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**Behavioral and Neurochemical Sensitization and Cross-Sensitization Elicited
by the Proinflammatory Cytokine Tumor Necrosis Factor- α : Peripheral and
Central Actions**

A Thesis submitted to the Department of Psychology and Faculty of Graduate
Studies and Research of Carleton University in partial fulfillment of the requirements for
the degree of

Doctor of Philosophy

Psychology

Specialization in Neuroscience

© Shawn Hayley

Department of Psychology

Carleton University (2002)

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Acceptance of the thesis:

"Behavioral and Neurochemical Sensitization and Cross-Sensitization Elicited by the
Proinflammatory Cytokine Tumor Necrosis Factor- α : Peripheral and Central Actions"

submitted by

Shawn P. Hayley

in Partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Chair

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Carleton University
May, 2002

Abstract

Cytokines, immune system messenger proteins, act as signaling molecules between the immune, endocrine and central nervous systems. Like traditional stressors, the proinflammatory cytokines, interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), promote behavioral and neurochemical changes and may influence psychiatric disorders. We reported previously that TNF- α provoked a time-dependent sensitization (Hayley et al., 1999), wherein mice reexposed to TNF- α 28 days following pretreatment with the cytokine displayed increased corticosterone levels and signs of illness. Interestingly, a sensitization of NE and 5-HT utilization within the prefrontal cortex and central amygdala was also apparent upon TNF- α reexposure after a 1-day interval.

The present studies further investigated the sensitizing actions of TNF- α and some potential mechanisms for these effects. Similar to TNF- α , the bacterial endotoxin, LPS, sensitized behavioral and neurochemical responses to subsequent endotoxin or TNF- α exposure. Interestingly, some of these sensitizing effects were mediated by peripheral macrophages. As reported for IL-1 β , TNF- α sensitized CRH and AVP expression within the hypothalamic median eminence, however, the temporal pattern of this expression (peaking after 7 days) did not correspond with the corticosterone sensitization, suggesting the independence of these effects. Further, both the human and murine forms of TNF- α elicited similar sensitization profiles. Murine TNF- α elicited a profound sensitization of serotonergic activity (and few dopamine effects) within extrahypothalamic regions, notably the amygdala. However, intracerebroventricular (icv) administration of the cytokine sensitized monoamine activity (1 and 7 day reexposure

intervals) primarily within the hypothalamus and involved robust dopamine changes. In contrast to the effects of systemic TNF- α , icv infusion of the cytokine did not sensitize behavioral or neuroendocrine activity, suggesting that the central and peripheral sensitization processes were governed by different mechanisms. In the case of the peripheral factors, histaminergic processes may have been fundamental to the observed effects, as antihistamine pretreatment (H_1 and H_2 receptor antagonists) blocked the plasma corticosterone and sickness sensitization effects elicited by systemic TNF- α . In fact, it is suggested that the sensitization of illness represents a shock reaction that is mediated, at least in part, by the vasoactive monoamine, histamine.

Taken together, these data suggest that tumor necrosis factor- α sensitizes behavioral, hormonal and neurotransmitter functioning through several distinct mechanisms. These findings may be important to clinical situations involving cytokine immunotherapy (e.g. TNF- α or IL-2 for cancers), wherein the side-effect profile may arise from time-dependent sensitizations provoked by the schedule of cytokine administration. Likewise, the cross-sensitization between LPS and TNF- α suggests that exposure to one immunogenic challenge may render individuals susceptible to the central consequences of an alternate insult.

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I am indebted to Hymie Anisman for his guidance and inspiration during the last few years. As with all research, when one is lucky enough to work with a good team the rewards gained are numerous. I was fortunate enough to have worked with a great team including, Zul Merali and Dan McIntyre, who always offered helpful and creative solutions to the many problems I faced while completing this thesis. I am also grateful to the many fellow team members whose technical assistance and personal support will not be forgotten. These include Nathalie Lukenbill, Charlene Dodd, Katia Ethier, Owen Kelly, Tom Borowski and of course, Kerry Sudom. In particular, I would also like to thank Jerzy Kulczycki whose biochemical expertise and humanity have aided me immeasurably during my graduate studies. Finally, the dedication of this thesis must go to my parents, who always believed in me and supported me throughout all the trials and tribulations.

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This thesis is primarily based on the following papers, which will be referred to in the text by their roman numerals. Each paper will represent a separate chapter in this thesis.

- I. Hayley S, Staines WM, Merali Z & Anisman H (2001). Time-Dependent Sensitization of Corticotropin Releasing Hormone, Arginine Vasopressin and c-fos Immunoreactivity within the Mouse Brain in Response to Tumor Necrosis Factor- α . *Neuroscience*, 106 (1), 137-148.
- II. Hayley S, Lacosta S, Merali Z, van Rooijen N & Anisman H (2001). Central Monoamine and Plasma Corticosterone Changes Induced by a Bacterial Endotoxin: Sensitization and Cross-sensitization Effects. *Eur. J. Neurosci.*, 13 (6), 1155-1165.
- III. Hayley S, Wall P & Anisman Z (2001). Sensitization to the Neuroendocrine, Central Monoamine and Behavioral Effects of murine Tumor Necrosis Factor- α : Peripheral and central mechanisms. *Eur. J. Neurosci.*, 15, 1-17.
- IV. Hayley S, Kelly O & Anisman H (2001). Murine tumor necrosis factor- α sensitizes plasma corticosterone activity and the manifestation of shock: modulation by histamine. *J. Neuroimmunol.*, submitted.

The following manuscript represents a work in progress by the author and as such is included in an appendix separate from the thesis proper. Further studies, aimed at investigating the role of TNF- α signaling in basal and pathological central states, are presently being conducted in knockout mice deficient for the p55 and p75 TNF- α receptors, as well as the closely related TNF- α super-family receptor member, FAS.

Appendix A. Hayley S, Kelly O & Anisman H (2001). Involvement of the p55 tumor necrosis factor- α in the acute and sensitizing effects of immune and non-immune stressors. Neuroendocrinology, in preparation.

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Abbreviations

5-HIAA	5-hydroxyindole acetic acid
5-HT	serotonin
ACTH	adrenocorticotropin
AVP	arginine vasopressin
BBB	blood brain barrier
CNS	central nervous system
CRH	corticotropin releasing hormone
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
HPA	hypothalamo-pituitary-adrenal
HPLC	high performance liquid chromatography
i.p.	intraperitoneal
icv	intracerebroventricular
IFN- γ	interferon-gamma
IL-1 β	Interleukin-1 beta
LPS	lipopolysaccharide
ME/ARC	median eminence/arcuate nucleus
MHPG	3-methoxy-4-hydroxyphenylglycol
NE	norepinephrine
NF κ B	nuclear factor kappa B

NTS	nucleus of the solitary tract
OVL	organum vasculosum laminae terminalis
PFC	prefrontal cortex
PGE ₂	prostaglandin E ₂
PVN	paraventricular nucleus of the hypothalamus
TNF- α	tumor necrosis factor-alpha
ZEME	external zone of the median eminence

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Glossary

Acute phase proteins: Serum proteins that increase during acute inflammation.

Acute phase response (APR): A nonspecific response by an individual stimulated by IL-1 β , IL-6, IFN and TNF- α . C-reactive protein may show a striking rise within a few hours. The liver produces acute-phase protein at an accelerated rate, and the endocrine system is affected with elevated glucogenesis and impaired thyroid function. Infection, inflammation, tissue injury, and often neoplasm may be associated with APR.

Adrenocorticotrophic hormone (ACTH): Polypeptide hormone secreted by the anterior pituitary; stimulates cells of the adrenal cortex to secrete cortisol.

Antigen: Any foreign material that is specifically bound by a specific antibody or specific lymphocytes.

Arginine vasopressin (AVP): A peptide hormone released from the posterior pituitary lobe but synthesized in the hypothalamus. Has anti-diuretic properties and can act on the HPA axis.

Autocrine: The action of a chemical on the same cell that synthesized it.

Cachexia: A wasting away syndrome (progressive loss of body weight accompanied by weakness) that characterizes the clinical course of many cancers. The cytokine, TNF- α is thought to be an important mediator of this syndrome.

Corticotropin releasing hormone (CRH): Hypothalamic hormone that stimulates ACTH secretion by the anterior pituitary.

Cytokines: Immune system proteins that are biological response modifiers. They coordinate antibody and T cell immune system interactions and amplify immune reactivity. Cytokines include monokines (e.g., IL-1, TNF- α) synthesized by macrophages, and lymphokines (e.g., interleukins, gamma interferon, lymphotoxin) produced by activated T lymphocytes and natural killer cells.

Endotoxin: A Gram-negative bacterial cell wall lipopolysaccharide (LPS) that is heat stable and causes neutrophils to release pyrogens. It may produce septic shock and causes blood coagulation, stimulates cytokine and complement cascades and can lethal consequences.

Glucocorticoid: One of a group of hormones (e.g., cortisol) of the adrenal cortex that are important in protein and carbohydrate metabolism, secreted especially in times of stress.

Intercellular adhesion molecule-1 (ICAM-1): Molecule found on the surface of endothelial cells that regulate cell adhesion and trafficking.

Interferon alpha (IFN α): Cytokine produced by monocytes, macrophages, lymphoblastoid and mesenchymal cells. It is recognized for its antiviral properties and its ability to inhibit the proliferation of B cells, monocytes, fibroblasts, and transformed cells.

Interferon gamma (IFN γ): Synthesized by activated T-helper cells. Activates killer T cells, NK cells and macrophages.

Interleukins: A group of cytokines synthesized by lymphocytes, monocytes and other cells, which promote growth of T and B cells and other hematopoietic stem cells, and have a variety of other biological functions.

Interleukin (IL)-1: Produced by monocytes and macrophages; it stimulates acute phase protein synthesis and promotes T-cell proliferation; it is also an endogenous fever-producing substance. IL-1 is comprised of two principal polypeptides: IL-1 α and IL-1 β .

IL-1 receptor antagonist (IL-1Ra): Molecule that binds to the IL-1 type I receptor and blocks the binding and biological activity of IL-1.

IL-6: (formerly known as B cell differentiation factor): produced by vascular endothelial cells, phagocytes, fibroblasts, activated T lymphocytes, and neoplasms. IL-6 is secreted in response to IL-1 or TNF. It mainly acts on B cells and hepatocytes. Its main function is to cause B lymphocytes to differentiate into cells that synthesize antibodies. IL-6 also induces hepatocytes to form acute-phase proteins including fibrinogen.

Janus kinase (JAK): Phosphorylating enzyme involved in gp130-dependent signal transduction by IL-6 family cytokines.

Leukocyte: White blood cell. The principle types include the granulocytes (neutrophils, eosinophils and basophils), lymphocytes (T and B cells) and monocytes (macrophages).

Lymphocyte: Small cell with virtually no cytoplasm, and bears antigen-specific receptors; found in blood, in all tissue, and in lymphoid organs, such as lymph nodes, spleen, Peyer's patches. Lymphocytes are divided into 2 principal groups: T and B lymphocytes. In addition, NK cells, which are large granular lymphocytes, comprise a small percentage of the lymphocyte population. Lymphocytes are distinguished by the expression of distinctive surface molecules that have precise roles in immune reaction.

Mitogen activated protein kinases (MAPK, MAP3K): Group of protein kinases that act in sequence during intracellular signaling by numerous families of receptors. Ligand binding and G-protein activation leads to activation of MAPK3, which phosphorylates MAPK2 (also known as MEK), which in turn phosphorylates MAPK.

Natural killer (NK) cells: Cells that attack and destroy tumor cells and certain virus-infected cells. NK cells induce lysis through the action of antibody. Immune memory is not involved, in that previous contact with the antigen is not necessary.

Nuclear factor-Kappa B (NF- κ B): A ubiquitous transcriptional factor activated by phosphorylation of its regulatory factor, I κ B. When activated, NF- κ B enters the nucleus and binds to transcription elements on the cell's DNA. Genes activated by NF- κ B are involved in immune or inflammatory responses and cell growth control.

Paracrine: Local effects of a chemical acting on cells in its immediate vicinity.

Plasma: Transparent yellow fluid that constitutes 50-55% of blood volume. It is 92% fluid, 7% protein, and 1% inorganic salts, sugars, lipids, hormones and gases.

Pleiotropy: The tendency of a cytokine to have multiple target cells and multiple actions.

Prostaglandin: Biologically active lipids, with hormonal actions, derived from arachidonic acid by actions of the enzyme, cyclooxygenase. Found in virtually all of mammalian tissues, prostaglandins have numerous actions including decreasing blood pressure, stimulation muscle contraction, regulating inflammation and blood clotting and modulating immune responses.

Pyrogen: A substance that induces fever. It may be endogenously produced, such as by IL-1 or may result from exogenous infection (e.g. bacterial or viral insults).

Sensitization: A process whereby an animal is exposed to some challenge and then re-exposed to a milder form of the insult after some time delay. A sensitization is manifested if the biological or behavioral response to the re-exposure stimulus is significantly greater than that which would be observed after a single exposure to the particular challenge. Thus, the underlying biological system(s) is said to be primed by the initial stimulus exposure and such effects often increase with the passage of time following the treatment. In immunological terms, sensitization refers to a situation in which exposure to an antigen primed the immune system such that a second exposure to the same antigen results in a greater response (e.g. elevated antibody levels).

Signal transduction and activators of transcription (STATs): Latent transcription factors that are activated following stimulation of gp130 by IL-6 superfamily cytokines.

Synergism: Situation in which the combined effects of two or more substances result in an effect greater than the additive effects of the individual actions of the substances. For instance, with respect to plasma corticosterone release, IL-1 + TNF- α results in a much greater rise (synergistic) of corticosterone than if the cytokines were administered individually and their effects summed (e.g. IL-1 alone = 10 units, TNF- α alone = 5 units, IL-1 + TNF = 15 units if they act in an additive fashion, but if IL-1 + TNF- α = 30 units

then they may be said to act in a synergistic fashion). Cytokines typically have numerous synergistic effects.

Transforming growth factor-beta (TGF- β): A cytokine that induces the proliferation of many cell types including epidermal and epithelial cells. It also facilitates the growth of selected transformed cells.

Tumor Necrosis Factor alpha (TNF- α): TNF- α is a monokine produced by macrophages stimulated with bacterial endotoxin; it participates in inflammation, wound healing and tissue remodeling. The cytokine also has potent anti-tumor properties and is associated with cachexia. More recently, this cytokine has been shown to be active in the central nervous system in terms of neurochemical and neuro-hormonal actions.

Vascular cell adhesion molecule (VCAM): Molecule on the surface of endothelial cells that regulate cell adhesion and trafficking.

General Introduction to the Central Effects of Cytokines

Multidirectional communication occurs between the immune, autonomic, hormonal and central nervous systems (Anisman et al., 1993; Blalock, 1994; Neta et al., 1992). It has even been suggested that the immune system may act as a sensory organ to inform the brain of viral or bacterial insults (Blalock, 1984, 1995). Stimulation of the immune system by various antigens, as well as viral and bacterial products, promotes dose-dependent increases of central monoamine activity (Besedovsky & Del Rey, 1991; Dunn, 1993; Tsumori et al., 1998) and elevates circulating levels of the stress-reactive hormones, corticosterone and adrenocorticotropin (ACTH) (Rivier et al., 1989; Dunn, 1993; Anisman et al., 1993). Just as immune activation affects CNS processes, it has been shown that stressors and pharmacologically induced changes of brain monoamine utilization may affect immune functioning, including alterations of lymphocyte proliferation and natural killer cell activity (Lysle, 1995; Pezzone et al., 1994).

Cytokines, which are protein messengers produced by circulating cells of the innate (e.g. macrophages and mast cells) and adaptive (e.g. T and B cells) immune system, appear to play an important role in the multidirectional communication between the neuro-immune-endocrine physiological systems. Indeed, while cytokines, such as interleukin-1 β (IL-1 β), IL-6 and tumor necrosis factor alpha (TNF- α), act primarily as signaling molecules of the immune system, they may also serve as immunotransmitters between the immune system and the central nervous system (CNS) (Anisman, Hayley & Merali 2000; Dunn, 1992; Hopkins & Rothwell, 1995). Recently, it has also become apparent that neurons and glial cells within the CNS synthesize cytokines and that their

expression is increased by brain insults or even stressors (Rothwell, 1999; Breder et al., 1993; Nyguen et al., 1998).

In view of the similarity between the central neurochemical actions of stressors and those of immune activation (Bernardini et al., 1990; Dunn, 1992; Lyson & McCann, 1992; Perlstein et al., 1993), it was suggested that immune challenge (and particularly cytokine activation) may be interpreted by the brain as if it were a stressor (Anisman et al., 1993; Dunn, 1990). Likewise, owing to the potent direct or indirect central actions of cytokines, increasing attention has been devoted to the possibility that cytokines may come to affect psychiatric illnesses, including depression and schizophrenia (Musselman et al., 2001; Maes, 1999; Leonard & Song, 1999; Kim et al., 2000). Alterations of cytokine expression may also contribute to a variety of neurodegenerative disorders, such as multiple sclerosis (Woodroffe, 1995; Merrill & Benveniste, 1996), Parkinson's disease (Mogi et al., 2000), Alzheimer's disease (Griffin, Sheng, Roberts & Mrak, 1995), as well as the progression of cell loss associated with stroke and ischemia (Barone et al., 1997; Rothwell, 1999).

Interestingly, like stressors, cytokines not only have immediate effects on central monoamine and HPA activity, as well as on some behaviors (e.g. sickness behavior), but may also result in sensitization of these processes, such that later responses to cytokine challenge (as well as stressors) may be enhanced. In fact, IL-1 β has been shown to provoke phenotypic changes in hypothalamic neuropeptide composition (Schmidt et al., 1995; Tilders & Schmidt, 1998), similar to that seen after a chronic psychosocial stressor (de Goeijet al., 1992; Schmidt et al., 1996; Bartanusz et al., 1993). Thus, when reexposed to IL-1 β or a footshock stressor several days following initial treatment with the cytokine,

circulating levels of corticosterone are markedly elevated (Schmidt et al., 1995), indicating that the cytokine induced a sensitization of HPA output.

While greatest attention concerning neuro-immune communication has focused on IL-1 β , it is clear that other cytokines, including TNF- α , may also be involved in this respect. Indeed, as in the case of IL-1 β , administration of TNF- α provoked a time-dependent sensitization of plasma corticosterone, as well as that of sickness behavior and central monoamine activity (Hayley et al., 1999, 2001a). While the augmented illness and corticoid activity was apparent upon reexposure to TNF- α after a 2-4 week interval, the elevated monoamine utilization was most pronounced at earlier times (1-7 days) following the initial cytokine challenge (Hayley et al., 1999, 2001a). The processes subserving such a sensitization effect have not been deduced, nor are the ramifications of a sensitization effect entirely clear. Yet, such behavioral and neurochemical plasticity may be important for clinical conditions, such as depression or multiple sclerosis, which potentially involve elevated cytokine activity. Indeed, it has even been suggested that protracted alterations of HPA functioning stemming from early-life stressor or immunological challenges may contribute to the organism's subsequent ability to contend with environmental challenges, and hence may contribute to the development of stressor-related psychopathology (Heim et al., 1997; Meaney et al., 1996; Shanks et al., 2000). The growing awareness that TNF- α is involved in the wasting away of body tissues (cachexia) associated with diseases, such as cancer and HIV (Uomoto et al., 1998; Ilyin et al., 1997, 1999; Lortholary et al., 1999), has prompted greater attention being devoted to the behavioral and metabolic effects of this cytokine. Moreover, the recent evidence implicating TNF- α in various brain pathologies such as multiple sclerosis, Parkinson's

disease and stroke (Rothwell, 1999; Mogi et al., 1994; Barone et al., 1997; Issazadeh et al., 1995), has prompted investigations into the neurochemical changes induced by the cytokine.

The present series of experiments were undertaken to assess in greater detail the immediate and protracted effects of TNF- α on neuroendocrine, central monoamine and neuropeptide functioning, as well as behaviors stemming from the treatments. Moreover, it was our intention to explore some of the mechanisms that might contribute to these TNF- α effects. In so doing we assessed the actions of both human and murine TNF- α , determined their effects when administered centrally and peripherally, established the time course for the sensitization and assessed the influence of various anti-inflammatory treatments, such as antihistamines, on such effects. As well, the contribution of the main receptor subtype of TNF- α (p55) to the sensitization effects of the cytokine was determined using genetically modified mice. Finally, it was of interest to determine if the gram negative bacterial endotoxin, lipopolysaccharide (LPS), which induces the co-release of several macrophage derived cytokines, would provoke sensitization effects similar to TNF- α .

