFN-α in female rats. In addition to the measures used in the second study, included here were evaluations of changes in thresholds for BSR following a systemic injection (Kentner et al., 2007). The results of the BSR tests suggest that the rewarding properties underlying the VTA are not disrupted by a single administration of this cytokine. This observation raises a couple of possibilities; one is that IFN-α, at least when singly administered, is unable to disrupt reward mechanisms. Perhaps this cytokine is a relatively mild challenge to the organism, insufficient to evoke a hedonic change. Another related possibility is that BSR is too robust and fails to be altered in the face of this IFN-α challenge unlike studies in which challenges such as IL-2 and LPS (Miguelez et al., 2004; Anisman et al., 1998; Borowski et al., 1998; Hebb et al., 1998; Anisman et al., 1996). It appears that alterations in BSR thresholds are dependent on the cytokine employed which may reflect different immune challenges to the organism.

Another measure that has frequently been used to assess anhedonic status is the sucrose test in which decreased sucrose preference or intake is thought to indicate anhedonia (Baker et al., 2006; Dalla et al., 2005; Duncko et al., 2001; Konkle et al.,

2003). It has been shown that repeated administration of either human or rat IFN-α in male Lister Hooded rats produces reductions in the intake of a 1% sucrose solution(Sammut et al., 2001; 2002). This was documented alongside an increase in consumption of the 32% solution in a three bottle sucrose test (Sammut et al., 2002), and thought to reflect a decrease in the rewarding value of the sucrose following cytokine challenge. Hedonic status was subsequently reinstated by the administration of antidepressant drugs such as desipramine or fluoxetine, which resulted in sucrose consumption returning to near baseline values (Sammut et al., 2002). In a more recent study, the investigators reported a decrease in saccharine preference following human IFN-α challenge in male Wistar rats (Fahey et al., 2007).

Notwithstanding the above studies, the sucrose test as an indicator of anhedonia has been shown to be unreliable (see Baker et al., 2006; see also Konkle et al., 2003). One explanation may be in the underlying confounds inherent to palatable-food intake paradigms. First, these measures rely on food and water restriction procedures that are in themselves stressful to the organism and may affect consumption levels, irrespective of the cytokine administration. Second, it is difficult to discern between the affects of anhedonia or anorexia (another sickness behaviour) elicited by cytokine challenge in the suppression of fluid and food intake. For these reasons, BSR may be a more adequate measure because it does not employ food as a motivator.

The overall finding of the first two studies is that IL-2 administration elevates BSR thresholds in a long-term manner while IFN-α does not. This result is shared with the clinical literature in that IL-2 has been reported to induce affective changes after a single treatment (Capuron et al., 2000; Maes et al., 2001), whereas IFN-α has required several

administrations before symptoms of major depressive disorder are apparent (Capuron et al., 2000). Future work is required to delineate the repeated effects of IFN-α on BSR thresholds in order to determine its validity to clinical treatment programs.

A Pot Hole Along the Way: Concerns of Mobility and Sex

Ambulatory behaviours such as those assessed by most forced swim, open field, and locomotor tests are typically used as markers of physical health following cytokine challenge. The general perspective is that the profile of a sick animal is one of reduced activity compared to that of a healthy one. IFN-α has been recognized as a cytokine that will depress motor activity (Segall and Crinc, 1990; Dunn and Crinc, 1993; Kumai et al., 2000); however it has also been shown to cause no change (Makino et al., 2000; Bethus et al., 2003). In the second study in which the effects of IFN-α were investigated in male rats, we report increased activity in response to the cytokine challenge whereas in the third study, motor activity was not altered in female rats. Inconsistent changes in the quantity of motor behaviours have also been noted with respect to the cytokine IL-6 in which Zalcman and colleagues (1998) observed increased activity in mice, while Dunn and Swiergiel (1998) saw no change. Still, our findings raise the possibility that cytokine challenge has sex specific consequences.

In studies in which animals were challenged with chronic mild stressors, male rats tended to have increased levels of anxiety behaviour, as measured on open field tests, compared to their female counterparts (Bowman et al., 2006). These stressors also significantly impaired males on a battery of memory tasks while either improving or not

affecting female rats on the same tasks (Bowman et al., 2003). Taken together, this work suggests that females may be more resilient to stressors, perhaps via better coping abilities than males on some measures (Bielajew et al., 2003; Bowman et al., 2003; 2006; Dalla et al., 2005; Drossopoulou et al., 2004; Karandrea et al., 2002; Konkle et al., 2003). Finally, in the second and third studies of the thesis, physiological indications of sickness behaviour such as elevations in temperature and piloerection were equally observed in both male and female rats.