Proinflammatory cytokines

Cytokines have typically been described as signaling molecules utilized by the immune system. Common functions of cytokines include induction of proliferation/differentiation of leukocytes, lymphocyte migration and accumulation at areas of trauma, production of fever and edema as well as regulation of antibody and cell mediated immunity in response to viral or bacterial infections. These messengers are

released from immune cells of either a myeloid lineage, such as macrophages and monocytes, or those of a lymphoid lineage, including T and B-lymphocytes. Classification of the cytokines has been based upon their molecular structure and common physiological actions, such as the production of fever or inflammation. For instance, macrophage derived IL-1, TNF- α and IL-6 are potent proinflammatory cytokines, whereas IL-4 and IL-10, which are released from T-cells, have anti-inflammatory actions (Fradelizi, 1998; Beutler, 1999; Pulendran et al., 1999).

In addition to the aforementioned actions, cytokines also affect central nervous system (CNS) functioning. Although the current thesis will primarily investigate the time-dependent effects of TNF- α on neurotransmission and behavioral processes, discussion of IL-1 β and its central actions will also be included since the majority of data concerning the involvement of cytokines on central processes has focused on IL-1 β . Furthermore, there is accumulating evidence suggesting that the effects of viral and bacterial insults on CNS processes involve the conjoint effects of several cytokines, including IL-1 β and TNF- α (Turrin & Plata-Salaman, 2000; Perlstein et al., 1993; Gayle et al., 1998; Anisman et al., 2000a; Brebner et al., 2000).

Interleukin-1

The mature 17 kDa forms of the polypeptides, IL-1 α and IL-1 β , are derived from a 31-33 kDa pro-IL-1 precursor after cleavage by the protease, interleukin-converting enzyme (ICE) (Thornberry et al., 1992; Higgins et al., 1994). Biologically active soluble and membrane-bound IL-1 has numerous actions, including modulation of inflammation, formation and remodeling of bone, appetite and fever regulation, insulin secretion, and

neuronal phenotype development (Dinarello, 1988; Plata-Salaman, 1994; Dodds et al., 1994; Ling et al., 1998; Dziegielewska et al., 2000). Signal transduction in response to IL-1 is dependent upon interactions between its type I receptor and the IL-1 receptor accessory protein, which is located on an adjacent portion of the cell membrane (Rothwell, 1999; Huang et al., 1997; Greenfeder et al., 1995). The actions of IL-1 are under tight regulation by the endogenous IL-1 receptor antagonist (IL-1ra), which competes with IL-1 for active receptor sites (Bresnihan & Cunnane, 1998; Dinarello, 1998) and the type II IL-1 receptor which acts as a false receptor, in that it does not transduce a signal upon binding the cytokine (Colotta et al., 1994).

Tumor necrosis factor- α

Tumor necrosis factor- α was originally named for its potent anti-tumor properties and has even been used clinically for the treatment of various cancers including melanoma, liver and breast cancer as well as glioblastoma (Lans et al., 2001; Wright et al., 1999; Lin et al., 1996; Lin & Vilcek 1987; Sidhu & Bollon, 1993). However, it soon became apparent that the dangerous side effects of systemically applied TNF- α limited the therapeutic usage of the cytokine. In fact, elevated-circulating levels of TNF- α can induce severe toxicity including delirium, chills, fever, kidney failure and ultimately death in certain cases (Sidhu & Bollon, 1993; Budd et al., 1991). Moreover, chronic TNF- α treatment can induce a wasting syndrome (cachexia) in rodents, similar to that observed in clinical conditions involving sustained infectious or inflammatory insults such as AIDS or malaria (Lortholary et al., 1999; Ilyin & Plata-Salaman, 1997). Promising recent reports indicate that it may be possible to dissociate the anti-tumor and

toxic effects of TNF- α (Barbara et al., 1996), however, such factors as dose and timing of administration will likely play a role in determining the usefulness of the cytokine in future clinical trials.

TNF- α is a pleiotropic cytokine, which exerts a wide array of actions on numerous cell types including bone osteoclasts/osteoblasts (important for rheumatoid arthritis), leukocytes, fibroblasts, skin keratinocytes, insulin sensitive adipocytes, as well as brain neurons and glial cells (Eigler et al., 1997). Moreover, a role in brain development has been suggested for TNF- α , given that immunoreactivity for the cytokine is increased in the sheep neocortex at developmental times of intense synaptogenesis (Dziegielwska et al., 2000). As is the case with other cytokines, TNF- α typically acts locally at the site of production, however, small amounts of the cytokine circulates through the vascular system. Healthy humans have been reported to have serum levels of TNF- α within the range of 1-7 pg/ml, while individuals suffering from cancer often have elevated levels of the cytokine (reports range from 2-20 to 30-30000 pg/ml) (Sheen-Chen et al., 1997; Warzoche et al., 1997; Ocvirk et al., 2000).

The cytokine initially exists as a transmembrane precursor (52 kDa), which is cleaved by the protease, TNF- α converting enzyme (TACE), to produce the mature 17 kDa soluble trimeric form of TNF- α (Brouckaert et al., 1993; Beutler, 1999; Mattson et al., 1997). Although TNF- α is mainly produced by peripheral macrophages and to a lesser extent brain glial cells, the closely related TNF- β isoform is primarily secreted from Th1 cells (Issazadeh et al., 1995; Beutler, 1999). Much like IL-1, strict endogenous regulatory mechanisms exist to keep levels of TNF- α in check. In this respect, TNF- α receptors are often shed and circulate freely in a soluble form, binding any excess ligand

(van Deuren, 1994; Moldawer, 1993). Moreover, various endogenous antagonists for TNF- α also play a role in the regulation of the cytokine, including the peptides, α -melanocyte stimulating hormone and vasoactive intestinal peptide, which block production and/or the actions of TNF- α after both central and peripheral administration (Rajora et al., 1997; Kim et al., 2000).

The actions of TNF- α are mediated by two distinct receptors subtypes, which are termed p55 and p75, with molecular weights of 55 and 75 kDa, respectively (Neta et al., 1992; Dinarello, 1988). Although there is some degree of overlap in the functions governed by these two receptor subtypes, they differ in several respects. For example, the p55 receptor is generally reported as playing an important role in apoptosis and cellular necrosis, while the p75 receptor may be more important for nutritive functions, such as the promotion of thymocyte proliferation (Beutler & van Huffel, 1994). Interestingly, the p55 receptor, has a unique intracellular region referred to as the "death domain", which is though to mediate the apoptotic actions of TNF- α (Tartaglia et al., 1993). Although stimulation of the p75 receptor may result in apoptotic consequences in a limited number of cell lines, the receptor generally stimulates cell proliferation rather than cell death (Grell et al., 1993; Tartaglia et al., 1991). TNF- α acts by binding to either the p55 or p75 receptor and causing the intracellular tails of the receptor to shift, resulting in a conformational change which subsequently triggers various intracellular cascades (Beutler, 1999). Intracellular pathways transduced by the p55 receptor include activation of the death domain and the subsequent recruitment of caspases, such as ICE and caspase-8, which may result in cytotoxic consequences (Ashkenazi & Dixit, 1998). As well, activation of second messenger systems may result in stimulation of phospholipase

A₂ and the production of prostaglandins or leukotrienes via an arachnidonic acid substrate (Townson & Pate, 1996). Many of the intracellular actions of p75 may be related to the stimulation of the transcription factor, nuclear factor- κ B (NF- κ B), which may stimulate the production of various factors which inhibit apoptosis, such as manganese superoxide dismutase (Rothe et al., 1994).

Acute phase response

The acute phase response is a generalized host reaction, which involves alterations of metabolic, neurological and endocrine functioning, that occurs in response to various environmental insults, including infection, inflammation as well as to extreme physical or psychological stress (Mandrup-Poulsen et al., 1995; Zhou et al., 1996). The proinflammatory cytokines IL-1 β , IL-6, and particularly TNF- α , are believed to be important mediators of the acute phase response and may act as mediators of the brain's response to such challenges (Mandrup-Poulsen et al., 1995; Galanos & Freudenberg, 1993; Berczi et al., 1998; Gelin, Moldawer, Iresjo, & Lundholm, 1993). For example, TNF- α has been found to modulate the firing of thermosensitive and glucose-responsive hypothalamic neurons, and has also been shown to alter feeding and nutrient metabolism in a manner similar to that observed during systemic infection (Breder et al., 1994; Plata-Salaman et al., 1988). It is thought that the acute phase response engenders profound central neurochemical changes, which may be of adaptive significance (Maness et al., 1995). However, these neurochemical alterations have also been proposed to act like stressors in eliciting behavioral pathologies such as depressive illness (Maes et al., 1995),

and is sufficiently severe and prolonged, acute phase response reactions may result in shock and ultimately death.

Systemic administration of high doses of LPS readily provoke septic shock which typically involves a rapid loss of blood pressure and volume (hypovolemia) and multiple organ failure (e.g. respiratory collapse and myocardial infarction) resulting in death (Berczi, 1993). Interestingly, pretreatment with a low dose of LPS may either reduce or increase (desensitization and sensitization, respectively) the shock inducing capacity of subsequently administered endotoxin (West et al., 1997), depending upon route of administration, timing of treatment and other variables. It has been suggested that TNF- α plays a primary role in the modulation of lethality resulting from LPS induced shock (Galanos & Freudenberg, 1993; Watanabe et al., 1995). In fact, antagonists of the TNF- α receptor reduce the severity of symptoms resulting from bacterial-induced shock (Watanabe et al., 1995; Berczi, 1993). Moreover, the shock induced by very large doses of systemically administered TNF- α was accompanied by elevated circulating IL-6, which correlated positively with the severity of symptomatology (Brouckaert et al., 1992). Given that IL-6 has marked effects on liver acute phase proteins (Van Bladel et al., 1991), and hepatic proteins have been reported to be essential in the sensitizing and desensitizing effects of cytokines on lethality (Wallach et al., 1988), it is likely that the liver plays a major role in the shock provoking properties of TNF- α and bacterial endotoxins. As well, the elevation of circulating glucocorticoids that normally follows systemic IL-1 or TNF- α administration, act in an anti-inflammatory capacity thereby potentially reducing the shock-like symptoms provoked by these cytokines. In fact, systemic corticosterone was reported to reduce splenic IL-1 mRNA, fever and anorexia

induced by LPS, but did not modulate the suppression of locomotion provoked by the endotoxin (Pezeshki et al., 1996). The importance of the acute phase response in the present investigation will become apparent during the course of discussion of the sensitization of sickness behavior induced by TNF- α . Indeed, the profound sickness symptoms provoked by the cytokine likely reflect a shock reaction.

Mechanisms of cytokine induced central effects

Cytokines are relatively large, hydrophilic polypeptides that do not readily cross the blood brain barrier (BBB). Although the particular route or mechanism involved in the cytokine actions is a matter of debate, it is certain that cytokines influence brain activity. For instance, systemically administered IL-1 β has been reported to stimulate *c-fos* expression in numerous discrete brain regions important for autonomic and endocrine functions, such as the PVN, dorsomedial hypothalamus, bed nucleus of the stria terminalis, central nucleus of the amygdala, lateral parabrachial nucleus and the ventrolateral medulla (Ericsson et al., 1997; Ericsson, Kovacs & Sawchenko, 1994; Day et al., 1999; Xu et al., 1999). Several hypotheses have been generated during the last few years to address the issue of how elevated peripheral cytokines may affect CNS functioning. These include the possibility that IL-1 β (Banks et al., 1989) and TNF- α (Guitierrez et al., 1993) have saturable carrier mediated transport mechanisms, which pump these cytokines into the brain. However, more attention has been devoted to the suggestion that cytokines gain entry into the brain through circumventricular areas, which lack an efficient BBB (Banks et al., 1989; Quagliarello et al., 1991). Indeed, the suggestion has been offered that cytokines may passively diffuse into the brain via the organum vasculosum laminae

terminalis (OVLT) at which time they may reach various brain nuclei through the process of volume diffusion (Dantzer, 2000). Other circumventricular organs, such as the area postrema, median eminence and subfornical organ have also been proposed to provide routes through which cytokines may traverse into the brain parenchyma (Lee et al., 1998; Laflamme & Rivest, 1999). Not only can cytokines penetrate these circumventricular regions, but also systemic administration of IL-1 β or TNF- α can lead to a disruption of the BBB (Quagliarello et al., 1991) making it easier for these substances to gain entry to the brain. The proposition has also been entertained that cytokines may affect the BBB by the induction of adhesion molecules, such as ICAM-1 and VCAM-1 in the brain endothelium and astrocytes, which may guide inflammatory leukocytes into the brain parenchyma (Merill & Benveniste, 1996).

Once within the brain, cytokines can provoke a functional response by binding to their receptors at hypothalamic and extrahypothalamic brain regions (Schobitz et al., 1994; Kinouchi, Brown, Paternak & Donner 1991; Raber & Bloom, 1994; Cunningham & De Souza, 1993; Laflamme & Rivest, 1999). Indeed, IL-1 and TNF- α may have functions similar to classical neurotransmitters through their modulation of neuronal Ca²⁺ channels and activation of intracellular second messenger systems (Tancredi et al., 1992). Also like neurotransmitters, IL-1 and TNF- α influence the electrophysiological firing patterns of neurons and has been implicated in long term potentiation (Plata-Salaman, 1988; Tancredi et al., 1992; Katfuchi et al., 1997; O'Connor & Coogan, 1999).

In addition to direct effects, cytokines may also influence CNS functioning without actually passing through the BBB into the brain parenchyma. For instance, afferent fibers of the vagus nerve may transmit information to the brain after stimulation

by cytokines or in response to stressors (which themselves may increase cytokine levels) (Dantzer et al., 1996; Hansen et al., 2000; Watkins et al., 1995). Indeed, receptors for IL-1 have been demonstrated on the nodose ganglion which sends afferent projections to the brainstem NTS, and *c-fos* expression is elevated in these regions in response to systemic IL-1 or LPS challenge (Ek et al., 1998; Gaykema et al., 1998). Activation of vagal branches may stimulate the *de novo* synthesis of cytokines within various brainstem and hypothalamic nuclei (Hopkins & Rothwell, 1995; Gaballec et al., 1995; Dantzer, 2000), thus increasing local brain concentrations of these messengers. Finally, it is possible that secondary molecules induced by cytokines, such as prostaglandins, may contribute to their central functions. Indeed, pretreatment with the cyclooxygenase inhibitor, indomethacin, attenuates at least some of the central actions of IL-1 (e.g., the NE variations observed within the hypothalamus) (Masana et al., 1990; Tsumori et al., 1998). As well, cytokine receptors are numerous at circumventricular and other vascular regions, and it has been suggested that circulating cytokines may interact with such receptors to generate secondary messengers, including prostaglandins, inside the brain (Laflamme & Rivest, 1999).

Central cytokine distribution

Although their levels are admittedly low under basal conditions, cytokines and their receptors are endogenous to the brain, having been identified within neuronal cell bodies, microglia and astrocytes, (Farrar et al., 1987; Katsuura et al., 1988; Nistico & De Sarro, 1991). Receptors for IL-1 β and IL-6 have been identified in several areas of the mouse and rat brain, including the hippocampus, choroid plexus, olfactory bulb,

cerebellum and hypothalamus (Cunningham & De Souza, 1993; Schobitz et al., 1994). Radioligand binding demonstrated brain TNF- α receptors have the highest concentration in the brainstem, thalamus and basal ganglia, while lower concentrations were present in the cerebellum and cortex (Kinouchi et al., 1991). Moreover, mRNA for the p55 TNF- α receptor was detected within the hypothalamus, amygdala, locus coeruleus, OVLT, subfornical organ as well around the lateral ventricles and other vascular associated sites (Laflamme & Rivest, 1999). In contrast, little basal expression of the p75 TNF- α receptor was detected (Laflamme & Rivest, 1999). As for the protein, TNF- α immunoreactivity was detected, mainly within neurons, across several brain regions including the hypothalamus, bed nucleus of the stria terminalis and the caudal raphe nuclei (Breder et al., 1993).

In addition to being expressed in brain under basal conditions, central cytokine expression is subject to alterations in response to stressors as well as various immunological and neurological insults. For example, elevated IL-1 β , IL-6 and/or TNF- α mRNA has been observed in the CNS following immobilization stress (Minami, Kuraishi, Yamaguchi, Nakai, Hirai, & Satoh, 1991; Yabuuchi, Maruta, Minami, & Satoh, 1996), systemic LPS administration (Eriksson et al., 2000; Buttini & Boddeke, 1995; Gatti & Bartfai, 1993; Breder et al., 1994; Gabellec et al., 1995; Laye et al., 1994; Liu et al., 1996), central LPS injection (De Simoni et al., 1995; Quan et al., 1994; Rajora et al., 1997), viral infection (Sato et al., 1997) and brain injury, cerebral ischemia and seizure (Buttini et al., 1994; Giulian & Robertson, 1990; Hopkins & Rothwell, 1995; Rothwell & Hopkins, 1995; Taupin et al., 1993; Minami et al., 1990; Yabuuchi et al., 1993). As well, peripherally administered cytokines may even stimulate their own expression within the

brain, thus potentially placing these molecules in the vicinity of their receptors (Cunningham & De Souza, 1993; Nyguen et al., 1998; Butinni & Boddeke, 1995).

Cytokine induced activation of the HPA axis

It is well established that stressors and various immunogenic challenges (particularly IL-1 β and LPS) stimulate the HPA axis (Del Rey & Besedovsky, 1992; Dunn, 1988,1992; Rivier, 2001; Brebner et al., 2000). Systemic and central administration of IL-1 β stimulates the expression and release of CRH and AVP secretion from PVN neurons of the hypothalamus (Mandrup-Poulsen et al., 1995; Tilders et al., 1993; Rivier, 2001; Lee & Rivier, 1994). Likewise, direct infusion of IL-1 β into the median eminence (site of CRH terminals from neurons originating within the PVN) has also been reported to increase AVP and CRH secretion as well as ACTH levels (McCoy et al., 1994; Watanobe & Takebe, 1993). Together, CRH and AVP synergistically stimulate the release of pituitary ACTH which, in turn, elicits adrenal glucocorticoid secretion (Johnson et al., 1992; Lightman, 1994; Plotsky, 1991; Rivier & Vale, 1985). The glucocorticoids stimulate hippocampal cells, terminating CRH release from the PVN (Rivier, 2001; Jacobson & Sapolsky, 1991; Meany et al., 1996; Sapolsky et al., 2000). Corticosterone acts in an adaptive manner in that it plays a permissive role in the release of free fatty acids (thus providing a source of energy), has anti-inflammatory actions, and prevents excessive immune activation, thereby precluding autoimmune disorders (Munck et al., 1984; Sapolsky et al., 2000).

Pretreatment with CRH antagonists, such as α -helical CRH or anti-CRH antiserum, have been shown to attenuate the IL-1 β induced ACTH changes (Saperstein et

al., 1992; Sapolsky et al., 1987, 2000). Interestingly, AVP antibodies attenuated the increased ACTH elicited by central but not systemically administered IL-1 β (Lee & Rivier, 1994). Moreover, intravenous and intracerebroventricular (icv) IL-1 β administration increased *c-fos* expression within PVN CRH neurons (Ericsson et al., 1994; Vellucci et al., 1995). As well, icv IL-1 treatment elevated *c-fos* expression within the supraoptic nucleus; while CRH but not AVP mRNA was increased within the PVN (Vellucci et al., 1995). Taken together, these data suggest that IL-1 β may act at the level of the hypothalamus to elicit the release of ACTH secretagogues. However, *in vitro* studies have shown that when IL-1 β is cultured with pituitary cells for several days, ACTH secretion may be provoked (Woloski et al., 1985). It was even suggested that CRH treatment may lead to the sensitization of pituitary cells, such that greater ACTH response is elicited upon subsequent IL-1 β challenge (Payne et al., 1994). However, other reports have reported that there was, in fact, no clear indication of such a sensitization effect nor that IL-1 β could act directly on the pituitary (Jessop & Lightman, 1994). Thus, data concerning the mechanism through which IL-1 β influences HPA activity is somewhat controversial.

In addition to IL-1 β , other cytokines, including IL-6 and TNF- α , influence HPA activity (Ando & Dunn, 1999; Wang & Dunn, 1999; Hayley et al., 1999, 2001a; Brebner et al., 2000; Bernardini et al., 1990; Zhou et al., 1996). As in the case of IL-1 β , both intravenous and i.c.v. administration of TNF- α activates the HPA axis, stimulating ACTH and corticosterone secretion in a dose dependent fashion, and such effects were blocked by CRH antiserum (Bernardini et al., 1990; Turnbull et al., 1997). In contrast, AVP antibodies did not affect the stimulation of ACTH provoked by the cytokine

(Kageyama et al., 1995), suggesting that CRH may play a more prominent role in the HPA activating effects of the cytokine. Likewise, we have shown that ip injected recombinant human or murine TNF- α at doses ranging from 1.0 to 4.0 μ g increased plasma corticosterone levels (Brebner et al., 2000; Hayley et al., 1999, 2002a). Moreover, TNF- α has been reported to provoke hypothalamic CRH release (Watanobe & Takebe 1992), suggesting that the cytokine may stimulate HPA activity through activation of the PVN. However, TNF- α has also been reported to have direct actions on corticotroph cells at the level of the pituitary (Kobayashi et al., 1997) and adrenal gland (Ritchie et al., 1996).

While the acute effects of IL-1 β and TNF- α on the HPA activity have been relatively well documented, much less attention has focused on the chronic effects of these substances. Continuous systemic infusion of IL-1 β over a 1 week period, using osmotic minipumps, resulted in a persistent increase of plasma ACTH and corticosterone levels (Sweep et al., 1992). It has also been shown that infusion of IL-1 β into the cerebral ventricles for 3 days elicited substantial elevations of plasma ACTH and corticosterone, even at relatively low doses (0.5 μ g/day) (van der Meer et al., 1996a), and the co-administration of IL-1 β and TNF- α synergistically increased ACTH and corticosterone (van der Meer et al., 1995). The HPA activation was inhibited by CRH immunoneutralization or systemic treatment with dexamethasone, supporting the notion of a hypothalamic site of action for IL-1 β which is sensitive to immunoregulatory glucocorticoid feedback mechanisms (van der Meer et al., 1996a).

The route of cytokine administration may also determine the mechanism by which they impact on HPA processes. It will be recalled that the suggestion was offered that

cytokines activate the HPA axis via vagal afferents. For instance, transection of the vagus nerve blocks many of the behavioral effects (e.g. social exploration and food motivated behavior) of intraperitoneally injected LPS or IL-1 β (Bluthe et al., 1996; Dantzer et al., 1996). In contrast, the effects of vagotomy on IL-1 β induced suppression of social exploration were absent when administered subcutaneously or intravenously (Bluthe et al., 1996).

Central neurochemical effects of immune activation or cytokine challenge

Antigenic challenge, such as administration of sheep red blood cells (SRBC) to mice, provokes altered hypothalamic unit activity which coincided temporally with the peak immune response (Besedovsky et al., 1983). As well, SRBC inoculation induced time-dependent variations of NE within the PVN and supraoptic nuclei of the hypothalamus (Zalcman et al., 1989, 1991; Carlson, Felten, Livnat & Felten, 1987), and increased the *in vivo* utilization of dopamine (DA) within the nucleus accumbens (Lacosta et al., 1994). Like SRBC, the bacterial endotoxin, LPS, also affected central monoamine utilization. *In vivo* studies indicated that ip LPS increased norepinephrine (NE) and DA concentrations within the medial prefrontal cortex and in the medial hypothalamus (Lavicky & Dunn, 1995), while postmortem analyses revealed that the endotoxin also increased NE, DA and serotonin (5-HT) activity within the PVN and arcuate nucleus of the hypothalamus (MohanKumar et al., 1999). Moreover, other immune challenges, such as viral infection, altered brain NE and 5-HT activity (Dunn 1993; Dunn et al., 1989). In fact, viral challenge (influenza) increased NE turnover in hypothalamus, as well as tryptophan concentrations in parietal cortex and in

hypothalamus (Dunn & Vickers, 1994; Dunn et al., 1989). Likewise, neonatal viral infection (Borna disease virus) provoked alterations of NE and 5-HT levels within the frontal cortex and cerebellum (Horning et al., 2001). However, the possibility should be considered that the mechanisms responsible for neurotransmitter changes may be dependent on the characteristics of the immune challenge.

As in the case of antigenic challenges, cytokine administration provokes changes in brain monoamines and their metabolites in hypothalamic and extrahypothalamic regions. Systemic IL-1 β administration augmented NE activity within the PVN, arcuate nucleus/median eminence, mediobasal and lateral hypothalamic nuclei as well as the locus coeruleus and prefrontal cortex (Dunn, 1988; Lacosta et al., 1998). Such effects, however, may be dependent upon the route of administration of the cytokine, as systemic but not intra-hypothalamic treatment with IL-1 β elevated extracellular NE levels within the medial hypothalamus (Kaur et al., 1998). Likewise, IL-1 β influences 5-HT activity, as indicated by elevations of the metabolite, 5-HIAA, in the hypothalamus (Dunn, 1992; Brebner et al., 2000) as well as prefrontal cortex and hippocampus (Zalcman et al., 1994; Brebner et al., 2000). Moreover, synthesis of 5-HT is likely affected by IL-1 β , given that tryptophan levels were increased in the brainstem, hypothalamus and prefrontal cortex (Dunn, 1988). Dunn & Welch, (1991) were able to block the effect of IL-1 β on brain tryptophan using the ganglionic antagonist chlorisondamine, suggesting a role for sympathetic involvement in this respect. In addition to NE and 5-HT, IL-1 β increased DA utilization within the hypothalamus and frontal cortex (Kabiersch et al., 1988; Masana et al., 1990; Lacosta et al., 1998).

As in the case of postmortem studies, cytokine treatments influence *in vivo* functioning of monoamine neurons. For instance, direct application of IL-1 β into the rat anterior hypothalamus markedly increased extracellular levels of NE, 5-HT and DA along with a concomitant increase in their metabolites MHPG, 5-HIAA, HVA and DOPAC (Shintani et al., 1995). Interestingly, the increase of NE and DA concentrations were evident for up to 6 hours, whereas the 5-HT levels only transiently remained above baseline (Shintani et al., 1995). Likewise, direct injection of IL-1 β into the medial basal hypothalamus augmented 5-HT and DA neuronal activity as reflected by increased interstitial DA, DOPAC and 5-HIAA levels in this region (Mohankumar & Quadri, 1993; Mohankumar et al., 1993). Recently, IL-1 β has been reported to elevate extracellular NE concentrations within the medial prefrontal cortex in response to local application of the cytokine (Kamikawa et al., 1998). In addition, intravenous IL-1 β administration increased anterior hypothalamic unit activity (Bartholomew & Hoffman, 1993), and increased 5-HIAA accumulation in this brain region (Carmelia et al., 1991). Intracerebroventricular administration of IL-1 β has also been shown to provoke an increase of *in vivo* extracellular concentrations of hippocampal 5-HT and 5-HIAA (Linthorst et al., 1995), and microdialysis experiments in rats conducted in our laboratory revealed that i.p. injections of IL-1 increased interstitial 5-HIAA levels in the nucleus accumbens and the hippocampus (Merali et al., 1997; Song et al., 1999).

In addition to IL-1 β , it appears that TNF- α and IL-6 may also provoke alterations of CNS monoamines, albeit to a lesser extent. Systemic administration of TNF- α caused an increase of hypothalamic NE activity (Mefford et al., 1991; Ando & Dunn, 1999), while turnover of the 5-HT precursor, tryptophan, was increased within the hippocampus

as well as the hypothalamus (Ando & Dunn, 1999). In addition, we found that TNF- α increased NE activity within the PVN, central amygdala, dorsal hippocampus and locus coeruleus, while 5-HT utilization was increased within the PVN, medial prefrontal cortex and central amygdala (Brebner et al., 2000; Hayley et al., 1999, 2001b). The effects of TNF- α on central DA activity were largely absent, with the exception of a modest increase of DOPAC within the nucleus accumbens (Brebner et al., 2000).

As the available data are limited, conclusions pertaining to some actions of cytokines can not be entirely deduced as yet. However, it seems likely that the effects of TNF- α on brain neurotransmission may not only be region specific, but may also vary across protracted time periods for certain monoamines. For instance, ip TNF- α was reported to elevate NE metabolism within the striatum after 2-12 hours and that of 5-HT within several brain regions 2 hours following injection, while DA metabolites within the hypothalamus were only increased after a 24 hour interval had elapsed (Cho et al., 1999). *In vitro* studies suggest that TNF- α may influence catecholamine release from cultured sympathetic neurons (Soliven & Albert, 1992), as well as NE release from electrically stimulated locus coeruleus and hippocampal tissue sections (Ignatowski et al., 1997). Furthermore, in slice preparations, TNF- α markedly reduced the firing rate of ventromedial hypothalamic neurons (Katfuchi et al., 1997) and the cytokine was reported to inhibit long-term potentiation in hippocampal sections (Tancredi et al., 1992).

Similar to TNF- α , IL-6 has been reported to elevate hypothalamic and hippocampal NE turnover, although these variations were appreciably lower than those elicited by IL-1 β (Zalcman, 1994). More recently, both systemic and central administration of IL-6 were shown to dose-dependently increase 5-HT turnover and

tryptophan accumulation within the hypothalamus and brainstem (Wang & Dunn, 1999); however, in this particular study the cytokine failed to influence NE activity. As well, it was reported that IL-6 increased DA utilization within the prefrontal cortex and 5-HT in the hippocampus in a manner similar to that of IL-1 β (Zalcman, 1994). In contrast, we found IL-6 to have limited effects on brain monoamines, although the cytokine did increase 5-HT and DA utilization within the central amygdala (Brebner et al., 2000), while profoundly inhibiting the *in vivo* release of DA within the nucleus accumbens (Song et al., 1999). Interestingly, IL-6 has also been proposed to have a neuroprotective role owing to its effects on amino acid neurotransmission. Consistent with potentially neurotoxic effects of elevated excitatory amino acids, IL-6 reduced the release of the excitatory amino acid, glutamate, from rat cortical slices, but increased depolarizing-induced release of the inhibitory transmitter, GABA, from the mediobasal hypothalamic explants (De Laurentiis et al., 2000).

Role of prostaglandins in the HPA and central monoamine responses to cytokines

Inasmuch as IL-1 β , IL-6 and TNF- α have potent pyrogenic effects, the possibility has been considered that the neuroendocrine and neurotransmitter changes induced by these cytokines were secondary to such effects. Intravenous or icv administration of these endogenous pyrogens results in a rapid rise of brain prostaglandin E₂ (PGE₂), which is known to cause fever and is released during inflammation (Dinarello et al., 1991; Rivest, 1999). In fact, induction of PGE₂ in the brain has been suggested as one possible factor by which cytokines influence HPA activity and central monoamine turnover (Breder et al., 1993). Consistent with this proposition, elevated expression of

prostaglandin receptors was demonstrated within the hypothalamus, brainstem nuclei and circumventricular organs in response to IL-1 β (Zhang & Rivest, 1999). Systemically applied IL-1 β provoked an early profound increase of extracellular PGE₂ levels within the OVLT and medial preoptic nucleus, followed by a later modest increase of the prostaglandin at the PVN and hippocampus (Komaki et al., 1992). Within the PVN, iv administered IL-1 β elevated the *in vivo* release of PGE₂ together with that of AVP and CRH (Watanobe & Takebe, 1994). Moreover, brainstem slices, obtained from rats treated with IL-1 β , exhibited potentated *ex-vivo* release of PGE₂ as well as that of CRH (Molina-Holgado et al., 1998). Interestingly, such an effect was apparent in animals receiving systemic but not central IL-1 β treatment (Molina-Holgado et al., 1998). Taken together, these data support the possibility that circulating IL-1 β may interact with circumventricular organs and/or brainstem nuclei to influence AVP and CRH activity within the PVN.

The effects of prostaglandins may be brain region specific, in fact, it has been suggested that they may contribute to NE turnover in the hypothalamus, but not in the cortex or medulla oblongata (Terao et al., 1993). However, subsequent studies, using the cyclooxygenase inhibitor, diclofenac, indicated that prostaglandins may mediate the late (after 40 minutes following cytokine challenge) but not the early (up to 20 minutes after challenge) increase of interstitial NE within the prefrontal cortex in response to IL-1 β (Kamikawa et al., 1998). Moreover, antagonism of the NMDA receptor blocked both phases of the augmented NE release, and it was suggested that IL-1 β may affect NE activity through the induction of glutamate which subsequently elevates local prostaglandins activity (Kamikawa et al., 1998).

In an attempt to ascertain the role prostaglandin may play in the central effects of cytokines, many studies have used inhibitors of the prostaglandin-converting enzyme, cyclooxygenase, such as indomethacin. When peripherally injected, indomethacin attenuated the IL-1 β -induced rise of plasma ACTH, as well as the elevated *c-fos* expression within the PVN, supraoptic nucleus, central amygdala and several brainstem nuclei (Buller et al., 1998). Pretreatment with indomethacin also blocked the LPS induced alterations of central NE and DA concentrations (Lavicky & Dunn, 1995) as well as IL-1 β induced cortical DA utilization (Masana et al., 1990). Likewise, indomethacin prevented the increased hypothalamic NE turnover associated with systemic and icv administration of IL-1 β (Terao et al., 1993) and reduced the rise of PVN extracellular NE provoked by the cytokine (Tsumori et al., 1998). However, subpyrogenic doses of IL-1 β were also found to elicit central amine alterations, indicating a minimal contribution of hyperthermia to the brain amine changes (Dunn, 1988; Kabiersch et al., 1988). Indeed, we found doses as low as 0.01-0.2 μ g of IL-1 β to influence NE turnover in the PVN, locus coeruleus, prefrontal cortex (Lacosta et al., 1998; Brebner et al., 2000) and DA in prefrontal cortex (Zalcman et al., 1994). Furthermore, Shintani et al (1993) showed that IL-1 β can directly enhance the release of NE from the anterior hypothalamus independent of prostaglandin production, and indomethacin failed to block this NE release. However, neuroanatomical studies demonstrated that microinjection of PGE₂ into the ventrolateral medulla provoked distinct *c-fos* staining within the brainstem and PVN reminiscent of that observed after IL-1 β administration (Ericsson et al., 1997).

The role of cytokines in the stress response

Interestingly, it has been suggested that some of the central monoamine and HPA variations associated with stressors, may stem from alterations of central cytokine activity (Dunn, 1993; Anisman et al., 2000). In fact, Shintani et al., (1995) demonstrated that pretreatment with the IL-1 receptor antagonist, IL-1ra, blocked the hypothalamic NE alterations and the plasma ACTH increases induced by immobilization stress. However, IL-1ra was only effective when administered shortly before the stressor onset and not after the stressor was applied (Shintani et al., 1995), suggesting that cytokines may play a role in the initial regulation of the stress response, but such a response may be difficult to control once initiated. As well, we have recently found that both IL-1 β and a predator stressor (fox odor) provoked comparable increases of plasma corticosterone and brain 5-HIAA accumulation, however, such effects may be strain-dependent (Brebner et al., 2000; Hayley et al., 2001c).

It is likely that the PVN may play an important role in the modulation of the brains response to immune activation as well as stressors. Indeed, augmented noradrenergic and/or serotonergic activity within the PVN or whole hypothalamus is evident after a variety of immune and non-immune associated stressors including restraint, footshock, predator exposure, viral infection, LPS, IL-1 β and TNF- α (Dunn, 1993; Hayley et al., 1999,2001c ; Brebner et al., 2000; Lacosta et al., 1998). Likewise, IL-1 β and various stressors have been found to increase *c-fos* expression in CRH-immunoreactive neurons within the PVN (Rivest & Rivier, 1994). Both footshock and IL-1 β also increased PVN mRNA levels for the immediate early gene, NGFI-B, as well as that of CRH (Lee & Rivier, 1998). Further support for a hypothalamic site of action

comes from studies demonstrating that lesions of the PVN attenuated stressor and cytokine induced increases of plasma ACTH and corticosterone as well as the altered immune activation associated with such treatments (Rivest & Rivier, 1991; Makara & Kovacs, 1997).

While a crucial role for the amygdala in processing traditionally stressful stimuli has long been proposed, only recently has evidence emerged to suggest amygdaloid involvement in the central response to cytokines and other immunogenic stimuli. In addition to the marked CRH changes in the PVN, it has been shown that stressors and IL-1 both increase CRH mRNA expression at extrahypothalamic sites, including the amygdala and bed nucleus of the stria terminalis (Sawchenko et al., 1996; Makino et al., 2000; Lee & Rivier, 1998). However, the effects of IL-1 were restricted to the central amygdala, as the cytokine failed to affect CRH mRNA within the lateral and oval nucleus of the bed nucleus of the stria terminalis (Day et al., 1999). Given that CRH mRNA is elevated in stressor reactive brain regions and anxiety is elicited by central CRH (Imaki et al., 1993), this peptide may be important in subserving anxiety (Davis, 1992; Gray, 1990), and hence, IL-1 may also influence anxiogenic processes.

Interestingly, it has been shown that, as in the case of stressors, administration of IL-1 β or TNF- α may elicit anxiogenic-like effects, although this has only been evaluated in a limited number of situations (Connor et al., 1998). It should be underscored, however, that there are now several investigators who have questioned the involvement of CRH in maintaining anxiety, as CRH knockout mice were not distinguishable from wild type mice in several behavioral tests (Dunn & Sweirgel, 1999). Yet, there is the belief that CRH, while not necessary for the maintenance of an anxiety response, may be

fundamental in the development of fear/anxiety (LeDoux, 2000). Further to this point, it is not unreasonable to suggest that the different phases of the anxiogenic response may be mediated by distinct stressor-responsive brain circuits. In particular, it has been proposed that, while the basolateral nucleus of the amygdala may play a prominent role in the initial processing of fearful stimuli, the central nucleus may be more important for the generation of behavioral outputs to contend with the challenge (Davis, 1992).

There is reason to believe that stressors and cytokines may differentially activate regions of the amygdala. Indeed, Ericsson et al., (1994) reported profound *c-fos* activation of the central amygdala in response to footshock, while systemic IL-1 provoked the strongest expression of the immediate early gene within the medial nucleus of the amygdala. Scant data exists with respect to the role the medial amygdala may play in the response to stressors and cytokines, however, one recent report suggested that the medial nucleus may play a more important role than the central nucleus in the neuroendocrine response to restraint stress (Dayas et al., 1999). Given that cytokines and stressors may contribute differentially to amygdaloid stimulation, these challenges may mediate different phases of anxiety/fear responses.

Despite the similarity of outcomes associated with stressors and immune challenge, it has been suggested that the neural pathways mediating the HPA effects of traditional stressors (e.g. footshock or restraint) may be dissociated from those subserving the neuroendocrine effects of systemic stressors (e.g. cytokines) (Herman & Cullinan, 1997). In this respect, Herman and Cullinan (1997) offered the view that while processive stressors (i.e., those that are involved in higher-order sensory processing, such as, exposure to predators) influence HPA activity via the limbic forebrain, systemic stressors

(e.g., respiratory, cardiovascular, bacterial and viral insults as well as cytokines) may do so primarily through stimulation of brainstem afferents. In support of this contention, exposure to a novel environment (procedural stressor) increased *c-fos* mRNA within limbic regions (e.g. lateral septum and medial amygdaloid nucleus), to a much greater extent than did inhalation of ether vapours (systemic stressor), although both stressors had comparable effects within the hypothalamus (Emmert & Herman, 1999). In the case of cytokines (systemic stressor), ablation of aminergic fibers originating from the medulla or removal of the area postrema (a brainstem, circumventricular organ) attenuated the HPA stimulating effects of IL-1 (Ericsson et al., 1994; Lee et al., 1998), supporting a role for brainstem nuclei in the HPA effects of cytokines. However, contrary to the notion of a minimal contribution of limbic sites in the HPA variations elicited by cytokines, recent studies have demonstrated that lesions of the central amygdala attenuated the plasma corticosterone and ACTH responses elicited by IL-1 β , suggesting that indeed amygdaloid-PVN communication may be important in regulating HPA responses to the cytokine (Xu et al., 1999).

Synergistic effects of IL-1 β , IL-6 and TNF- α

Studying the mechanisms which underly the effects of any one particular cytokine is complicated by the fact that numerous complex interactions occur between different cytokines and their target cells. These interactions may be of either an additive, synergistic (greater than the sum of the individual cytokine effects) or antagonistic nature (see Turrin & Plata-Salaman, 2000). In this respect, IL-1 β and TNF- α can induce each other's production as well as that of IL-6, IL-12 and IFN- γ , all of which may interact so

as to potentiate or inhibit each other's actions. For instance, coadministration of IL-1 β and TNF- α synergistically reduced sexual behavior, body weight and food consumption and increased adrenal gland weight (van der Meer et al., 1995; Sonti et al., 1996; Avitsur & Yirmiya, 1999). Likewise, TNF- α and IL-1 β (Perlstein et al., 1993) and IL-6 and IL-1 β (Zhou et al., 1996) have been reported to increase plasma ACTH and/or corticosterone levels in a synergistic fashion. In agreement with these findings, we have also demonstrated that IL-1 β and TNF- α increased corticosterone and reduced consumption of a palatable solution, and both effects were of a synergistic nature (Brebner et al., 2000). However, co-administration of IL-1 and TNF- α did not synergistically affect brain monoamine activity (Brebner et al., 2000). In contrast, IFN- γ had an inhibitory effect on the synergistic induction of TNF- α mRNA produced by combined IL-1, IL-6 and TNF- α treatment in C6 glioma cultures (Gayle et al., 1998). Interestingly, some evidence exists suggesting that the kinetics of cytokine synergisms may be important in determining their effects on the HPA axis. For instance, the synergistic effects of IL-1 β and IL-6 on ACTH release was present at 30-60 minutes after injection but, at a later time (2-3 hours post administration) the effect was similar to that of IL-1 β alone (Perlstein et al., 1991).