The Scenic Route: A Circuit of Methodological Concerns with Cytokine Dose

One concern is the report that the human and rat IFN-α subtypes elicit different behavioural effects, particularly with respect to motor activity (Dunn and Crinc, 1990; Saphier et al., 1994; Makino et al., 2000) suggesting that the two forms are in fact not equivalent. This idea would not be unforeseen given that cytokines elicit pleiotropic effects that are dependent on species and individual differences (see Walker et al., 1999). In our studies, we employed the rat form of the cytokine, thought to be more potent than the human subtype (Crinc and Segall, 1992; Saphier et al., 1994), which is the most widely used since it is known to bind in the rat brain (Janicki, 1992).

Another issue surrounding our data is the appropriateness of our IFN-α drug doses, and protocols for administration (source, route, and timing). Varying administration schedules between studies might be a reason underlying inconsistent results of physiological and behavioural alterations in response to IFN-α challenge. In our studies we employed one systemic injection of IFN-α based on work suggesting that one

exposure to this cytokine can elicit disruptions in cognitive performance (Smith et al., 1988). Nonetheless, most recent studies have used repeated drug administration schedules (Bethus et al., 2003; De La Garza and Anis, 2003; De La Garza et al., 2005). It is possible that the repeated administrations of IFN-α that occur for approximately one week in most studies (Makino et al., 1998; Makino et al., 2000; De La Garza et al., 2005; Sammut et al., 2001) may represent a 'sub-chronic' regime and that longer dosing periods, reflecting actual clinical schedules, are necessary (see Fahey et al., 2007).

Another procedural variation includes the use of administration routes that range from intraperitoneal (Sammut et al., 2001, De La Garza et al., 2005b), and intravenous (Makino et al., 2000) to intracerebral (Blatteis et al., 1991; Plata-Salamán, 1992; Kamata et al., 2000; De La Garza and Anis, 2003) injections, the latter of which is not clinically relevant. Different routes will affect the kinetics of drug absorption, and the dynamics of the cytokine in and/or outside of the brain, and have a consequence on behavioural outcomes.

An important consideration is the specific cytokine dose level employed. In our studies, we evaluated low doses of IFN-α and report consequent physiological disruptions; however we did not observe any evidence of anhedonia. Typically in work of this nature, larger doses of IFN-α are employed (Makino et al., 2000; Fahey et al., 2007), although anhedonia has been reported using cytokine doses comparable to ours (Sammut et al., 2001; 2002).

Other behavioural deficits associated with IFN-α administration, such as decreased motility, and food/water intake, have also been elicited by employing large dose quantities (Segall and Crinc, 1990; Dunn and Crinc, 1993; Plata-Salamán et al., 1992;

Reyes-Vazquez et al., 1994;Kumai et al., 2000), although this is not always the case (Makino et al., 2000; Bethus et al., 2003).

Because of the conflicting reports of behavioural and physiological changes for any dose of IFN-α, the importance of studying lower doses is valuable. For example, low doses of LPS, which are unquantifiable in the blood, are nonetheless able to stimulate fever in an organism (see Watkins et al., 1995). Note that, in the first study of this thesis, it was the lowest dose (0.5 μg) of IL-2 which led to the development of anhedonia, as measured by increased BSR frequency thresholds.

With respect to IFN-α, Saphier and colleagues (1994) reported that 1 and 10 units were able to inhibit corticosterone whereas higher and lower doses did not. This suggests that the effective dose windows for this cytokine have not been determined. Anisman and contributors (1998) have commented that cytokines, specifically IL-2, have differential effects depending on the supplier and activity of the drug, and that this factor may account for this problem.

In April 1995, the World Health Organization held a meeting and determined that there was a lack of reliability in the suitability of IFN-α preparation, and recommended that the international standards to assess IFN-α activity be re-evaluated (Meager et al., 2001). Of the data from the different subtypes of IFN-α assessed (human recombinant versus leukocyte IFN-α 2a, 2b, 2c etc), 25% did not demonstrate reliable dose-response relationships; this also occurred with preparations within the same assay (Meager et al., 2001). The use of different viruses or cells resulted in varying estimates of potency even between similarly structured subtypes (varying by two amino acid changes) indicating different biological activities (Meager et al., 2001). These findings are important given

the vast array of behavioural and physiological effects due to IFN-α reported in the literature, especially since different subtypes and suppliers are used between studies.