There is reason to believe that cytokines and stressors synergistically influence central processes, suggesting the possibility that these challenges are activating common neural substrates. In particular, Zhou et al., (1996) demonstrated a synergistic increase of plasma ACTH and corticosterone levels in response to IL-6 plus a novelty stressor, but curiously a similar effect was not seen with respect to IL-6 plus restraint. In addition, IL-1 β has been found to act synergistically with a mild stressor (air puff) to increase 5-

HIAA, HVA and MHPG concentrations at several limbic brain regions (Merali et al., 1997; Song et al., 1999). Moreover, as IL-1 β and IFN- γ levels are positively correlated in hippocampal cultures, addition of an anoxic insult reversed this relationship, such that IL-1 β concentrations rose while that of IFN- γ fell (Pellegrini et al., 1996). Thus, stressors may disturb the balance of cytokine networks, such that the role of certain cytokines may be favoured.

Cytokine and stressor sensitization effects

Some degree of behavioral and neurochemical plasticity is necessary for an animal to cope effectively with environmental demands. In this respect, stressful events not only elicit acute responses, but may also invoke mechanisms that influence the organism's responses to later challenges. Indeed, exposure to a variety of stressors, including immunological stimuli, have the effect of priming (sensitization) biological systems so that an augmented response is elicited by later exposure to the same or somewhat different challenges (Tilders & Schmidt, 1998; Anisman et al., 2001). The phenomenon of sensitization refers to an increased response of a biological system to a particular stimulus, given prior exposure to that stimulus. With respect to the immune system, exposure to a particular antigen may initially have limited effect, but upon reexposure to this same antigen a marked immunological reaction (e.g. antibody response) may occur. Similarly, in the fields of behavioral neuroscience or psychopharmacology, the term implies that some challenge (e.g., a stressor or drug treatment) elicits an enhanced response (e.g., neurochemical, neuroendocrine or behavioral) in animals previously exposed to that stimulus. In this instance, however, the stimulus needn't be identical to

the one the animal had initially encountered. Indeed, it has been demonstrated that exposure to a particular stimulus may enhance the response to a subsequently applied stimulus of a different form (cross-sensitization) (Anisman et al., 1993; Tilders & Schmidt, 1999; Hayley et al., 2001b).

There have been numerous reports showing that exposure to stressors will enhance behavioral and neurochemical responses to subsequently applied stressors (Anisman et al., 1993; Finlay et al., 1997; Gresch et al., 1994; Jordan, Kramer, Zukas & Petty, 1994). For instance, a single exposure to footshock provoked exaggerated behavioral signs of anxiety or fearfulness (e.g. reduced social exploration coupled with increased freezing and defecation) upon reexposure to the stressor several weeks later (van Dijken et al., 1993). In terms of central monoamine activity, stressor-induced sensitization effects have been demonstrated within the hypothalamus, hippocampus and amygdala with respect to NE activity, and within the prefrontal cortex for DA utilization (Anisman & Sklar, 1979; Finlay et al., 1997; Gresch et al., 1994; Irwin et al., 1986). Medial prefrontal cortex monoamine activity may be particularly vulnerable to stressor induced sensitization effects, as rats reexposed to swim stress, several days or weeks following previous encounter with the stressor, displayed profound *in vivo* increases of extracellular NE, 5-HT and DA within this brain region (Jordan et al., 1994).

In addition to the monoamine variations, stressors have been reported to provoke a phenotypic change in the co-localization of the hypothalamic neuropeptides, AVP and CRH, such that median eminence CRH terminals increase their co-expression of AVP (Bartansz et al., 1993). Interestingly, the shift in neuropeptide composition of CRH terminals in the ZEME was not stressor-specific and similar increases in AVP stores in

CRH terminals were reported after immobilization or social defeat stressors (Bartansz et al., 1993; De Goeij et al., 1991) and after adrenalectomy (Bertini & Kiss, 1991; Sawchenko et al., 1987; Whitnall et al., 1987). Furthermore, the changes in CRH terminals in the ZEME were paralleled by an increased proportion of CRH/AVP coproducing neurons within the mediodorsal PVN (De Goeij et al., 1992), as well as an increased AVP mRNA expression in the parvocellular portion of the PVN (Bartanusz et al., 1993).

At this juncture it should be underscored that the immediate and protracted effects of stressors are influenced by chronicity of such treatments. In this respect, the monoamine reductions ordinarily provoked by acute stressors may be absent following protracted or repeated insults (Deutch et al., 1985; Herman & Cullinan, 1997; Irwin et al., 1986; Roth et al., 1988). Such an "adaptation" involves active processes, since monoamine levels in chronically stressed animals do not simply return to prestress levels, but exceed basal values (Puglisi-Allegra et al., 1991). Evidently, chronic stress promotes a compensatory increase of amine synthesis, or moderation of excessive utilization, hence leading to the increased concentrations (Herman et al., 1982). Moreover, there have been numerous studies showing that chronic predictable and unpredictable stressors may differentially influence neurochemical functioning (Anisman et al., 1993; Zacharko et al., 1989).

Much like acute insults, chronic stress also proactively influences responses to later stressors. In particular, chronic cold stress sensitized NE release within the hippocampus and prefrontal cortex and DA release from cortically projecting neurons in response to later exposed to a tail shock (Jedema et al., 1999; Gresch et al., 1994;

Nisenbaum et al., 1991; Nisenbaum & Abercrombie, 1992). Furthermore, exposure to intermittent cold for a week augmented the effects of subsequent restraint, in terms of enhanced HPA activity coupled with elevated *c-fos* immunoreactivity within the PVN, amygdala, bed nucleus of the stria terminalis, prefrontal cortex, nucleus accumbens and paraventricular nucleus of the thalamus (Bhatnagar & Dallman, 1998). More recent studies demonstrated that repeated restraint sensitized freezing behavior, systemic corticosterone release and *c-fos* immunoreactivity within the central amygdala in response to subsequent social defeat (Chung et al., 2000). Chronic exposure to a series of different stressors also sensitized *in vivo* dopamine release from the frontal cortex (Cuadra et al., 1999), suggesting that although chronic predictable stressor exposure may often result in behavioral/neurochemical habituation, repeated unpredictable stressors may have the opposite effect (i.e. sensitization). These sensitization effects elicited by chronic stressors may result, in part, from attempts of CNS circuits to adapt to the repeated challenge which may lead to wear and tear of endogenous systems. Such repeated perturbations of stressor-sensitive systems has been described in terms of allostatic load, whereby sufficiently severe deviations from homeostasis may eventually result in adverse behavioral repercussions (McEwen, 1998).

In addition to stressors, pharmacological agents such as antidepressant or psychostimulant treatment, also produce behavioral and neurochemical sensitization effects. Indeed, chronic or intermittent amphetamine or cocaine administration sensitized rodent exploratory and motoric behaviors, as well as that of central NE and DA turnover (Kalivas et al., 1993; Gaytan et al., 1999; Flores et al., 2000). The behavioral sensitization elicited by chronic amphetamine treatment has been reported to be blocked

by infusion of neutralizing antibodies to basic fibroblast growth factor into the ventral tegmental area (Flores et al., 2000). Moreover, the amphetamine treatment elevated basic fibroblast growth factor immunoreactivity within the ventral tegmental area and the substantia nigra to an extent that was correlated with the magnitude of the behavioral sensitization, strongly suggesting involvement of the cytokine in the sensitizing actions of amphetamine (Flores et al., 2000). As well, a single dose of amphetamine resulted in a time-dependent sensitization (manifested after 1-3 weeks), of locomotor behavior and HPA activity as well as dopamine release from slices of the nucleus accumbens, caudate putamen and prefrontal cortex and that of acetylcholine from the accumbens and caudate slices (Vanderschuren et al., 1999).

Clinically used antidepressants, such as imipramine, also induce neurochemical sensitization effects several weeks after either a single dose or repeated treatment regimens (Antelman, 1988). Interestingly, the hypothesis has been offered that such sensitization effects may underlie the clinical efficacy of these drugs (Antelman, 1988; Antelman & Gershon, 1998; Schmidt et al., 1995). In fact, contrary to popular belief, the time interval following the initial antidepressant dose has been proposed to be more important than the actual chronicity of such treatment (Antelman & Gershon, 1998; Tilders & Schmidt, 1999). In keeping with this point of view, it is reasonable to suggest that some critical time interval must elapse after initial the drug treatment in order to allow for a sufficient degree of neuroplasticity, manifested in receptor or transmitter changes.

Sensitization in response to cytokines

It will be recalled that cytokines induce neurochemical and neuroendocrine effects reminiscent of those associated with stressful events (Anisman et al., 1993; Blalock, 1994; Dunn, 1992). As was the case for stressors, administration of proinflammatory cytokines (e.g. IL-1 β and TNF- α) may result in a sensitizing effects, such that subsequent responses to these cytokines are increased upon their reintroduction some time following the initial exposure (Schmidt et al., 1995; Tilders et al., 1993; Hayley et al., 1999, 2001a, 2002a). In fact, a single exposure to either IL-1 β or footshock has been found to result in a long-term hyper-responsiveness (sensitization) of the HPA axis (de Goeij et al., 1991; Schmidt et al., 1995; Tilders et al., 1994; Uehara et al., 1987). Moreover, it appeared that the emergence of such a sensitization was dependent on the passage of time following the initial challenge (Schmidt et al., 1995).

Tilders and his associates (de Goeij et al., 1992a; Schmidt et al., 1995) reported that following a chronic stressor regimen, or several days after a single stressor or IL-1 β treatment, characteristic changes were observed in hypothalamic CRH terminals in the external zone of the median eminence (ZEME). Ordinarily approximately 40-50% of the CRH terminals in the ZEME co-store AVP (Whitnall et al., 1987a); however, following stressor or cytokine treatments there is a progressive increase of AVP stores in these CRH terminals (Bartanusz et al., 1993; de Goeij et al., 1992a; Schmidt et al., 1995). Although CRH stores within the ZEME were not appreciably effected by IL-1 β treatment, AVP levels substantially increased within CRH terminals, presumably allowing for an enhanced co-release of these ACTH secretagogues (Schmidt et al., 1995). The increased colocalization of these neuropeptides only became apparent 4 days after

IL-1 β administration, peaked 7-11 days following the treatment and did not return to control levels until 40 days later (Schmidt et al., 1995). Commensurate with the notion that IL-1 β treatment results in sensitization of HPA functioning, a second administration of IL-1 β (eleven days after the initial challenge) resulted in a significant reduction of the AVP stores in the ZEME coupled with elevated plasma ACTH and corticosterone levels (Schmidt et al., 1995). Thus, in this instance, the augmented co-storage of AVP and CRH was translated into an exaggerated corticosterone response, upon reexposure to IL-1 β , presumably reflecting increased release of these peptides from the hypothalamus. Similarly, we have observed a progressive time-dependent sensitization of plasma corticosterone release induced by TNF- α . Indeed, exaggerated corticoid responses were evident in mice reexposed to the cytokine 14 and 28 days following the initial TNF- α pretreatment, however, such a sensitization was absent upon reexposure after earlier times (1 and 7 days) (Hayley et al., 1999).

It has been suggested that IL-1 β ordinarily activates non-AVP producing PVN CRH neurons in naive rats; however, after a period of several days or weeks following a single challenge with the cytokine there may be a shift toward the activation of CRH hypothalamic neurons which coproduce AVP (Schmidt et al., 1995; Tilders & Schmidt, 1999). One possible mechanism that may account for the augmented activity hypothalamic AVP and CRH activity is that the IL-1 β somehow reduced the degree of corticoid negative feedback, which typically keeps HPA activity in check. As already mentioned hyper-responsive AVP/CRH and hormonal responses occur after adrenalectomy (Plotsky et al., 1987), and reduced glucocorticoid inhibitory actions on AVP production has been suggested to mediate some of the effects of stressors on HPA

activity (Sawchenko et al., 1987). Alternatively, it was proposed that pretreatment with IL-1 β sensitized peptide synthesis and/or release at PVN neurons resulting in enhanced accumulation of AVP, together with CRH, in ZEME terminals (Tilders & Schmidt, 1998, 1999). Subsequent increased co-release of these peptides into the hypophyseal portal plexus may result in a synergistic increase of pituitary ACTH and adrenal corticosterone release (Rivier & Vale, 1983; Whitnall et al., 1988).

The possibility also needs to be considered that the cytokine induced corticosterone and ACTH sensitization effects may stem from direct actions upon the pituitary or adrenal glands. Indeed, high densities of TNF- α receptors have been demonstrated within the anterior pituitary and adrenal and the cytokine may even directly provoke ACTH release from this region (Kobayashi et al., 1997). Moreover, recent reports have demonstrated that another cytokine, IL-6, can increase plasma ACTH and corticosterone levels in the absence of CRH (Bethin et al., 2000), raising the possibility of a peripheral site of action or mediation by alternate brain peptides. Finally, the neuroendocrine consequences of various challenges, including pathogenic, chemical or other immunogenic insults (e.g. cytokine), may be influenced by the immediate state of the microenvironment. For example, hyperthermia sensitizes tumor cells to the well-documented cytotoxic effects of TNF- α (Wang et al., 1996). Thus, it is possible that treatment with a cytokine may affect subsequent thermogenic responses to this treatment, which may come to influence later HPA responses induced by re-exposure to the cytokine or to stressors.

In addition to the aforementioned sensitization effects of cytokines on HPA activity, only a limited amount of data exists with respect to the sensitizing actions of

cytokines on brain neurotransmission and various forms of behavior and illness. Small amounts of endotoxin have been reported to lower the effective dose of TNF- α required to elicit signs of illness (Heremans et al., 1990), thereby indicating a cross-sensitization between these substances. Likewise, bacterial infection (e.g. *Bacillus Calmette-Guerin*) sensitized the induction of lethality in response to subsequently applied TNF- α and this effect was believed to be mediated by the cytokine, interferon- γ (Cauwels et al., 1995). Systemic administration of TNF- α also had sensitizing effects on various indices of illness which varied with the passage of time. Indeed, reexposure to TNF- α 2-4 weeks following initial treatment with the cytokine, markedly augmented sickness behaviors (e.g. reduced consumption, social exploration and locomotion), while such effects were absent at earlier reexposure times (1-7 days) (Hayley et al., 1999, 2001a, 2002b). Interestingly, cytokines have been suggested to modulate dopaminergic and other CNS systems involved in reward and motivation. Indeed, repeated IL-6 treatment was found to augment the locomotor inducing effects of subsequently applied amphetamine (Zalcman et al., 1999). As well, enhanced expression of the trophic cytokine, fibroblast growth factor, was reported to be essential for the behavioral sensitizing effects of amphetamine (Flores et al., 2000). Apart from data collected in our laboratory, no published reports exist with respect to the sensitization effects of cytokines on brain monoamines.

In addition to the immediate effects of TNF- α on central monoamine utilization (Hayley et al., 1999; Brebner et al., 2000; Ando & Dunn, 1999), reexposure to TNF- α following initial treatment increased monoamine metabolite accumulation relative to that of animals that had received only a single acute injection of the cytokine. Within the PVN, TNF- α induced a sensitization of NE activity, as reflected by accumulation of

MHPG, which followed a time course similar to that of the corticosterone and sickness sensitization effects. Moreover, the elevated MHPG levels were further enhanced upon reexposure to the TNF- α , particularly at progressively longer intervals following initial treatment with the cytokine (Hayley et al., 1999). In contrast, an earlier sensitization of MHPG accumulation was observed in the medial prefrontal cortex and central amygdala wherein reexposure to TNF- α 1 day following pretreatment with the cytokine elicited the effect. However, a TNF- α provoked sensitization of 5-HT activity, as reflected by accumulation of 5-HIAA, was apparent within the central amygdala upon reexposure to the cytokine after an intermediate interval of 7-days following pretreatment. Variations of 5-HIAA within the prefrontal cortex were maximal upon reexposure to TNF- α 14 days following the initial cytokine injection (Hayley et al., 1999).

Conclusions

It appears that cytokines are involved in normal as well as impaired CNS functioning. A limited number of studies have demonstrated the presence of IL-1, IL-6 and TNF- α and their receptors in discrete brain regions under normal conditions, and these cytokines may even play a role in brain development. Brain levels of these cytokines are markedly upregulated in a number of neurological conditions (e.g. multiple sclerosis and cerebral infections), as well as in response to traumatic events (e.g. head injury or stroke). Likewise, increased circulating levels of IL-1 and TNF- α , as well as IL-2, are often associated with depressive or schizophrenic conditions, attesting to their potential involvement in psychiatric illnesses. Furthermore, IL-1 and TNF- α provoke

HPA and central monoamine variations reminiscent of those observed in response to stressful or anxiogenic stimuli.

In addition to these immediate effects, cytokines also appear to exert long lasting behavioral and neurochemical consequences. Indeed, a single exposure to TNF- α has been shown to prime CNS activity, such that later reexposure to the cytokine provoked profound alterations of sickness, HPA activation and brain monoamine utilization. Such sensitization effects may potentially stem from increased receptor sensitivity or the augmented release of various endogenous factors, such as other cytokines (e.g. interferons), prostaglandins or acute phase proteins. In this respect, enhanced co-activity of these factors would be expected to result in synergistic actions on central processes under certain conditions. Cytokine- and stressor-induced sensitization of CNS activity suggests that a certain degree of neuroplasticity may be evident in response to stressful or immunogenic stimuli, and hence may influence the response to later challenges. In particular, intermittent stressors, bacterial or viral infections may predispose individuals to behavioral disturbances by virtue of the sensitizing effects of liberated cytokines on neurochemical functioning. Finally, with the increased use of cytokine immunotherapy for a variety of cancers and other conditions, the temporal dosing schedule of such treatment may impact greatly upon the success of the therapy and likelihood of toxic side effects (i.e. time-dependent sensitization of anti-tumor vs sickness inducing properties).

Aim of the present thesis

In light of the immediate and protracted actions of cytokines, and their similarity to the actions of stressors, the present investigation sought to assess

1. the immediate influence of TNF- α on neuroendocrine and central neurotransmitter functioning,
2. whether the murine form of TNF- α (mTNF- α), which affects two types of TNF- α receptors (termed p55 and p75), has behavioral and neurochemical effects comparable to that of human recombinant TNF- α (rhTNF- α), a form of the cytokine which only influences the p55 receptor in the mouse.
3. whether mTNF- α induces a sensitization effects with respect to sickness behaviors, plasma corticosterone and central monoamines, just as rhTNF- α has such effects.
4. the dose-response relationship for the mTNF- α induced hormonal sensitization
5. the sensitization effects of TNF- α on central *c-fos*, AVP and CRH expression
6. the immediate and sensitizing effects of intracerebroventricularly administered TNF- α in an effort to establish whether TNF- α directly influences CNS processes, hence affecting central monoamine and plasma corticosterone levels.
7. whether cross-sensitization occurs between LPS pretreatment and subsequent endotoxin, TNF- α , IL-1 β or stressor administration.
8. the involvement of peripheral macrophages in any cross-sensitization effects between LPS and TNF- α

9. the role histamine may play in mediating the sensitization of illness and neurochemical activity
10. whether the acute and/or sensitizing effects of $\text{TNF-}\alpha$ are selectively mediated by its p55 receptor, as determined using mice genetically deficient for this receptor

Chapter I

Time-Dependent Sensitization of Corticotropin Releasing Hormone, Arginine Vasopressin and *c-fos* Immunoreactivity within the Mouse Brain in Response to Tumor Necrosis Factor- α .

Abstract

Stressor or cytokine treatments, such as interleukin-1 β , promote time-dependent alterations of hypothalamic-pituitary-adrenal functioning, including increased arginine vasopressin stores within corticotropin releasing hormone terminals in the external zone of the median eminence. Likewise, we have previously shown that the proinflammatory cytokine, tumor necrosis factor- α , provoked a time-dependent sensitization of neuroendocrine and brain monoamine activity. To further explore the protracted consequences of tumor necrosis factor- α , the present investigation determined whether the cytokine sensitized activity of neuroendocrine regulatory brain regions, as assessed by *c-fos* expression, and had protracted consequences on amygdaloid corticotropin releasing hormone, as well as hypothalamic corticotropin secretagogues. Indeed, immunoreactivity for arginine vasopressin and corticotropin releasing hormone, and their co-localization within cell terminals of the median eminence, varied over time following an initial 4.0 μ g tumor necrosis factor- α treatment, peaking after 7 days and normalizing within 28 days. Within the central amygdala, a sensitization effect was evident as reflected by increased corticotropin releasing hormone immunoreactivity, but this effect required reexposure to the cytokine, unlike the median eminence changes that simply evolved with the passage of time. As well, tumor necrosis factor- α provoked a marked sensitization of *c-fos* staining within the paraventricular nucleus of the hypothalamus, supraoptic nucleus and the central amygdala.

Taken together, these data suggest that tumor necrosis factor- α influences responsiveness of stressor-reactive brain regions and has protracted effects on central

neuropeptide expression within the hypothalamus and central amygdala, although the time course for the effects vary across brain regions. Evidently, exposure to tumor necrosis factor- α may promote neuroplasticity of brain circuits involved in mediating neuroendocrine, sickness or inflammatory responses. It is suggested that such a sensitization may influence the response to immunological and traumatic insults and may thus be relevant to behavioral pathology.

Introduction

Elevated expression of brain mRNA and/or protein for proinflammatory cytokines, particularly interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), is associated with immunological or traumatic insults, neurodegenerative diseases and even stressors (Feuerstein et al., 1998; Griffin et al., 1995; Mogi et al., 1994; Nguyen et al., 1998; Quan et al., 1999). Moreover, expression of IL-1 β and TNF- α receptors have been detected within the numerous brain regions, such as the paraventricular nucleus of the hypothalamus (PVN), circumventricular organs, brainstem and hippocampus (Alheim and Bartfai 1998; Ericsson et al., 1995; Kinouchi et al., 1991; Licinio et al., 1991; Nadeau and Rivest, 1999). Further, immune activation, involving leukocyte-derived cytokines, induces stressor-like effects upon various brain processes (Anisman et al., 1993; Dunn, 1995). As well, administration of IL-1 β and TNF- α provoke sickness behaviors, including reduced locomotion, exploration and social interaction, piloerection, ptosis, hypovolemia and anhedonia (Dantzer et al., 1996; Kent et al., 1992; Hayley et al., 2002b). Much like the effects of typical stressors (restraint, footshock), IL-1 β stimulates corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) expression within neurons of the PVN (Lee and Rivier, 1994; Madrup-Poulsen et al., 1995; Tilders et al., 1993) and their terminals within the median eminence (McCoy et al., 1994; Watanobe and Takebe, 1992). Similarly, intravenous and centrally applied TNF- α elicit hypothalamic CRH activity, as well as ACTH and corticosterone secretion (Bernardini et al., 1990; Johnson et al., 1992; Turnbull et al., 1997).

In addition to hypothalamic neuropeptide alterations, cytokines also induce CRH changes in other stressor sensitive limbic regions, such as the amygdala (Kasckow et al., 1997; Raber et al., 1998; Raber et al., 1995). Given that TNF-immunoreactive fibers have been reported to innervate the amygdala (Breder et al., 1993), the possibility exists that the cytokine modulates amygdaloid neurochemistry through actions on TNF- α positive neurons (e.g. within the hypothalamus, bed nucleus of the stria terminalis, caudal raphe and ventrolateral medulla) which project to this region. Moreover, IL-1 β and TNF- α increased mRNA for the immediate early gene, *c-fos*, within the central amygdala, PVN, as well as circumventricular and brainstem sites (Buller et al., 1998; Lee et al., 1998; Nadeau and Rivest, 1999). It appears that cytokine or immunological (e.g. endotoxin) challenges stimulate Fos immunoreactivity within discrete subcellular regions of neuroendocrine regulatory nuclei, suggesting central metabolic activation (Matsunaga et al., 2000).

Interestingly, bacterial endotoxin, cytokine and stressor treatments have not only been shown to have immediate effects on hypothalamo-pituitary-adrenal (HPA) activity, but may have protracted actions as well (Hayley et al., 2001b; Tilders & Schmidt, 1998). In this respect, such treatments increase the co-localization of AVP and CRH within the external zone of the median eminence, but this effect only evolved with the passage of time, becoming apparent only after several days (Schmidt et al., 1995, 1996; Tilders and Schmidt, 1998). In addition, upon subsequent IL-1 β or stressor challenge 11 days following the initial IL-1 β treatment, circulating ACTH and corticosterone were markedly elevated (Schmidt et al., 1995), suggesting that the initial treatment provoked the sensitization of HPA responsivity. It was similarly demonstrated that TNF- α had

protracted effects with respect to sickness behavior as well as HPA and central neurotransmitter activity (Hayley et al., 1999, 2002b). A single systemic TNF- α injection induced a time-dependent sensitization of sickness symptoms and plasma corticosterone release, which only became evident upon reexposure to the cytokine 14 or 28 days later (Hayley et al., 1999). Systemic TNF- α also increased utilization of norepinephrine (NE) and serotonin (5-HT) within the prefrontal cortex, PVN and amygdala (Ando and Dunn, 1999; Brebner et al., 2000; Hayley et al., 1999, 2002a), and these effects were likewise subject to a time-dependent sensitization, the characteristics of which varied across brain regions and the specific neurotransmitter being assessed.

The present investigation assessed whether systemic TNF- α treatment provoked an increase of central AVP and CRH immunoreactivity (ir), as well as co-localization of these peptides, and whether such effects increased with the passage of time. Given the stressor-like actions elicited by cytokine treatment, CRH-ir was also assessed within the central amygdala, a stressor-sensitive brain region, which may modulate HPA activity (Sawchenko et al., 1996; Xu et al., 1999). Finally, as *c-fos* expression occurs within several neuroendocrine regulatory brain regions (PVN, central amygdala and supraoptic nucleus) following TNF- α and IL-1 β treatment (Buller et al., 1998; Lee et al., 1998, Nadeau and Rivest, 1999), immunoreactivity for the immediate early gene was assessed in order to determine the extent to which reexposure to TNF- α sensitized neuronal responses.

Materials and Methods

Adult, male, CD-1 mice (3-4 months of age), obtained from Charles River Inc, Laprairie Quebec, were housed in groups of four in standard propylene cages. After arrival, mice were given at least two weeks to acclimate to the laboratory. Animals were maintained on a 12-h light dark cycle (lights on 0800 -2000 h), and all experimental procedures were conducted between 0900 and 1200 h in order to avoid variations due to diurnal rhythms. An ad libitum diet of Ralston Purina (St. Louis, MO) mouse chow and tap water was available. All protocols were approved by the Animal Care Committee of Carleton University and met the guidelines provided by the Canadian Council on Animal Care. In addition, all efforts were made to minimize animal suffering and used only the minimal necessary number of mice given the variability associated with the experimental procedures.

Recombinant human TNF- α (specific activity of 1.1×10^5 U/ μ g, and negligible endotoxin levels) was obtained from R & D Systems (Minneapolis, MN) in lyophilized form and was reconstituted in sterile non-pyrogenic PBS and stored in 10 μ l aliquots at -80°C.

Procedure

Time-dependent corticotropin releasing hormone and arginine vasopressin variations: Mice were injected intraperitoneally (ip) with either 4.0 μ g of TNF- α (dissolved in 0.4 ml of sterile non-pyrogenic saline) or an equivalent volume of the vehicle. Either 1, 7, 14 or 28 days later mice ($n = 5$ per group) were reexposed to a second 1.0 μ g dose of TNF- α or saline. The doses of TNF- α were selected on the basis of

earlier studies conducted in this laboratory (Brebner et al., 2000; Hayley et al., 1999), which indicated that unlike the 4.0 μg TNF- α dose, 1.0 μg produced suboptimal behavioral and neurochemical changes, thus precluding ceiling effects from obscuring a potential sensitization effect.

One hour following the final TNF- α or saline injection, animals were perfused intracardially with 0.9 % saline followed by a solution of 4% paraformaldehyde and 0.4 % -saturated picric acid in 0.16 M phosphate buffer (pH 6.9). Brains were removed and placed in the fixative for 90 minutes at 4°C and subsequently transferred to a solution of 10% sucrose in 0.1 M phosphate buffer (pH 7.2), containing 0.01% sodium azide, until sectioning.

Sensitization of c-fos immunoreactivity 7 and 14 days following initial tumor necrosis factor- α treatment: A second experiment assessed the course of *c-fos*-ir variations over a 24 hr period following TNF- α administration among naïve mice and following reexposure to the cytokine. To this end, CD-1 mice ($n = 3$ per group) were injected ip with 4.0 μg of TNF- α or vehicle and then reexposed to the 1.0 μg dose of the cytokine after a period of 7 days. In order to determine the course for the acute effects of TNF- α and that seen after reexposure to the cytokine (sensitization effect) 7 days after initial treatment, these animals were perfused either 1, 3, 8 or 24 h following the final rhTNF- α treatment and tissue sections assayed for *c-fos*-ir (see procedure below).

A third experiment evaluated the long-term sensitization effects of TNF- α on *c-fos*-ir. To this end, CD-1 mice ($n = 3$ per group) received i.p. injection of TNF- α (4.0 μg) or vehicle, and then 14 days later exposed to either TNF- α (1.0 μg) or to vehicle. Three

hours following the latter treatment mice were perfused and the *c-fos*-ir determined. As well, double labeling of AVP- and *c-fos*-ir was determined for PVN sections (see procedure below).

Immunohistochemistry

Brains were cut on a cryostat (Reichert-Jung Frigocut, model 2800) and stored at -40°C until processing, except for sections examined for *c-fos* which were reacted immediately. Coronal sections were cut at 14 µm thickness throughout hypothalamic and amygdaloid nuclei and *c-fos*-ir was determined within the PVN, supraoptic nucleus and amygdala. Alternate 5 µm sections through median eminence were reacted for AVP-ir and CRH-ir, while CRH-ir was evaluated across sections containing the central amygdala. As well, double labeling was conducted to determine the extent of AVP/CRH-ir colocalization within terminals of the external zone of the median eminence.

Slides were rehydrated with PBS (normal saline in 10 mM sodium phosphate) for 2 min and then further washed for 20 min in the buffer. For double labeling, sections were incubated, overnight at 4°C in a humid chamber, with anti-CRH antiserum (1:400 dilution; polyclonal rabbit, Peninsula Laboratories, CA) combined with anti-AVP antiserum (1:800; polyclonal guinea pig, Peninsula Laboratories, CA). All antibodies were diluted with 10 mM PBS containing 0.3% Triton-X. After further rinsing with PBS, slides were incubated at 37 °C for 45-60 min with secondary, biotinylated goat anti-guinea pig (Vector laboratories, CA; 1:50 dilution) and fluorescein conjugated donkey anti-rabbit (Amersham, UK; 1:20) immunoglobulins. Finally, sections were incubated a

further 1 h at 37 ° C with Texas Red conjugated streptavidin (Amersham, UK; 1:100 dilution).

For sections double labeled for *c-fos* and AVP immunoreactivity, rabbit polyclonal antibodies directed against the N-terminus portion of the Fos protein (Vector laboratories, CA; 1:1000) were incubated together with guinea pig anti-AVP antiserum (Pennisula Laboratories, CA; 1:400) overnight at 4 ° C. Biotinylated donkey anti-guinea pig (1:50 dilution) and fluorescein labeled goat anti-rabbit antibodies (Amersham, UK; 1:20 dilution) were applied for 4-6 h at room temperature and then sections were incubated for 1 h at 37 ° C with the Streptavidin-Texas red (Amersham, UK; 1:100). Slides were cover-slipped with an anti-fade medium containing 0.1 % p-phenylenediamine in phosphate buffered glycerol.

Immunohistochemical quantification

Given the time-consuming procedure required to process the numerous tissue sections from each experiment, sections from one animal from each experimental group were analyzed daily over a period of 5 days. In essence, a series of replications were performed in which sections were processed in parallel under identical conditions. Coronal sections were collected across the extent of the hypothalamus and amygdala consistent with plates 34 through to 43 of the mouse stereotaxic atlas of Franklin and Paxinos (1997). In all cases, three successive sections, separated by an interval of 50 µm, were sampled at each level of the regions used for quantitative analyses. Digital images were acquired using Northern Eclipse (Empix Inc.) imaging software and a Sony XC75 PAL CCD camera in conjunction with a Matrox frame grabber with integration chip. A

standard number of integrations were used for each image depicted within a given figure and no individual image manipulations were performed. After image acquisition, gray level and density measurements were made using Image Tool software (UTHSCSA Image Tool for Windows, version 2.00).

Integrated density (mean gray level x pixel area) was determined for AVP and CRH fluorescence within the median eminence and CRH within the central amygdala of digitized images. Median eminence, cryostat sections were cut at levels corresponding to plates 44-49 in Franklin and Paxinos (1997). After digitization of images, objects within a section were defined by manual thresholding, such that background fluorescence and dimly immunofluorescent terminals were excluded from the analysis. Briefly, using Image Tool software for each section, a sliding scale was adjusted so that discrete punctate labeled terminals were selected for analysis. Given the high density of median eminence CRH immunoreactivity, this thresholding procedure was employed to prevent "bleeding" of numerous terminals together since standard epifluorescence, unlike confocal microscopy is subject to optical interference from planes slightly out of focus. It should be underscored that all brain sections were analyzed with the rater blind to the experimental treatment, thus reducing any subjective bias towards terminal selection in any particular group. After terminal selection, a computer generated algorithm determined relative density based upon pixel size and gray level. Quantification of AVP and CRH within the median eminence was made at rostral (-1.7 mm), medial (-1.94 mm) and caudal (-2.06 mm) levels relative to bregma. These measures were then pooled for each animal in order to obtain a relative measure of peptide immunoreactivity across the entire extent of the brain region. For the central amygdala, quantitative measures were

taken 1.34 mm caudal to bregma, corresponding to plate 42 in Franklin and Paxinos (1997), at the emergence of the arcuate nucleus. Labeled punctate CRH varicosities were sharply defined, hence automatic computer detection was used to select objects for density measures.

Within the PVN, plates 26, 38 and 40 were taken as sections representative of the rostral, medial and caudal portions of the nucleus, respectively. Indeed, previous studies reported differences in peptide sensitivity to stressors across the rostral-caudal extent of the PVN (Dijkstra et al., 1998). Differences in *c-fos* immunoreactivity within the PVN, supraoptic nucleus, and the central amygdala were quantified by counting the number of Fos positive cells using a 40x objective lens. Likewise, determination of the degree of co-localization of *c-fos* within AVP-ir containing neurons was made by visual microscopic inspection in a similar fashion.

Statistical Analyses

The *c-fos*, AVP or CRH immunoreactivity differences between treatments were evaluated using one- or two-factor ANOVAs as appropriate. Significant main effects or interactions were followed by Tukey-HSD post hoc multiple comparisons ($\alpha = 0.05$).

Results

At the time of sacrifice, mice acutely treated with TNF- α showed few signs of illness. However, as we previously observed (Hayley et al., 1999), mice reexposed to TNF- α 14 or 28 days following initial exposure to this cytokine generally displayed marked sickness, characterized by reduced motor activity and social exploration, curled body

posture, piloerection, and ptosis. In addition, mice showed clear signs of cyanosis of the ears, nose and tail, and hypovolemia reflected by a pronounced decrease of blood flow at the time of decapitation.

Arginine vasopressin and corticotropin releasing hormone double labeling

As shown in Figure 1.1, a single injection of TNF- α had protracted time-dependent effects on CRH and AVP immunoreactivity within terminals of the median eminence. Specifically, among animals previously treated with saline, AVP/CRH-ir within the median eminence was not visibly altered 1 h after a single 1.0 μ g ip injection of TNF- α , and there was only a slight non-significant elevation of CRH- and AVP-ir 1 day after initial cytokine administration (4.0 μ g). However, a marked increase of neuropeptide immunoreactivity was observed within the median eminence 7 days after cytokine treatment, and was more pronounced for AVP than CRH. This effect was still apparent 14 days after the initial treatment, but was absent 28 days after TNF- α administration. Quantitative analyses revealed significant variations of AVP-ir and CRH-ir among mice initially treated with TNF- α , F's (4, 24 and 4, 23) = 4.82 and 8.48, P's < .05. As depicted in Figure 1.1, and confirmed by the multiple comparisons, 7 and 14 days after cytokine treatment the CRH and AVP density was elevated relative to that of vehicle treated mice or mice that had received the cytokine, on a single occasion, 1 hr earlier ($P < 0.05$). It is important to emphasize that reexposure to TNF- α was not necessary for the induction of the increased CRH-ir and AVP-ir. It seems that the effects observed within the median eminence were dependent simply upon the passage of time, as the CRH-ir and AVP-ir at 7 and 14 days exceeded that seen 1-day after initial TNF- α

treatment. As these results were somewhat surprising given our previous reports demonstrating a sensitization of plasma corticosterone activity 28 days following TNF- α (a time in which the AVP/CRH-ir were comparable to control in the present investigation), a partial replication of this study was undertaken ($n = 4/\text{group}$). Once again, at 28 days following TNF- α administration (4.0 μg) coexpression of CRH and AVP was indistinguishable from that of saline-treated mice.

Insert Figure 1.1 about here

In a subset of animals ($n = 3/\text{group}$), visual inspection, using 100x magnification, of double-labeled median eminence sections revealed that approximately 20-30% of CRH terminals (mean $\pm$ SEM = 171 ± 23 , total CRH terminals) were immunoreactive for AVP (mean $\pm$ SEM = 44 ± 7 , AVP positive terminals) in vehicle treated mice. In contrast, an estimated 70-80% of the CRH-ir terminals (mean $\pm$ SEM = 235 ± 31 , total CRH terminals) stained positive for AVP (mean $\pm$ SEM = 169 ± 35 , AVP positive terminals) in mice pretreated 7 days earlier with TNF- α (Figure 1.2). In each case, terminals were counted from the entire external zone of the median eminence. Sections were obtained from the rostral aspect of the median eminence as shown in plate 45 (-1.70 from bregma) in Franklin and Paxinos (1997), thereby ensuring that the same level of the region was

Figure 1.1 Immunoreactivity for AVP and CRH within the median eminence of mice treated with saline on two occasions 7 days apart (left), TNF- α (4.0 μ g) 7 days earlier (middle) or TNF- α (4.0 μ g) 14 days earlier (right). The top panels depicts AVP immunoreactivity, as labeled using Texas Red, while the bottom panels show CRH positive terminals (visualized using the FITC fluorophore), within the same double labeled section. Reexposure to the cytokine 1 or 28 days after initial TNF- α treatment was without effect, and the data for these time points are not shown in the photomicrographs. IZ denotes the internal zone of the median eminence. EZ denotes the external zone of the median eminence. Enhanced AVP and CRH immunoreactivity was evident within EZ 7 and 14 days after a single TNF- α injection, (B,C and E,F, respectively). However, AVP-ir was somewhat diminished within IZ after the 14 day interval (C). Magnification x 20. $n = 5$ / group.

sampled in each experimental group. Thus, $\text{TNF-}\alpha$ appeared to provoke a time-dependent increase in the co-localization of AVP and CRH within these median eminence terminals.

Insert Figure 1.2 about here

Within the internal layer of the median eminence, AVP-ir did not vary appreciably between vehicle-treated mice and those that received $\text{TNF-}\alpha$ 1 and 7 days earlier. However, among mice pre-exposed to $\text{TNF-}\alpha$ 14, and particularly 28 days earlier, AVP-ir in the internal zone was somewhat diminished and had a more patchy distribution (see Figure 1.1). It should be underscored that the results observed in the 28-day group appeared more variable than that of the other groups. Also notable was that reexposure to $\text{TNF-}\alpha$ at these later times provoked a further reduction of AVP internal fiber band staining in approximately half of the median eminence sections examined from the 14 and 28 day reexposed mice.