Clinically, the relevant dose levels for IFN-α may not be comparable to what is used in most animal studies. Low dose IFN-α therapy ranges between 300,000 to 600,000 units/m^2 while the high dose spans from 1 to 50 million units/m^2 in humans. Although many of these studies use dose levels of several hundred thousand units, these ranges are typically scaled to reflect body weight (kg) as opposed to body surface area (m^2) – which is typically used in humans in oncology work. The estimated body surface area of a human (63.5 kg and 161.544 cm tall) is 1.713 m^2; using a typical weight of a female rat (350 g) in the present studies results in an estimated body surface area of 0.045 m^2, and different recommended drug dose levels (see US Drug and Food Administration).

Studies that examine IFN-α in order to discern its mechanisms of action also tend to employ high doses (for example, 1 million units/body to prevent hepatocellular carcinoma) (Nakaji et al., 2004). However, in an *in vivo* study investigating the role of vesicular somatitis virus in treating hepatocellular carcinoma in rats, prophylactic treatment with 66 units/g of IFN-α was able to counter the toxic effects of the virus to the organism while allowing it to replicate and effectively treat the cancer (Shinozaki et al., 2005). In vitro work demonstrated that the viral replication was not attenuated by IFN-α levels below 10 units/ml, but that 1000 units/ml significantly halted the replication ability of the virus (Shinozaki et al., 2005). Emerging studies such as this prompt questions regarding the potential ability of IFN-α to treat disease at lower doses. Notably, Suzuki and colleagues (2003) have developed an adenovirus vector expressing rat IFN-α gene to treat liver cirrhosis in rat by intravenous administration. They report that this procedure

induced an elevation of IFN-α in the liver, but not in serum, perhaps resulting in a clever way to circumvent systemic toxicity and sickness behaviours. Work such as this provides hope that the use of high and toxic doses of cytokines will become obsolete in the treatment of disease, in exchange for more target-specific approaches.

A Fork in the Road

At this 'junction' in the thesis, the focus of investigating sickness behaviours in response to IFN-α challenge was changed following the observation that this cytokine induced piloerection, but less so in animals that received BSR. In our laboratory, we have previously observed a protective effect of BSR with respect to its ability to circumvent the anorextic consequences of paroxetine treatment in rats (Konkle et al., 1999). Other laboratories have also noted this phenomenon. For example, BSR can attenuate the development of stomach ulcers (Freimark, 1973; Marshall and McCutcheon, 1976). Continual tail shock, with sporadic episodes of lateral hypothalamic stimulation, resulted in fewer and less severe gastric lesions than animals that received the tail shock alone (McCutcheon et al., 1986). In addition, exposure to BSR has been associated with increases in peripheral NK cytotoxicity (Wrona et al., 2004; Wrona and Trojniar, 2003; Wenner et al., 2000; Wenner et al., 1996) and humoral responses (Vlajković et al., 1993; Šakić and Vlajković, 1990; Janković et al., 1988).

In the final study of the thesis, we investigated the possible protective role of BSR and another form of reward, specifically environmental enrichment, on sickness behaviours in response to two lipopolysacharide challenges in female Sprague-Dawley rats. The study

was undertaken to differentiate between reward and possible stimulation effects in modulating immunity. We also measured the endogenous levels of cytokines in order to determine the magnitude of their involvement in offsetting the effects of endotoxin challenge.

In some studies of this nature, additional groups that are evaluated may include animals that receive non-contingent stimulation of the relevant structure, or stimulation applied to another structure not associated with reward. In our case, we elected to not apply non-contingent VTA stimulation as this procedure has been shown to evoke aversive responses in some animals (for example, escape) (see Steiner et al., 1969; Sudakov et al., 1989; Cantor et al., 1971). We chose not to include a group that received 'non-rewarding' stimulation based on the findings that such stimulation does not influence the immune markers measured (Wrona et al., 2004; Wrona and Trojniar, 2003; Wenner et al., 2000; Wenner et al., 1996).

During the BSR screening period in our studies, all animals were exposed to the stimulation regardless of their final group designation. The rats that did not respond appropriately received minimal exposure to the screening procedure (maximum 20 minutes). These animals did not receive electrical stimulation the entire screening duration; instead it was sporadically distributed across the session in order to determine the presence of either appetitive, neutral, or aversive behaviours. If any of the latter two behaviours were displayed, animals were automatically assigned to either the environmentally enriched, or one of the control groups.