As shown in Figure 1.3, $\text{TNF-}\alpha$ influenced CRH immunoreactivity within the central nucleus of the amygdala. Indeed, quantitative analysis revealed significant variations of amygdaloid CRH-ir among the $\text{TNF-}\alpha$ treatment groups, $F(9,25) = 2.76$; $p < 0.05$. The multiple comparisons indicated that a single acute $\text{TNF-}\alpha$ treatment ($1.0 \mu\text{g}$ administered 1 hr before sacrifice) elevated peptide immunoreactivity relative to that seen in saline treated mice. Although CRH-ir was also elevated above control levels 1 and 7 days following a single $4.0 \mu\text{g}$ $\text{TNF-}\alpha$ treatment, these changes were greatly enhanced

upon reexposure to the cytokine at these times. Indeed, among mice pretreated with TNF- α , reexposure to the cytokine 1 and 7 after initial treatment increased CRH-ir over and above that observed in mice that were initially treated with TNF- α and then treated with saline at these times. In contrast to the effects seen at 1 and 7 days after initial cytokine administration, relative to vehicle controls, significant differences were not evident in mice reexposed to either TNF- α or saline 14 or 28 days following initial treatment. It is noteworthy that the CRH-ir was confined to a dense plexus of punctate varicosities that surrounded spherical unstained regions, likely representing local cell bodies. A small number of AVP-ir fibers were detected within the amygdala, but these were distinct from the CRH-ir swellings.

c-fos immunoreactivity

Administration of TNF- α influenced *c-fos* immunoreactivity measured within several stressor-sensitive brain nuclei, including the PVN, supraoptic nucleus, and central amygdala, F 's(7,16) = 8.55, 3.76 and 13.73, P 's < 0.05. As shown in Figure 1.4, acute TNF- α treatment in cytokine naïve mice (vehicle pretreatment 7 days earlier) provoked an increased number of PVN and supraoptic Fos positive cells at 3 and 8 h following the cytokine injection, relative to that observed after 1 and 24 h. However, no such acute cytokine effects were observed within the central amygdala. Moreover, as shown in Figure 1.4, among mice pretreated with TNF- α 7 days earlier, at 1,3 and 8 h following

Figure 1.2 Immunoreactivity for AVP (top) and CRH (bottom) within the external zone of the median eminence (EZ) of mice treated with TNF- α (4.0 μ g) 7 days earlier. Both peptides were imaged within the same double-labeled section, using Texas Red and FITC fluorophores as in previous figure. Arrows denote examples of cells co-reactive for both peptides (magnification x 100). In fact, the majority of AVP and CRH EZ terminals were double labeled for both neuropeptides. $n = 5$ / group

reexposure to the cytokine the number of PVN and supraoptic Fos immunoreactive cells was increased relative to vehicle pretreated mice that subsequently received the cytokine. Within the central amygdala, such a sensitization effect was only evident at 3 h following the mTNF- α reexposure treatment. In neither case, did the increased *c-fos*-ir persist for the 24 h interval, suggesting that a transient sensitization was engendered upon TNF- α reexposure after the 7 day interval. .

Insert Figures 1.3 and 1.4 about here

In the experiment where mice were reexposed to 1.0 μ g of TNF- α 14 d following an initial 4.0 μ g cytokine dose, the increase of *c-fos*-ir within the PVN, supraoptic nucleus and central amygdala was greater than among mice that had not previously been treated (Figure 1.5). A significant Initial treatment x Reexposure treatment interaction was apparent for the number of immunoreactive Fos cells within these regions, F's (1,8) = 16.93, 13.19, 6.56; P's < 0.05, respectively. Multiple comparisons indicated that 3-h following acute TNF- α administration a small, but significant increase of *c-fos*-ir was evident in the PVN, supraoptic nucleus and central amygdala ($p < 0.05$). However, as shown in Figure 1.5, relative to animals that had received only a single exposure to the cytokine, reexposure to TNF- α 14 days after pretreatment with the cytokine, increased the number of Fos positive cells in all three of these brain regions. This sensitization was most pronounced at the PVN and was more robust than that observed in the

Figure 1.3. Immunoreactivity for CRH within the central nucleus of the amygdala. The panel at the left shows the immunoreactivity in the central amygdala (arrow) at a 10 x magnification. A. CRH-ir in mice treated with saline on two occasion 14 days apart: B. CRH-ir in mice that received saline followed 14 days later by TNF- α (1.0 μ g): C. CRH-ir in mice that received TNF- α (4.0 μ g) followed 7 days later by saline: D. CRH-ir in mice that received TNF- α (4.0 μ g) followed 7 days later by a second lower-dose injection of TNF- α (1.0 μ g). It is clear that mice reexposed to TNF- α after initial pretreatment with the cytokine displayed enhanced CRH-ir (D), suggesting a sensitization of expression of the peptide in these amygdaloid fibers. Magnification x 40. $n = 5$ / group

Figure 1.4. Number of *c-fos* positive cells within the supraoptic nucleus (left), central amygdala (middle) and PVN (right) in response to TNF- α treatments. Mice were initially treated with either TNF- α (4.0 μ g: hatched bars) or vehicle (grey bars), and then reexposed a lower dose of the cytokine (1.0 μ g) 7 days later. Mice were perfused and brain tissue collected 1, 3, 8 or 24 hr after the second injection. A sensitization of *c-fos* immunoreactivity was evident in each of these brain regions in mice perfused 3 hr following the reexposure challenge. * $p < 0.05$ relative to mice initially treated with saline. $n = 3$ / group.

7-day reexposure experiment. Interestingly, double labeling revealed that PVN *c-fos* was approximately 90 % co-localized with AVP-ir neurons (Figure 1.6).

Insert Figures 1.5 and 1.6 about here

Discussion

Median eminence co-localization of arginine vasopressin and corticotropin releasing hormone

Over time following acute stressor or IL-1 β treatment, phenotypic changes occur within CRH neurons terminating in the external zone of the median eminence, such that AVP and CRH expression increases (Schmidt et al., 1995). In the present investigation, a single dose of TNF- α likewise provoked a time-dependent shift towards increased AVP/CRH co-immunoreactive terminals within this site, largely attributable to increased AVP within CRH terminals. This effect peaked 7-14 days following cytokine treatment, but normalized after 28 days. Given that TNF- α was previously shown to sensitize plasma corticosterone responses in mice reexposed to the cytokine 28 days after pretreatment, but not after earlier intervals (Hayley et al., 1999), it appears likely that the augmented median eminence AVP/CRH-ir does not play a primary role in this HPA sensitization effect. The possibility ought to be considered that the sensitization with

Figure 1.5. Number of *c-fos* positive cells within the supraoptic nucleus (top), central amygdala (middle), and the PVN (bottom) in response to TNF- α treatments. Mice were initially treated with either TNF- α (4.0 μ g) or vehicle (solid and hatched, respectively), and then 14 days later reexposed to either vehicle or a lower dose of TNF- α (1.0 μ g). Again, a pronounced sensitization of *c-fos* immunoreactivity was observed in these brain regions. * $p < 0.05$ relative to mice initially treated with vehicle. ^o $P < 0.05$ relative to mice that received vehicle and then treated with TNF at the reexposure session. $n = 3$ / group.

Figure 1.6. *c-fos*-ir (A) and AVP-ir (B) within the PVN. The figure depicts a section from an animal treated with TNF- α (4.0 μ g) and reexposed to a 1.0 μ g dose of the cytokine 14 days later. At this time the coexpression of *c-fos*-ir and AVP-ir was greatly increased relative to that of acutely treated mice. The sensitization with respect to *c-fos*-ir was predominantly localized within AVP neurons of the PVN. $n = 3$ / group.

Reexposure TNF- α Treatment

Vehicle

TNF- α (1.0 μ g)

respect to corticosterone involved direct glandular actions, given that TNF- α may directly influence the pituitary (Kobayashi et al., 1997) or the adrenal (Ritchie et al., 1996). Such a proposition is in accord with the recent report that IL-6 stimulated ACTH secretion independent of hypothalamic CRH contribution (Bethin et al., 2000). Alternatively, as reexposure to the cytokine may induce sensitization of sickness behavior (Hayley et al., 1999), it is possible that the elevated corticosterone was secondary to the stress associated with illness or related factors (e.g. activation of acute phase proteins, histamine), rather than effects directly attributable to altered AVP/CRH expression.

The increased median eminence AVP/CRH accumulation 7-14 days after TNF- α may stem from enhanced PVN neuropeptide synthesis. Indeed, at the 14 day interval, TNF- α provoked augmented PVN *c-fos*-ir, which occurred largely within AVP positive neurons. Although these AVP-ir neurons are presumed to be predominately magnocellular, the possibility exists that some of these cells may co-express other peptides, including CRH. Moreover, parvocellular CRH and magnocellular AVP neurons in mice do not display well-defined patterns of distribution as observed in rats (Dijkstra et al., 1998). Thus, although the external zone of the median eminence largely comprises terminals originating from parvocellular PVN, some of the *c-fos* responsive PVN AVP-ir neurons observed in the present investigation may project to this region, accounting for the concomitant increase of AVP together with CRH staining observed. It is also possible that a leaky blood brain barrier at the median eminence makes this region a target for circulating TNF- α , hence potentially leading to direct actions of TNF- α on these terminals. Indeed, in the case of IL-1 β , direct infusion of the cytokine into the median

eminence increased local release of both AVP and CRH (McCoy et al., 1994; Watanobe and Takebe, 1992).

The internal band of the median eminence comprises AVP-ir fibers of passage, originating from magnocellular AVP synthesizing neurons within the supraoptic nucleus and PVN (Holmes et al., 1986; Whitnall and Gainer, 1988). These vasopressinergic fibers terminate at the posterior lobe of the pituitary, from which the peptide is released directly into circulation where it has osmoregulatory functions (Szczepanska-Sadowska, 1996). The AVP-ir within the internal band was reduced at 14 and 28-days following TNF- α administration, an effect that was still more pronounced upon reexposure to the cytokine. The possibility exists that over protracted periods TNF- α may influence AVP transport to the posterior pituitary, hence affecting water balance and blood flow. In fact, upon TNF- α reexposure 28 days following initial cytokine treatment, mice appeared to be hypovolemic and exhibited marked signs of illness, including cyanosis of the extremities (Anisman et al., 2000; Hayley et al., 1999, 2001a). These sickness symptoms are consistent with disturbed blood flow and/or pressure associated with systemic shock (Hardaway, 2000; Kettelhut et al., 1987).

The sensitization effects of TNF- α were not restricted to brain processes, as we recently observed that the time-dependent AVP/CRH alterations were paralleled by elevations of immunoreactivity for *c-fos*, thymidylate synthase, IL-1 α and heme oxygenase-1 within the ileum and colon of mice reexposed to TNF- α (Paire et al., 2001). Although speculative, the temporal correspondence between the gut and the brain AVP/CRH-ir variations may be related to activation of peripheral vagal afferents, given

that visceral projections to brain stem nuclei influence hypothalamic neurotransmission (Laye et al., 1995; Maier et al., 1998).

c-fos activation following tumor necrosis factor- α : acute and reexposure effects

Consistent with the enhanced central *c-fos* mRNA expression elicited by intravenous TNF- α (Nadeau and Rivest, 1999), intraperitoneal administration of this cytokine had an activating effect on several stressor reactive brain regions, including the PVN, supraoptic nucleus and central amygdala. While acute TNF- α had limited effects on immunoreactivity for the Fos protein, upon cytokine reexposure 7 or 14 days after initial TNF- α pretreatment, *c-fos-ir* was markedly increased in each of these regions. The increased immunoreactivity for the immediate early gene protein, Fos, may represent altered intracellular calcium, cAMP or other cellular variations associated with transcriptional events (Bravo et al., 1987; Sheng et al., 1990). Thus, the elevation of *c-fos-ir* elicited by TNF- α reexposure may reflect intracellular changes primed by the initial cytokine administration. Indeed, TNF- α altered synaptic transmission and modulated Ca²⁺ currents in hippocampal and sympathetic neurons (Soliven and Albert, 1992; Tancredi et al., 1992).

Administration of IL-1 β was previously shown to induce the expression of Fos-ir within parvocellular (Rivest and Rivier, 1994), as well as magnocellular PVN neurons (Chang et al., 1993). Recently, icv administered IL-1 β was also reported to induce the transcription factor, nuclear factor kappa B, within the basolateral amygdala as well as several vascular associated sites, while *c-fos* immunoreactivity was elevated within the PVN, supraoptic nucleus and central amygdala (Konsman et al., 2000). It will be noted

that icv IL-1 β provoked a pattern of *c-fos* upregulation indistinguishable from that observed after ip TNF- α treatment in the present investigation. Interestingly, Konsman et al., (2000) suggested that IL-1 β diffused through the brain parenchyma to reach these activated nuclei through a process termed "volume transmission". It is conceivable that peripherally administered TNF- α likewise activates a central diffusible factor (including IL-1 β itself) that ultimately comes to provoke the Fos changes.

Double labeling revealed that TNF- α increased *c-fos* reactivity within approximately 90% of the AVP immunopositive neurons of the PVN, provided that mice were reexposed to the cytokine (in this instance 14 days after initial TNF- α administration). Similar to the distribution of PVN CRH- and AVP-ir neurons reported for mice (Dijkstra et al., 1998), the double labeled Fos/AVP-ir neurons in the current investigation were scattered throughout the rostrocaudal and mediolateral extent of the PVN. However, neither AVP- nor *c-fos*-ir was detected in appreciable amounts within the perivascular region of the PVN and the strongest labeling tended to be localized at lateral aspects of the PVN. Systemic LPS, which increases central and peripheral cytokine activity (Hadid et al., 1999), has likewise been reported to activate *c-fos* immunoreactivity within magnocellular AVP neurons of the PVN and supraoptic nucleus, as well as parvocellular AVP neurons within the PVN, albeit to a lesser degree (Matsunaga et al., 2000).

It will be recognized that sensitization of neuronal activation within the PVN, supraoptic nucleus and central amygdala required reexposure to the cytokine, which is distinct from the time-dependent changes of CRH/AVP co-localization at the external zone of the median eminence (the latter being independent of the reexposure condition).

In effect, the sustained *c-fos*-ir following initial TNF- α treatment may reflect plasticity of neuroendocrine regulatory AVP-ir neurons. Of course, Fos protein may also have been colocalized with PVN CRH neurons, but since colchicine was not used in the present investigation to block fast axonal transport (owing to the stressor-like effects and induction of Fos-like immunoreactivity attributable to this synthesis inhibitor) (Ceccatelli et al., 1989), CRH-ir neurons were difficult to detect.

Corticotropin releasing hormone varicosities and c-fos-ir within the central amygdala

The dense plexus of CRH-ir terminals in the amygdala may originate from cell bodies within the lateral hypothalamus, dorsal raphe or intrinsic amygdaloid neurons (Gray, 1993). The route through which TNF- α increased CRH-ir varicosities within the central amygdala is unclear. The limited amount of TNF- α likely to penetrate the brain parenchyma (not discounting *de novo* central TNF- α synthesis) makes conclusions concerning direct ligand-receptor interactions highly speculative. Although the phenotype of the *c-fos*-ir amygdala cells have not been determined, Fos protein has been implicated in the regulation of CRH production and release (Chastrette et al., 1991). However, as *c-fos*-ir peaked 14 days after TNF- α , whereas CRH-ir peaked at 7 days, the *c-fos*-ir likely involves factors other than, or in addition to CRH.

The processes associated with the median eminence and amygdala CRH variations were distinguishable from one another. In particular, as already indicated, the CRH/AVP changes at the median eminence evolved simply with the passage of time following cytokine treatment, and were independent of the reexposure conditions. In contrast, changes of CRH-ir at the central amygdala were only evident upon reexposure

to the cytokine, and were maximal 1-7 days following initial treatment. In effect, the potential for increased CRH-ir within the central amygdala could be elicited by TNF- α , but an actual increase of immunoreactivity was only realized upon reexposure to the cytokine.

The sensitizing effects of TNF- α on amygdaloid CRH-ir and *c-fos*-ir may reflect the engagement of biological cascades, involving numerous chemical messengers, essential for dealing with the insult over protracted intervals. Activation of CRH within the amygdala as well as the anatomically closely related bed nucleus of the stria terminalis (BNST), has been proposed to play an important role in behavioral responses to stressful or fear-related stimuli (Koob, 1999; Lee and Davis, 1997). These behavioral effects appear to be independent of HPA activation, but may involve interactions between CRH and noradrenergic projections between the amygdala and BNST as well as the locus coeruleus (Koob, 1999; Van Bockstaele et al., 1998). Indeed, tracing studies demonstrated that the central amygdala sends CRH projections to tyrosine hydroxylase immunoreactive dendrites within the locus coeruleus (Van Bockstaele et al., 1998), which may affect behavioral arousal in response to psychogenic or neurogenic stressors. There is reason to believe that TNF- α may influence central NE activity as well as that of CRH. Indeed, TNF- α was shown to alter pre-synaptic noradrenergic activity, and pharmacological manipulation of NE levels influenced central expression of TNF- α (Ignatowski et al., 1997). Likewise, TNF- α was previously shown to provoke an increase of NE and MHPG levels within the locus coeruleus, while the cytokine sensitized MHPG accumulation within the central amygdala upon reexposure to TNF- α 1 day following its pretreatment (Hayley et al., 1999).

Finally, it is important to note that the cellular actions of TNF- α may be mediated by two distinct receptor subtypes, p55 and p75. The present investigation involved the recombinant human form of TNF- α , which selectively binds to the p55 receptor in the mouse (Beutler, 1999). These p55 and p75 receptors may subserve different effector functions, and "cross-talk" between these receptors may result in either synergistic or antagonistic interactions (Peschon et al., 1998; Tartaglia et al., 1993). Thus, the possibility exists that some of the TNF- α induced peptide and Fos variations may result from lack of activation of the p75 receptor, rather than stimulation of the p55 receptor per se. However, this possibility is unlikely, at least for the *c-fos* variations, given that expression of the immediate early gene is taken to reflect cellular excitation (Morgan and Curran, 1991), rather than inhibitory processes and that Fos levels were virtually negligible in saline injected mice in the present investigation. As well, previous studies revealed that the both the murine and human forms of TNF- α provoked virtually identical acute and sensitization effects with respect to sickness behavior and plasma corticosterone activity (Hayley et al., 1999, 2002a).

Conclusions

Taken together, neuroplasticity of AVP and CRH neuroendocrine systems may be encouraged in response to TNF- α treatment. Indeed, it appears that TNF- α may sensitize neuronal responses, as reflected by Fos induction, in brain regions important for neuroendocrine regulation after exposure to a variety of immunological and non-immunological stressors. These protracted consequences of TNF- α may have important implications for psychopathology given that cytokines and their soluble receptors may be

elevated in depressive illness (Maes, 1999), and particularly in chronic forms of depression (Griffiths et al., 2000). Thus, the possibility ought to be considered that systemic cytokine alterations may provoke phenotypic changes of central CRH and AVP processes, which contribute to the provocation of affective disturbances (Tilders et al., 1999). In effect, TNF- α may act as a neuromodulator within the brain and may have long-term effects on central biochemical activity important for functional responses to future challenges.

Chapter II

Central Monoamine and Plasma Corticosterone Changes Induced by a Bacterial Endotoxin: Sensitization and Cross-sensitization Effects.

Abstract

Low doses of lipopolysaccharide, tumor necrosis factor- α , interleukin-1 β , or exposure to a stressor (restraint) increased plasma corticosterone levels. In animals pretreated with lipopolysaccharide a marked sensitization of the corticosterone response was evident upon subsequent exposure to lipopolysaccharide, tumor necrosis factor- α , and restraint, one day later. As well, the sickness-inducing effects of lipopolysaccharide, tumor necrosis factor- α , and interleukin-1 β were markedly increased in mice pretreated with lipopolysaccharide. The sensitization effects were marked when the second treatment was administered one day after lipopolysaccharide administration, but not when a 28 day interval elapsed. In a second experiment, tumor necrosis factor- α influenced monoamine functioning in the paraventricular nucleus of the hypothalamus and within extrahypothalamic regions, including the central amygdala, locus coeruleus, prefrontal cortex. Moreover, serotonin activity within the central amygdala, as well as dopamine activity within the prefrontal cortex, were subject to a sensitization effect in animals pretreated with lipopolysaccharide one day earlier. Macrophage depletion by a suspension of clodronate liposomes attenuated the plasma corticosterone changes induced by acute tumor necrosis factor- α , but did not affect the sensitization. In contrast, the acute effects of tumor necrosis factor- α on central neurotransmitters were unaffected by the liposome suspension, but this treatment prevented the sensitization. These data may be relevant to clinical situations in which individuals exposed to bacterial infections may be rendered more susceptible to the behavioral and neurochemical effects of subsequently encountered stressors and immunological challenges.

Introduction

Stressors and immunological stimuli may prime biological systems, such that augmented neurochemical or behavioral responses are elicited upon later exposure to similar or dissimilar challenges (sensitization and cross-sensitization, respectively). While chronic stressor treatments provoke a progressively greater sensitization, a single exposure to the insult may also promote such an outcome (Anisman et al., 1993; Tilders & Schmidt, 1998). Like neurogenic stressors, the proinflammatory cytokine, interleukin- 1β (IL- 1β), increased the co-localization of arginine vasopressin (AVP) and corticotropin releasing hormone (CRH) within CRH terminals of the median eminence, leading to increased corticosterone release upon subsequent challenges (Schmidt et al., 1995). Interestingly, this outcome only became apparent after 4 days and peaked 1-2 weeks following the initial cytokine challenge (Schmidt et al., 1995). Similarly, the macrophage derived cytokine, tumor necrosis factor- α (TNF- α), induced a progressive sensitization of sickness behavior (e.g. ptosis, curled body posture, anorexia), plasma corticosterone and norepinephrine (NE) utilization within the paraventricular nucleus of the hypothalamus (PVN), which was maximal 28 days following initial treatment. However, the sensitization of NE and 5-HT utilization within the prefrontal cortex and central amygdala occurred after a 1-day interval (Hayley et al., 1999).

The gram negative bacterial endotoxin, lipopolysaccharide (LPS), ordinarily provokes sickness, elevated circulating glucocorticoids and central monoamine activity (Bluthe et al., 1994; Dunn, 1992; Lacosta et al., 1998; Kent et al., 1996), likely mediated by IL- 1β and TNF- α (Hadid et al., 1999; Linthorst & Reul, 1998; Ebisui et al.,

1994). While chronic endotoxin treatment may result in diminished effects (Porter et al., 1998; Hadid et al., 1996), limited attention has been devoted to the protracted consequences of acute LPS administration. In the main, acute LPS induces tolerance to the lethal effects of subsequently administered endotoxin (West et al., 1997); however, sensitization effects have also been reported (Hereman et al., 1990) depending on the dose, route of administration, and interval between the two injections. The sensitizing effects of LPS, as well as viral and bacterial infections, may be mediated by the individual or synergistic actions of cytokines, including IL-12, interferon- γ (IFN- γ) and TNF- α (Berg et al., 1995; Brouckaert et al., 1995; Cauwels et al., 1995; Nansen et al., 1997). Further, bacterial infection may result in a cross-sensitization, wherein the behavioral and physiological effects of subsequently encountered stressors, including nitric oxide activity within the PVN (which may have regulatory actions on AVP and CRH) are augmented (Yang et al., 1999).

In light of the time-dependent neurochemical effects of TNF- α and IL-1 β , coupled with the finding that LPS provokes release of TNF- α and IL-1 β from macrophages, it was of interest to establish whether a cross-sensitization was evident between the endotoxin and these cytokines with respect to sickness behaviors and circulating corticosterone levels. Moreover, given that TNF- α has been shown to sensitize monoamine activity within brain regions ordinarily affected by stressors (PVN, PFC and central amygdala), it was of interest to determine whether LPS would augment monoamine utilization within these regions upon subsequent TNF- α exposure.

Plasma corticosterone levels and sickness behaviors were assessed in LPS treated animals that were subsequently challenged with low doses of either LPS, TNF- α or IL-

1 β , or were exposed to a mild stressor. As well, we determined whether TNF- α elicited central NE, 5-HT, and dopamine (DA) activity would be augmented in LPS pretreated mice. Finally, involvement of endogenous macrophages (producers of IL-1 β , IL-6 and TNF- α) in the cross-sensitization was assessed evaluating neurochemical alterations following macrophage depletion induced by a liposome encapsulated clodronate suspension (Biewenga et al., 1995).

Materials and methods

Subjects

Male, CD-1 mice, obtained from Charles River Canada Inc. (Laprairie, Quebec) at approximately 7 weeks of age, were allowed 4 weeks to acclimatize to the laboratory before serving as subjects. Mice were housed in groups of 4 in standard (27 x 21 x 14cm) polypropylene cages, and were maintained on a 12 hour light-dark cycle (light 0700-1900 h) with unrestricted access to food and water. The study received ethical approval from the Carleton University Animal Care Committee and the experimental test paradigms met the guidelines set forth by the Canadian Council on Animal Care.

Procedure

Time-dependent Sensitization associated with LPS: Challenge with LPS, TNF- α , IL-1 β , restraint

In order to minimize variability attributable to diurnal variations, testing was conducted between 0800 and 1200 h. In the initial experiment, half of the mice received

intraperitoneal (i.p.) treatment with sterile, non-pyrogenic, physiological saline, while the remaining mice received 5.0 μg of lipopolysaccharide (LPS) (Sigma L-3755 from *Escherichia Coli* Serotype 026:B6) in a volume of 0.4 ml. Mice were tested at one of two times afterward; 1 or 28 days following initial treatment. At these times, mice of each group received i.p. administration of either saline, a low dose of LPS (0.125 μg) or recombinant human TNF- α (1.0 μg ; obtained from R & D Systems, 1.1. $\times 10^5$ U/ μg). After the second injection, sickness behaviors were rated at 15-minute intervals for a 1 h period using a 4-point scale (see below). Mice were then rapidly decapitated and trunk blood collected for plasma corticosterone determinations. The time of decapitation was based on earlier studies showing that both the LPS and TNF- α effects on neuroendocrine activity were apparent at this time (Borowski et al., 1998; Brebner et al., 2000).

Two subsequent experiments assessed the effects of LPS on the later response to systemic IL-1 β and restraint stress treatment, respectively. As the initial study had indicated that the sensitization effects following initial LPS treatment were only evident when the challenge was applied 1 day later, only this time point was assessed in the latter two experiments. In the first of these studies, CD-1 mice were given an i.p. injection of either saline or LPS (5.0 μg) and returned to their home cages. These groups were subdivided and 24 h later given a second injection of either a low dose of IL-1 β (0.05 μg) or saline ($n = 10/\text{group}$). Following the second injection mice were returned to their home cages, and sickness behavior was recorded (see later) over a 1 h period, after which blood was collected as described earlier. The IL-1 β , kindly supplied by Dr. Craig Reynolds (Biological Response Modifiers Program, National Cancer Institute), had a specific

reactivity of 1.9×10^6 U/50 μ l, protein concentration of 2.1 mg/ml, and contained endotoxin of less than 2.5 EU/mg protein.

In the next study mice were again randomly assigned to one of four treatment mice received either 5 μ g of LPS or saline, and then one day later these animals were further subdivided such that half received an additional challenge in the form of a brief, 10-minute restraint stressor, while the remaining mice were left undisturbed in their home cages ($n = 10$ /group). Restraint involved placing the mouse in an acrylic tube (inside diameter of 4.5 cm, and an overall adjustable length of 4.5 cm to 5.7 cm), securing the barriers such that they provided a snug fit, and the mouse's tail which protruded from the tube was taped down. Consequently lateral, as well as forward movement of the animal, was prevented, and only limited paw and head movement was possible. Five minutes following the stressor treatment (15 minutes following the beginning of the restraint procedure) the mice were decapitated and trunk blood and brains were collected. Earlier studies had indicated that the peak corticosterone response was apparent at this time following the commencement of the stressor treatment.

A final experiment ($n = 10$ /group) assessed the effects of macrophage depletion on the neuroendocrine and monoamine effects of TNF- α , as well as the sensitization effect elicited by LPS pretreatment in animals later exposed to TNF- α . As well, in this experiment the levels of NE, DA and 5-HT, and their respective metabolites MHPG, DOPAC and 5-HIAA, were assessed in response to the LPS and cytokine treatments. As the sensitization of the central neurochemical effects elicited by TNF- α administration (in mice that had been pretreated with TNF- α) were apparent 1 day following initial treatment (Hayley et al., 1999), this time frame was also used in the

present investigation. Mice were pretreated with saline or LPS (5.0 μ g, i.p.) and 1 day later exposed to either saline or TNF- α (1.0 μ g). Half the animals in each condition had previously been treated with the macrophage-depleting agent, liposome encapsulated clodronate (0.2 ml, i.p.), while the control animals received saline. These treatments were administered on two occasions, 3 days apart, and the LPS (or saline) treatment was administered 1 day afterward. Previous studies revealed this protocol to produce the maximal degree of macrophage depletion using the intraperitoneal route of administration (Biewenga et al., 1995).

As in the preceding studies, animals were decapitated 1-h following the final treatment and trunk blood and brains collected for corticosterone and monoamine determinations. Verification of the macrophage depletion was determined by histological inspection of macrophage staining within the liver and spleen using a rabbit anti-mouse macrophage antibody (1:20; Accurate Chemical & Scientific Corporation, NY).

Preparation of Liposome suspensions

Multilamellar liposomes were prepared as previously described (Van Rooijen et al., 1989). Briefly, 86 mg of phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany) and 8 mg of cholesterol (Sigma Chemical Co., St Louis, MO) were dissolved in 10 ml chloroform and dried at 27 °C using low vacuum rotary evaporation. Addition of 10 ml of PBS containing 2.5 g Cl₂MDP, also referred to as clodronate (a generous gift from Roche Diagnostics GmbH, Mannheim, Germany), was followed by shaking, sonication and swelling of the liposomes. Centrifugation at 10,000 x g was followed by three washings to remove non-encapsulated clodronate. The liposomes were resuspended

in 4 ml PBS to give a final solution containing 10 mg/ml Cl_2MDP . As described above, mice were injected intraperitoneally with two 0.2 ml treatments of this suspension spaced three days apart.

The clodronate and the liposomes (composed of phosphatidylcholine and cholesterol) themselves are not toxic. The liposomes (containing the clodronate) are readily ingested by macrophages during which time macrophage phospholipases disrupt the liposomal phospholipid bilayers resulting in release of clodronate. Intracellular accumulation of clodronate within the macrophage proceeds until a critical threshold concentration is reached, whereupon the cell is irreversibly damaged and dies by apoptosis.

Behavioral Analysis (Sickness Rating)

Commencing 15 minutes after the final injection and at three successive 15 minute intervals thereafter, the overall appearance of animals, as an index of sickness behaviors, were rated, for a ten second period, on a four point scale (1 = comparable to untreated animals exhibiting locomotor activity, exploration and/or social interaction; 2 = slightly lethargy, particularly with respect to diminished motor activity, rearing and exploration; 3 = lethargy coupled with ptosis and/or piloerection, and 4 = pronounced lethargy, curled body posture, labored breathing, and general nonresponsiveness). Earlier studies had indicated better than 90% agreement between raters blind as to the treatments animals received (Hayley et al., 1999).

Plasma Corticosterone Assay

Trunk blood was collected in tubes containing 10 μ l EDTA, centrifuged, and plasma was stored at -80°C for later corticosterone determination. Plasma corticosterone levels were assayed, in duplicate, by commercial RIA kits (ICN Biomedicals, California, USA). The intra-assay variability was less than 10%, and interassay variability was avoided by assaying all samples within a single run.

Brain Dissection Technique

One hour following the final injection, mice were decapitated, trunk blood collected, and brains removed and flash-frozen in isopentane placed on dry ice. Brains were sectioned into a series of coronal slices using a stainless steel dissecting block with adjacent slots spaced approximately 0.5 mm apart. The paraventricular nucleus of the hypothalamus (PVN), locus coeruleus and central amygdala were obtained by micropunch using a hollow 16 gauge microdissection needle with a beveled tip. The medial prefrontal cortex (PFC) and dorsal hippocampus were dissected out in their entirety using razor blades. Brain punches were taken according to the mouse brain atlas of Franklin and Paxinos, 1997. The tissue was stored at -80°C until determination of monoamine and metabolite concentrations using HPLC.

HPLC procedure for analysis of brain amine and metabolite levels

Levels of DA, NE and 5-HT, and their respective metabolites, MHPG, DOPAC and 5-HIAA were determined by HPLC using a modification of the method of Seegal et al. (1986). Tissue punches were sonicated in a homogenizing solution that was comprised

of 14.17 g monochloroacetic acid, 0.0186g disodium ethylenediamine tetraacetate (EDTA), 5.0 ml methanol and 500 ml H₂O. Following centrifugation, the supernatants were used for the HPLC analysis. Using a waters M-6000 pump, guard column, radial compression column (5m, C18 reverse phase, 8mm x 10cm), and a three cell coulometric electrochemical detector (ESA Model 5100,A) 20 ml of the supernatant was passed through the system at a flow rate of 1.5 ml/min (1400-1600 PSI). The mobile phase used for the separation was a modification of that used by Chiueh et al. (1983) each liter consisted of 1.3g of heptane sulfonic acid, 0.1g disodium EDTA, 6.5ml triethylamine, 35ml acetonitrile. The mobile phase was then filtered (0.22mm filter paper) and degassed following which the pH was adjusted to 2.5 with phosphoric acid. The area and height of the peaks was determined using a Hewlett-Packard integrator. The protein content of each sample was determined using bicinchoninic acid with a protein analysis kit (Pierce Scientific, Brockville, Ontario) and a spectrophotometer (Brinkman, PC800 colorimeter).

Statistical Analysis

For the initial experiment, the neuroendocrine data were analyzed by a 2 (LPS vs saline pretreatment) x 3 (Reexposure to saline, LPS or TNF- α) x 2 (time of reexposure; 1 vs 28 days) three-factor ANOVA. The behavioral data were similarly analyzed except that a within-group measure was included (i.e., behavioral assessments over the four sampling periods). The two ensuing experiments were analyzed as 2 (initial treatment: LPS or saline) x 2 (saline vs IL-1 β , or restraint vs no treatment) factorials. In the final study the neuroendocrine and central monoamine data was analyzed as a 2 (liposome vs saline pretreated) x 2 (LPS vs saline pretreatment) x 2 (Reexposure to saline or TNF- α)

three-factor ANOVA. Significant interactions were followed by Tukey's Honestly Significant Difference (HSD) test ($\alpha = 0.05$) of the simple effects comprising the interaction. During the course of tissue dissection and HPLC analyses several samples were lost, and as a result the degrees of freedom for the statistical analyses varied across brain regions and/or neurochemical substrate.

Results

Time-dependent Sensitization of LPS and TNF- α : Behavioral and corticosterone alterations

The ANOVA indicated that the overall sickness profile of animals was determined by the initial injection mice received, the second injection administered, as well as the time of the second injection, F 's (1, 80) = 19.29, 3.63 and 8.97, P 's < 0.05. Moreover, the sickness profile became progressively more pronounced over the course of the 1 h session, $F(3, 240) = 10.34$, $P < 0.01$. The interaction between these variables also approached significance, $F(3, 240) = 1.89$, $P = .08$. As *a priori* predictions had been made concerning the temporal changes associated with the reexposure treatment, separate analyses were performed regarding sickness behavior at 1 and 28 days following treatment. As seen in Figure 2.1, at the 28 day reexposure time the overall level of illness was relatively low with most animal exhibiting no signs of illness. However, among animals that had initially received the LPS treatment, the degree of illness (albeit slight) was higher than among vehicle treated mice, $F(1, 38) = 4.02$, $P = 0.052$. In contrast, at the 1 day reexposure time, illness was more pronounced, and varied as a function of the Initial treatment x Reexposure treatment x Blocks of time interaction, $F(3, 126) = 2.64$, P

< 0.01 . The multiple comparisons of the simple effects comprising this interaction confirmed that among animals treated with saline, there was no effect of subsequent LPS or TNF- α treatment during the initial 3 sampling times, while a modest rise of sickness was produced by the LPS treatment during the fourth sampling period. In contrast to these results, in mice initially treated with LPS, later reexposure to a low dose of either LPS or TNF- α appreciably increased the overall sickness profile (see Figure 2.1). Moreover, these effects were apparent as early as 15 min following endotoxin or cytokine administration, and persisted over the course of the testing session.

Insert Figure 2.1 about here

Figure 2.1 depicts the plasma corticosterone levels among mice treated with saline or LPS and then at 1 or 28 days afterward given either saline, LPS or TNF- α . The analysis of variance revealed that plasma corticosterone concentrations varied as a function of the Initial injection x Reexposure treatment x Time of Treatment interaction, $F(2, 100) = 3.67$, $P < 0.01$. The multiple comparisons confirmed that in animals that were initially treated with saline and then subsequently exposed to either LPS or TNF- α (at 1 or 28 days) the concentrations of corticosterone increased relative to that of saline treated

Figure 2.1 Mean ($\pm$ SEM) ratings of sickness symptoms (e.g. ptosis, curled body posture, reduced locomotion and social interaction) as determined using a 4-point scale (top) and concentrations of plasma corticosterone (bottom) as a function of the LPS and TNF- α treatments. Mice were pretreated with saline (left bars) or LPS (5.0 μ g; right bars) and either 1 day (gray bars) or 28 days (hatched bars) later exposed to saline, LPS (0.125 μ g) or TNF- α (1.0 μ g). * $P < 0.05$ relative to mice treated with saline on two occasions, $\circ P < 0.05$ relative to animals receiving a single injection of LPS or TNF- α .

mice. If mice were initially treated with LPS and then reexposed to LPS or treated with TNF- α 28 days later, a rise of corticosterone was evident which was marginally smaller than that seen after acute endotoxin or cytokine treatment. Interestingly, if animals treated with LPS were exposed to either the LPS or TNF- α 1 day after initial treatment, then a pronounced increase of corticosterone was apparent significantly exceeding that of animals that had initially received saline and then treated with LPS or TNF- α on the latter occasion.

Cross –sensitization between LPS and IL-1 β : Behavioral and corticosterone alterations

In general, the overall appearance of mice varied as a function of the Initial injection x the Reexposure treatment x Blocks of time interaction, $F(3, 108) = 4.75$, $P < 0.01$. The multiple comparisons of the simple effects confirmed that among mice treated with saline subsequent administration of the low dose of IL-1 β was without effect. However, among those mice treated with LPS, later IL-1 β treatment provoked a marked increase of sickness relative to mice that were treated with saline on the second occasion or mice treated with vehicle and then exposed to IL-1 β on the test day. These effects were evident as soon as 15 minutes after treatment, peaked at the 30 minute time point, and were still evident 1 h after cytokine administration (see Table 2.1).

Insert Table 2.1 about here

Table 2.1 Ratings of sickness behaviors (mean $\pm$ SEM) over time after IL-1 β (0.05 μ g) injection among mice pretreated with Saline or LPS (5.0 μ g).

	<i>Time Following Treatment (min)</i>			
	15	30	45	60
Sal/Sal	1.0 $\pm$ 0	1.1 $\pm$ 0.1	1.3 $\pm$ 0.5	1.5 $\pm$ 0.7
Sal/IL-1 β	1.4 $\pm$ 0.6	1.4 $\pm$ 0.2	1.2 $\pm$ 0.3	1.0 $\pm$ 0
LPS/Sal	1.4 $\pm$ 0.2	1.4 $\pm$ 0.2	1.2 $\pm$ 0.1	1.2 $\pm$ 0.2
LPS/IL-1 β	2.1 $\pm$ 0.2*	2.7 $\pm$ 0.3*	2.6 $\pm$ 0.3*	2.5 $\pm$ 0.3*

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*p < 0.05 relative to Sal/IL-1 β treated mice (n = 8-10 animals per group)

Analysis of variance indicated that IL-1 β significantly increased plasma corticosterone levels, $F(1, 36) = 65.91$, $p < .01$, ($x \pm \text{SEM} = 19.44 \pm 1.08$, 9.07 ± 0.69 for IL-1 β and saline treated mice respectively). Although the corticosterone concentrations were slightly higher in response to the IL-1 β in the LPS pretreated mice ($x \pm \text{SEM} = 20.85 \pm 1.02$) relative to saline pretreated animals receiving the cytokine ($x \pm \text{SEM} = 18.02 \pm 1.89$), neither the main effect nor the interaction involving the LPS pretreatment reached significance.

Cross-sensitization between LPS and restraint

Plasma corticosterone concentrations varied as a function of the interaction between Initial Pretreatment x Stressor Reexposure $F(1,33) = 9.39$, $p < 0.05$. The multiple comparisons revealed that restraint increased plasma corticosterone levels relative to non-treated mice ($x \pm \text{SEM} = 19.84 \pm 1.45$, 3.14 ± 0.36 , respectively). Moreover, this effect was augmented in mice pretreated with LPS and subsequently exposed to the stressor ($x \pm \text{SEM} = 23.72 \pm 2.10$), such that levels of corticosterone exceeded those seen in saline pretreated animals exposed to the stressor ($x \pm \text{SEM} = 16.39 \pm 1.19$).

Macrophage involvement in LPS and TNF- α cross-sensitization: Plasma Corticosterone

As predicted, administration of the liposome-clodronate suspension induced a greater than 90% depletion of macrophages within the liver and spleen.

Plasma corticosterone levels varied as a function of the interaction between the Initial treatment (LPS or saline) and the subsequent Cytokine treatment (TNF- α or

saline), $F(1,65) = 12.51$; $P < 0.01$. As in the initial experiment, the multiple comparisons indicated that administration of $\text{TNF-}\alpha$ on test day increased corticosterone levels above those of animals that received saline on this occasion. However, among LPS pretreated animals subsequently exposed to $\text{TNF-}\alpha$ corticosterone levels were greater than those of saline pretreated mice subsequently injected with the cytokine, indicating a cross-sensitization effect. The interaction between macrophage depletion, LPS treatment and later $\text{TNF-}\alpha$ administration was not statistically significant. Yet, since specific predictions had been made concerning the actions of the liposome encapsulated clodronate treatment, multiple comparisons were conducted of the means comprising this interaction. It was indeed found that the macrophage-depleting compound significantly attenuated the corticosterone response observed in saline pretreated mice that subsequently received $\text{TNF-}\alpha$ on test day (i.e. acute $\text{TNF-}\alpha$ treatment). Interestingly, as seen in Figure 2.2, if mice received LPS and then were exposed to $\text{TNF-}\alpha$, macrophage depletion did not alter corticosterone levels. Thus, the influence of macrophage involvement in the corticoid stimulating properties of $\text{TNF-}\alpha$ may depend upon the animal's prior history of immunogenic challenges or upon the magnitude of the corticosterone response elicited.