Generally, BSR and environmental enrichment were shown to have modest effects on immunity based on the observation of lower sickness behaviour scores in these animals,

compared to the control group. PCR quantification demonstrated that there was large within-group variation of the environmentally enriched rats after the second challenge. However, the receptors appeared to be stable across groups, and LPS challenges, suggesting that ligand levels were implicated in the expression of sickness.

The multiplex bead based assay also revealed some variation in cytokine levels within and across groups, specifically with respect to IL-10, IL-1β, and IFN-γ in the periphery and IL-6 in the VTA. Together, these data indicate that the experience of reward influences immune activity, but that not all rewards are equal in this context. Overall, the influence of the cytokine milieu is not clear, and it may be that we failed to sample the real variables responsible for the attenuated sickness behaviours in the two 'reward' groups.

It has been shown that exercise affects immune activity as a function of intensity and duration (reviewed in Gleeson and Bishop, 2005). Thus we cannot discount the possibility that our observed changes in immune parameters might be due to the elevated activity levels present in both reward conditions.

The Roads Less Traveled

Treatment with immunotherapy such as IL-2 and IFN-α may alter the course of different cancers as well as hepatitis C. However, these treatment regimes are often accompanied by several neuropsychiatric side effects including lethargy, motor, cognitive impairments, and depression. New emerging treatment protocols, some of which were mentioned above, will hopefully eliminate these complications in the future. For now,

clinical use of pegylated IFN-α and adjuvant therapy (Ribavirin) are at the forefront of clinical use although they too are associated with depression (Dieperink et al., 2003; Asnis and De La Garza II, 2004); in the case of Ribavirin treatment, these rates may be higher than that with IFN-α treatment alone (see Asnis and De La Garza II, 2004). These reports suggest the importance of evaluating the anhedonic potential of these new treatments in addition to IFN-α and IL-2 combination therapy.

Also affiliated with treatment choice for depression is the recognition that most studies modeling the clinical effects of cytokine challenge do so in healthy non-compromised animals. It would be more appropriate to evaluate depression treatments in animals that are comparable to the clinically sick condition – for example, animals displaying liver disease in order to properly assess the kinetics of antidepressant treatment for IL-2/IFN-induced depression. Conversely, validating the existence of cytokine-induced depression would be better studied in healthy animals in order to differentiate between major depression disorder induced by cytokine treatment and depression-like effects instigated by hemolytic or thyroid consequences of the cytokine exposure (see Asnis and De La Garza II, 2004).

Recently, the Anisman laboratory has developed a model in which they potentiate the effects of IFN-α (Anisman et al., 2007) or poly I:C (Gandhi et al., 2007) treatment by introducing mice to a social stressor in combination with the cytokine challenge. Their results demonstrate increased sickness behaviours, plasma corticosterone, and cytokine levels, as well as norepinephrine turnover; this is thought to be comparable to the clinical condition in which patients are being treated with immunotherapy in addition to undergoing psychosocial distress.

In addition to the psychosocial variables, it would be important to expand and develop the role of circadian timing, genetic and age effects, as well as sex differences in studying cytokine insults and ensuing sickness behaviours. For example, photoperiod is implicated in the effectiveness of the immune system against pathogens; the immune response of a rat challenged at night, corresponding to its active phase, is more responsive than during the day (see Ucar et al., 1983). Photoperiod is also involved in the development of LPS tolerance. Franklin et al (2007) gave consecutive injections of LPS to male Long Evans rats at the onset of either their light or dark period. Animals injected during the light period demonstrated the classical tolerance profile, whereas the animals that were injected in the dark did not. Instead, dark-tested animals displayed decreased levels of mobility in an open field test (Franklin et al., 2007), suggesting roles for circadian effects on immunity.

Genetic differences are also important, with reports of some strains being more vulnerable to immune challenges than others. A comparison between Sprague-Dawley and Fawn Hooded rats in response to an acute systemic injection of IL-1α demonstrated increased motor activity in both strains, more so in the Fawn Hooded strain (Simmons and Broderick, 2005). Additionally, Fischer 344 rats had attenuated temperature values compared to Sprague-Dawley rats during the second tier of the biphasic febrile response to LPS challenge; however the Fischer 344 male rats had higher corticosterone and IL-1β serum levels than Sprague-Dawleys, and females of the same strain (Taylor et al., 2005).

A final element to consider is the dynamics of action for endogenous levels of cytokines. The unique variations of these immune parameters may explain individual differences in anhedonic and other sickness consequences of immunotherapy or LPS

challenge via additive, or synergistic mechanisms. Evaluating these inter-individual differences in vulnerability to the sickness effects of immune challenge may lead to the development of better animal models to describe this phenomenon, as well as more effective treatment options to counteract it. And so we press on . . .

Humor was another of the soul's weapons in the fight for self-preservation. It is well known that humor, more than anything else in the human make-up, can afford an aloofness and an ability to rise above any situation, even if only for a few seconds. . . The attempt to develop a sense of humor and to see things in a humorous light is some kind of a trick learned while mastering the art of living.

-- Viktor Frankl
Man's Search for Meaning, 1963

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APPENDIX A: Cognitive and Affective Disruptions Associated with Immunotherapy

Reference	Drug	Dose	Administration Schedule (Cycle)	Patient Demographics	Cognitive and Emotional Disruptions in Response to Cytokine Therapy
Dutcher et al., 2000	IL-2 IFN-α IL-2 + IFN-α 5-Fluorouracil IFN-α	$10\ MIU/m^2$ $6\ MIU//m^2$ $5\ MIU/m^2$ $6\ MIU//m^2$ $750\ mg/m^2$ $9\ MIU//m^2$	Weeks 1-4 2x daily, days 3-5 s.c. Day 1, s.c. Weeks 2 & 3 Days 1, 3, 5, s.c. Days 1, 3, 5, s.c. Weeks 5-8 1x weekly, i.v 3x weekly, s.c. Two week rest.	X = 54 (31-77 yrs) N = 50 Metastic renal cell carcinoma Male and female	To assess the efficacy of immunotherapy on cancer growth. Followed treatment for four cycles. 5-Fluorouracil failed to increase efficacy, and resulted in more toxicity. Quality of Life Evaluations: Eastern Organization for Research and Treatment of Cancer (QLQ-C30). McCorkle and Young Symptom – Distress Scale – assessed three times. -CNS disruption scores on anxiety, depression, and other mood agititations: 11/50 patients scored Grade 2, and 5/50 scored Grade 3 -Fatigue 18/50 scored Grade 2, and 7/50 scored Grade 3. -Anorexia, 5/50 scored Grade 2, and 3/50 scored Grade 3. -Fever, 18/50 scored Grade 2, and 5/50 scored Grade 3.
Capuron et al., 2000	IL-2 IL-2 + IFN-α	$18\ MIU/m^2$ $18\ MIU/m^2$ $6\ MIU//m^2$	5 days, s.c. 2x daily 5 days sc Days 1, 3, 5, s.c	X = 56.3 ± 11.6 yrs X = 56.2 ± 11.2 yrs	To assess depression and anxiety in immunotherapy patients during the first days of therapy using the Montgomery and Asberg Depression Rating Scale (MADRS) and Covi Scale (anxiety)

	IFN-α IFN-α	18 MIU/m^2 20 MIU/m^2	Days 1, 3, 5, s.c. 5 days, i.v	X = 60.0 ± 12.9 yrs X = 41.2 ± 13.9 yrs N = 48 Renal cell carcinoma or melanoma Male and female	during baseline, and days 3 and 5 of treatment. All test completed prior to daily cytokine treatment, except occasionally for combination treatment, IL-2 given in morning, and IFN-α in afternoon. IL-2 alone had increased MADRS scores on day 3, with three patients having severe symptoms. IL-2 alone, or in combination with IFN-α had increased MADRS scores on days 5. Scores on the MADRS did not vary for IFN-α patients. Combination therapy patients had increased Covi scores, but was associated with increased somatic complaints, therefore, no anxiety.
Reference	Drug	Dose	Administration Schedule (Cycle)	Patient Demographics	Cognitive and Emotional Disruptions in Response to Cytokine Therapy
Maes et al., 2001	IL-2 IL-2 + IFN-α IFN-α IFN-α	18 MIU/m^2 18 MIU/m^2 6 MIU//m^2 18 MIU/m^2 20 MIU/m^2	5 days, s.c. 5 days s.c Days 1, 3, 5, s.c Days 1, 3, 5, s.c. 5 days, i.v	X = 58.2 ± 11.1 yrs X = 44.0 ± 9.0 yrs X = 48.6 ± 12.0 yrs N = 26 Metastic cell carcinoma Male and female	To assess depressive symptoms any cytokine levels in patients treated with immunotherapy. The Montgomery and Asberg Depression Rating Scale (MADRS) was used to assess patients at baseline, and again on days 3 and 5 after treatment began. Increased depression scores on MADRS were observed in patients receiving IL-2 alone, or in combination with IFN-α. -No change in patients receiving IFN-α alone. -increased IL-6 serum levels in patients

					receiving IL-2 alone, or the combination treatment.
Reference	Drug	Dose	Administration Schedule (Cycle)	Patient Demographics	Cognitive and Emotional Disruptions in Response to Cytokine Therapy
Capuron et al., 2001	IL-2 IL-2 + IFN-α IFN-α IFN-α	18 MIU/m^2 18 MIU/m^2 6 MIU//m^2 18 MIU/m^2 20 MIU/m^2	5 days, s.c. 2x daily 5 days sc Days 1, 3, 5, s.c Days 1, 3, 5, s.c. 5 days, i.v	X = 50.0 ± 14.0 yrs N = 47 Metastic renal cell carcinoma Male and female	Assessed time course of symptoms in response to immunotherapy with the Cambridge Neuropsychological Test Automated Battery on days 1, 5, 1 month. Patients receiving IL-2 alone, had impaired spatial working memory 5 days following treatment. Patients receiving IFN-α only, had slowed reaction time. Depressive symptoms were present 5 days after treatment in patients receiving IL-2 alone, or in combination with IFN-α, in addition to sleep disturbances. All symptoms persisted until the one month experimental end point without worsening.
Eton et al., 2002	IL-2 IL-2 IL-2 IL-2	6 MIU//m^2 9 MIU//m^2 12 MIU//m^2 15 MIU//m^2	Patients received at least 2 courses of their assigned dose. Daily injections 5 times per week, s.c. Two weeks off. -Acetaminophen was taken every 3-4 hours to	N = 19 X = 55 (36-65 yrs) Melanoma, metastatic disease – not brain. Male and Female	To assess the effects of medium to high doses of IL-2 on side effects and efficacy. Side effects monitored using the NCI Common Toxicity Critera scaled from Grade 1 to 4. At the two highest doses, patients had increased temperature 2-4 hours after injection, lasting 1-2 hours. Grade 1 and 2 anorexia, nausea, diarrhoea, fatigue, headache, blurry

			combat fever.		vision, confusion, anxiety, depression, sleep disturbances in week one. Some hallucinations. This was resolved 24-48 hours after treatment cessation.
Reference	Drug	Dose	Administration Schedule (Cycle)	Patient Demographics	Cognitive and Emotional Disruptions in Response to Cytokine Therapy
Gez et al., 2002	 IL-2 IFN-α IFN-α 5-Fluorouracil Vinblastine	 10 MIU//m^2 6 MIU//m^2 9 MIU//m^2 600 mg//m^2 6 mg//m^2	Weeks 1-4 3x weekly, s.c 1x weekly, s.c Weeks 5-7 3x week, s.c Day 1, weeks5&7 Day 1, weeks5&7 2 weeks off. At least 2 cycles	N = 62 X = 63 yrs Metastatic renal cell carcinoma	Tumor growth assessed for 34 months to assess efficacy of treatment. Three year survival rate was 88%. Common side effects included flu-like symptoms, nausea, and depression. World Health Organization Criteria: 18% had WHO Grade 1 depression 4% had WHO Grade 2 depression during first treatment cycle. 16% Grade 1, 4% Grade 2 drowsiness 47% Grade 2, 51% Grade 3 fever and chills. 36% Grade 2, 34% Grade 3 asthenia (loss of strength). 18% Grade 1, 16% Grade 2, 4% Grade 3 prutitus (itching, burning).
Capuron et al., 2004	IL-2 IL-2 + IFN-α	18 MIU/m^2 9 MIU/m^2 18 MIU/m^2 6 MIU//m^2	5 days, s.c. Reduced to, for 2 days other weeks. 5 days week 1, 3 sc Days 1, 3, 5, s.c weeks 1-3. 2 weeks rest	N = 32 X = 57.0 ± 9.0 yrs X = 46.0 ± 8.0 yrs X = 41.0 ± 14.0 yrs	To assess pre-existing factors that determine depressive effects of immunotherapy. Depressive symptoms assessed via Montgomery and Asberg Depression Rating Scale (MADRS) during baseline, and one month after treatment. Social support, sleep quality and somatic complaints also evaluated.

	IFN-α	20 MIU/m^2	5 days, i.v	Metastic renal cell carcinoma Male and female	Patients with higher to moderate MADRS scores at end of study had scored higher, non-significant scores at baseline (particularly sadness) 50% had mild, and 22% had moderate depression.