Insert Figure 2.2 about here

Figure 2.2 Plasma corticosterone levels (mean $\pm$ SEM) among mice that were treated with saline or LPS, and then 1 day later exposed to saline or TNF- α (sal/sal, sal/TNF- α , LPS/sal, LPS/TNF- α). Mice had been pretreated with either a liposome encapsulated clodronate suspension (hatched bars) or saline (gray bars) at 1 and 3 days prior to LPS treatment. * $P < 0.05$ relative to saline only treated mice, $\circ P < 0.05$ relative to mice receiving saline pretreatment followed by acute TNF- α one hour prior to decapitation.

Central Monoamine Determinations

The effects of the liposome pretreatment varied across brain regions, and appeared to be dependent upon the prior treatments that mice received. Within the PVN, the concentration of NE varied as a function of the Liposome Treatment x Initial Pretreatment (LPS or saline) x Reexposure Treatment (TNF- α or saline) interaction, $F(1,63) = 6.86$; $p < 0.05$. As depicted in Figure 2.3 and confirmed by the multiple comparisons, among the saline pretreated animals that had not been exposed to the liposomes, administration of TNF- α on the test day increased NE levels relative to mice that received saline on this occasion. However, among mice that received the LPS treatment and then 1 day later given TNF- α , the elevated levels of NE were attenuated. Likewise, among mice that were pretreated with the liposome encapsulated clodronate the increase of NE elicited by TNF- α was prevented.

Analysis of the MHPG concentrations within the PVN revealed a significant Liposome Treatment x Reexposure Treatment interaction, $F(1,62) = 4.16$, $p < 0.05$. As shown in Figure 2.3 and confirmed by multiple comparisons, TNF- α provoked an increase of MHPG within the PVN. Pretreatment with the liposome encapsulated clodronate suspension reduced the increase of MHPG levels otherwise provoked by the cytokine. Although the interaction between the Liposome pretreatment x Initial LPS treatment x TNF- α reexposure was not significant, it is noteworthy that TNF- α elicited increase of MHPG was entirely eliminated by the liposome encapsulated clodronate suspension in mice that had not received the LPS treatment. Among mice that had

received both LPS and TNF- α , the elevated MHPG levels were still apparent, albeit reduced relative to that of mice that had not received the liposome treatment.

Insert Figure 2.3 about here

Within the locus coeruleus NE levels were not significantly altered by any of the treatments, whereas TNF- α administration 1 hr prior to decapitation, increased MHPG accumulation relative to saline treatment, $F(1,66) = 29.91$; $p < 0.01$ ($x \pm \text{SEM} = 5.58 \pm 0.23$, 3.87 ± 0.19 , respectively). Neither liposome nor LPS pretreatment further modified this effect, although the liposome treatment itself elicited a modest, but significant increase of MHPG accumulation relative to animals not treated with the macrophage depletor, $F(1,66) = 5.26$; $p < 0.05$ ($x \pm \text{SEM} = 5.13 \pm 0.27$, 4.34 ± 0.23 , respectively).

Central amygdala NE concentrations were not significantly affected by the liposome, LPS or TNF- α treatments. However, levels of MHPG were increased by TNF- α administered 1 h earlier, $F(1, 69) = 17.94$, $P < 0.01$ (see Figure 2.4), but this effect was not modified by liposome or LPS pretreatments. Unlike the changes of NE activity, neither DA nor DOPAC within the central amygdala were reliably influenced by the liposome, LPS or TNF- α treatments. Finally, the liposome treatment also increased levels of amygdaloid 5-HT relative to non-liposome pretreated mice, $F(1, 64) = 3.99$, $p < 0.05$ ($x \pm \text{SEM} = 29.50 \pm 3.62$, 23.21 ± 2.12 , respectively), and once again neither LPS nor the liposome pretreatment modified this effect. In contrast, 5-HIAA accumulation varied as a function of the interaction between Liposome Treatment x Initial Pretreatment (LPS or saline) x Reexposure Treatment (TNF- α or saline) reached significance, $F(1, 64) = 3.84$,

Figure 2.3 Mean (+SEM) concentrations of NE (top) and MHPG (bottom) within the PVN as a function of the liposome, LPS and TNF- α treatments. As in Figure 2, among half of the mice peripheral macrophages were depleted using systemic application of a liposome clodronate suspension (hatched bars) while the remaining animals received saline (gray bars). Following liposome treatments, mice were pretreated with either saline (left bars) or LPS (5.0 μ g) (right bars) and 1 day later exposed to saline or TNF- α (1.0 μ g). * $P < 0.05$ relative to mice receiving only saline injections.

$P = 0.05$. As shown in Figure 2.4 and confirmed by the multiple comparisons, among non-liposome challenged mice, $\text{TNF-}\alpha$ increased 5-HIAA in LPS pretreated animals relative to those that had received $\text{TNF-}\alpha$ but had not been pretreated with LPS (i.e., a cross-sensitization effect was induced). However, as depicted in Figure 2.4, among liposome preexposed mice, $\text{TNF-}\alpha$ did not significantly alter levels of the metabolite, and this was the case regardless of whether animals were pretreated with LPS or saline.

Treatment with $\text{TNF-}\alpha$ was found to appreciably influence monoamine activity within the PFC. While NE levels were not affected by the cytokine, the accumulation of MHPG, as shown in Figure 2.5, within this region was increased by $\text{TNF-}\alpha$ administration, $F(1, 69) = 18.00$, $P < 0.01$. Moreover, levels of 5-HIAA were elevated, $F(1, 69) = 9.24$, $P < 0.01$ (5-HIAA: $\bar{x} \pm \text{SEM} = 5.53 \pm 0.46$, 3.86 ± 0.28 , for $\text{TNF-}\alpha$ and saline test day treatments, respectively), in the absence of any variations of 5-HT. These effects were not influenced by either the LPS or the liposome pretreatments. In contrast to the MHPG and 5-HIAA changes, the accumulation of DOPAC varied as a function of the Liposome Treatment $\times$ $\text{TNF-}\alpha$ interaction, $F(1, 60) = 5.43$, $P < 0.05$, and the LPS $\times$ $\text{TNF-}\alpha$ interaction, $F(1, 60) = 4.10$, $P < 0.05$. The multiple comparisons confirmed that LPS sensitized DOPAC accumulation in that the metabolite levels among mice pretreated with LPS and then given $\text{TNF-}\alpha$ exceeded that of mice that received only one of these treatments (see Figure 2.5). Furthermore, the elevated levels of DOPAC in the mice that received $\text{TNF-}\alpha$ was precluded in mice that had been pretreated with the liposome.

Insert Figures 2.4 and 2.5 about here

Figure 2.4 Concentrations of 5-HIAA (top) and MHPG (bottom) within the central nucleus of the amygdala (mean $\pm$ SEM) among mice receiving the liposome, LPS and TNF- α treatments. Following liposome clondronate (hatched bars) or saline (gray bars) administration, animals received ip injection of LPS (right bars) or saline (left bars) followed 1 day later by exposure to TNF- α or saline. * $P < 0.05$ relative to saline only treated mice, $\circ P < 0.05$ relative to mice receiving saline pretreatment followed by acute TNF- α one hour prior to decapitation.

Figure 2.5 Mean ($\pm$ SEM) concentrations of DOPAC (top) and MHPG (bottom) within medial prefrontal cortex among mice receiving the liposome, LPS and TNF- α treatments. Following liposome clondronate (hatched bars) or saline (gray bars) administration, mice were treated with LPS (right bars) or saline (left bars) and 1 day later exposed to TNF- α or saline. * $P < 0.05$ relative to mice receiving saline as the final injection, $\circ P < 0.05$ relative to mice receiving saline pretreatment followed by acute TNF- α one hour prior to decapitation.

As shown in Table 2.2, administration of TNF- α provoked a modest, but significant, increase of NE concentrations within the dorsal hippocampus relative to animals treated with saline on the test day, $F(1,70) = 8.64$, $p < 0.05$. Likewise, as shown in Table 2.2, levels of MHPG were increased by TNF- α administered 1 h earlier, $F(1, 69) = 8.57$, $P < 0.01$. However, neither the NE nor MHPG variations were influenced by the liposome or LPS pretreatment 1 day earlier. Although TNF- α failed to influence hippocampal 5-HT, levels of 5-HIAA were increased in response to the cytokine $F(1,59) = 11.71$, $P < 0.01$ (see Table 2.2). Interestingly, administration of the liposome-clondronate suspension reduced 5-HT levels relative to mice not treated with the selective macrophage depleting compound, $F(1,60) = 9.98$, $p < 0.01$ ($\bar{x} \pm \text{SEM} = 3.05 \pm 0.38$, 4.44 ± 0.72 , respectively). Finally, no significant interactions were evident between the liposome, LPS and TNF- α treatments with respect to 5-HT activity.

Insert Table 2.2 about here

Discussion

Behavioral and Neuroendocrine effects

Repeated endotoxin treatment results in a desensitization with respect to hypophagia (Porter et al., 1998), satiety normally elicited by cholecystokinin (Cross-Mellor et al., 1999), myocardial infarct size following coronary artery occlusion (Eising

Table 2.2 Concentrations ($\mu\text{g}/\text{mg}$ protein) of hippocampal NE, MHPG, 5-HT and 5-HIAA (mean $\pm$ SEM) in TNF- α (1.0 μg) treated mice (collapsed over liposome and LPS treatment).

	NE	MHPG	5-HT	5-HIAA
Sal	5.71 $\pm$ 0.44	3.56 $\pm$ 0.19	3.87 $\pm$ 0.54	4.09 $\pm$ 0.31
TNF- α	6.80 $\pm$ 0.52*	4.11 $\pm$ 0.18*	3.61 $\pm$ 0.55	6.43 $\pm$ 0.45*

*P < 0.05 relative to mice receiving saline 1 h prior to decapitation, (n = 8-10 animals per group)

et al., 1996), febrile responses (Soszynski et al., 1998), and circulating TNF- α and IL-1 β (Nangano et al., 1999). Although it has been repeatedly observed that low doses of systemically administered LPS may protect against the effects of subsequently applied lethal doses of the endotoxin (Freudenberg & Galanos, 1988; Quagliarello et al., 1991), when LPS is initially injected into the footpad and then re-administered iv 24 h later, a sensitization of lethality is evident (generalized Shwartzman reaction) (Heremans et al., 1990), possibly mediated by cytokines such as IL-12 or IFN- γ (Heremans et al., 1990; Ozmen et al., 1994). Thus, the route of injection, chronicity of treatment, as well as the particular dose of LPS administered, interact to determine whether the endotoxin acts in a sensitizing or de-sensitizing fashion. In the present investigation, a sensitization effect was provoked by ip LPS, such that plasma corticosterone levels and the sickness response were augmented in animals subsequently treated with TNF- α . In particular, at low doses, TNF- α itself did not induce sickness (reflected by overall appearance, reduced locomotion, diminished social interactions, ptosis and piloerection), but, increased circulating corticosterone levels as previously reported (Hayley et al., 1999; Lenczowski et al., 1997; van der Meer et al., 1996). The behavioral effects were appreciably increased, and further elevations of plasma corticosterone were apparent, among TNF- α mice that had been pretreated with LPS.

While these data are reminiscent of those observed among mice pretreated with TNF- α and then reexposed to the same cytokine 28 days later (Hayley et al., 1999), the LPS-induced sensitization was only evident upon subsequent exposure to either LPS or TNF- α 1 day later. In view of the divergent temporal profiles associated with LPS and

TNF- α pretreatment, different processes likely subserve these sensitizing effects. Interestingly, however, LPS pretreatment also resulted in the sensitization of sickness behavior in response to later IL-1 β challenge, as well as the corticosterone response elicited by a restraint stressor. Given that the sensitization elicited by LPS was evident upon subsequent exposure to various insults, raises the possibility that the endotoxin elicited a generalized priming effect involving several central (e.g. hypothalamus) and/or peripheral (e.g. liver, gut) mechanisms. Studies of LPS and TNF- α lethality suggest that these may include acute phase proteins and other factors liberated from the liver (Libert et al., 1991; Wallach et al., 1988), complement proteins, as well as blood coagulation factors (Berczi, 1998). As well, the hypothalamic-pituitary-adrenal (HPA) activation and sickness behaviors elicited by LPS and TNF- α likely involve central mechanisms as they can be elicited by icv administration of these compounds (Dantzer et al., 1998; Johnson et al., 1997; Turnbull et al., 1997; Wan et al., 1993). Of course, sickness involves a constellation of behavior changes and the specific symptoms may involve diverse central mechanisms (Dantzer et al., 1998; Linthorst & Reul, 1998).

The sickness profile elicited by IL-1 β was appreciably enhanced in animals pre-exposed to LPS one day earlier, suggesting some common actions of LPS and the cytokine. While LPS stimulates IL-1 β release, and IL-1 β potently increases corticosterone levels (Dunn, 1988,1990; van der Meer et al., 1996) the endotoxin did not further modify plasma corticosterone responses to IL-1 β administered 1 day later. These data are consistent with the view that the hormone and sickness involve different mechanisms (Anisman et al., 2000; Brebner et al., 2000). The absence of a sensitization with respect to plasma corticosterone levels was not a result of a ceiling effect, as only a

modest increase of the hormone level was elicited by the particularly low dose of IL-1 β used (0.05 μ g). However, as only a single dose of IL-1 β was used and corticosterone was only sampled at a single post-treatment interval, a sensitization effect might have been detected at other doses or times following treatment. Yet, the possibility should not be dismissed that TNF- α plays a more prominent role than IL-1 β in the neuroendocrine sensitizing effects of the endotoxin, just as TNF- α may be the primary cytokine involved in endotoxic shock induced by LPS (Galanos & Freudenberg, 1993).

It has been reported that cerebrovascular insults (e.g., cerebral artery occlusion) enhanced TNF- α brain mRNA likely originating from microglia or infiltrating blood-borne macrophages (Gregersen et al., 2000). As well, TNF- α immunoreactive macrophages detected within the pituitary were upregulated in response to LPS challenge (Arras et al., 1996). While selective macrophage depletion by the liposome-clondronate suspension, blocked the corticosterone rise induced by acute TNF- α in the present investigation, this treatment did not attenuate the LPS-induced sensitization effect. Thus, it is possible that the effects of macrophage depletion were unique to the immediate actions of LPS or TNF- α , and mechanisms independent of macrophages, were responsible for the sensitization. Alternatively, the effectiveness of the liposome treatment may depend upon the magnitude of the corticosterone changes elicited by the challenge, being absent in response to treatments that ordinarily promote particularly marked hormonal increases. In fact, liposome-clondronate pretreatment attenuated the rise of ACTH induced by subpyrogenic, but not by pyrogenic LPS dosages (Derijk et al., 1991). Likewise, *in vivo* macrophage depletion failed to affect the corticosterone

elevation induced by the bacterial superantigen, staphylococcal enterotoxin B (Shurin et al., 1997).

Although centrally derived CRH mediates plasma ACTH increases elicited by IL-1 β (van der Meer et al., 1996; Watanobe & Takebe, 1992), the effects of the neuropeptide on TNF- α induced HPA activation are less clear (van der Meer et al., 1996). Moreover, these cytokines may affect central processes by stimulating peripheral mechanisms (e.g. macrophages and/or vagal afferents) or by direct actions at the brain. In the case of LPS, both icv and iv administration of the endotoxin increased HPA responses (Habu et al., 1998) and central endotoxin treatment also elevated brain as well as peripheral TNF- α levels (Kalehua et al., 2000). Moreover, while iv administration of the endotoxin increased hypothalamic IL-1 β levels (Ma et al., 2000), central application of IL-1 receptor antagonist (IL-1ra) attenuated plasma ACTH increases elicited by icv, but not the effect induced by iv LPS treatment (Habu et al., 1998). The finding that macrophage depletion limited the corticosterone rise elicited by acute TNF- α , implicates macrophage functioning in the HPA activation elicited by the cytokine. Although macrophages are primary producers of TNF- α under basal conditions, and production is increased in the face of many immunological and metabolic challenges (Chancellor-Freeland et al., 1995; Liu et al., 2000), scant data exist concerning the direct signal transducing actions of TNF- α on macrophages. Nevertheless, receptors for the cytokine have been demonstrated on macrophages and it was postulated that the cytokine may act in an autocrine fashion regulating its own production (Beutler, 1999; Welborn et al., 1993; Witsell et al., 1992).

Central Monoamine variations

In agreement with previous studies (Ando & Dunn, 1999; Hayley et al., 1999), central monoamine alterations were elicited by systemic TNF- α at doses below those used to elicit illness, making it unlikely that the monoamine variations were secondary to malaise. Moreover, the effects of the cytokine and endotoxin treatments were not restricted to hypothalamic monoamine variations, but also occurred at extrahypothalamic sites, including MHPG accumulation within the PVN, locus coeruleus, PFC, hippocampus and central amygdala, DOPAC within the PFC, and 5-HIAA in the PVN and hippocampus.

Although systemic LPS augments NE, DA and/or 5-HT activity at hypothalamic and extrahypothalamic sites (Lavicky & Dunn, 1995; Linthorst et al., 1995; Molina-Holgado & Guaza, 1996), few data are available concerning the central monoamine effects induced by central endotoxin application, and icv administered TNF- α provoked only modest effects on central monoamine functioning (Connor et al., 1998; Pauli et al., 1998). Thus, although considerable data support a central role for the behavioral and neurochemical effects of LPS and TNF- α , a peripheral site of action may also be important in this respect. Further to this point, although vagal afferents may be important for the modulation of HPA activity provoked by cytokines or endotoxin (Maier & Watkins, 1998), the function of vagal afferents in mediating LPS-elicited central monoamine effects remain obscure (MohanKumar et al., 2000). The finding that depletion of macrophages by liposome encapsulated clondronate limited monoamine utilization at the PVN suggests the involvement of peripheral macrophages (e.g., release of cytokines, nitric oxide or other proteins) in some of the central actions of the cytokine.

Yet, it ought to be underscored that cytokines, as well as LPS, increase blood brain barrier permeability (Banks et al., 1999; Quagliarello et al., 1991), and thus blood-borne macrophages infiltrating the CNS may be responsible for the monoamine alterations.

Unlike the effects within the PVN, the liposome clondronate suspension did not attenuate the TNF- α elicited increases of MHPG accumulation within the PFC, central amygdala, hippocampus and locus coeruleus, nor that of 5-HIAA within the PFC and hippocampus. It is unclear why the liposome treatment differentially influenced monoamine activity across brain regions. It may be the case that macrophage activity may be fundamental to the cytokine-induced monoamine variations within hypothalamic sites, possibly owing to its greater accessibility from peripheral circulation. Yet, elevated levels of cytokine immunoreactive macrophages have been detected in the meninges and ventricular spaces after LPS challenge (Garabedian et al., 2000), placing these cells in close proximity to cytokine receptor sites (Nadeau & Rivest, 1999). Activation of such receptors, or other blood brain barrier-associated cells, may induce brain cascades involving various messengers, such as prostaglandins, phospholipase A₂, and the TNF- α inducible transcription factor, nuclear factor kappa B, which may then influence central neurochemical activity. Accordingly, altered neurotransmitter activity involving blood-borne products might be expected at other sites.

In mice pretreated with LPS, subsequent TNF- α increased 5-HIAA accumulation within the central amygdala, and DOPAC within the PFC, even though acute TNF- α treatment was without effect in this respect. In other regions, including the locus coeruleus and PVN, where TNF- α increased either NE and/or 5-HT activity, there was no evidence of a sensitization effect. In contrast to the lack of effect of the liposomes on the

corticosterone sensitization, as well as the aforementioned acute effects on central monoamine activity, the macrophage-depleting agent attenuated the 5-HIAA and DOPA sensitization within the central amygdala and PFC, respectively. It will be recalled that in mice treated with TNF- α and then reexposed to the same cytokine, the corticosterone sensitization was absent one day after initial treatment, but became progressively greater with the passage of time (e.g., at 28 days effects are marked) (Hayley et al., 1999). However, the sensitizing effects of the cytokine on monoamine activity at extrahypothalamic sites did not follow such a time course, and in certain cases (e.g. PFC and central amygdala) were only evident at the one-day interval. Thus, the corticosterone effects and the increased monoamine activity within the central amygdala and PFC were independent of one another.

It is possible that the initial LPS treatment induced macrophage hyper-responsivity, such that subsequent challenges provoked more robust cellular responses. Indeed, LPS increased subsequent macrophage functions, including tumor cytotoxicity, bactericidal activity and cytokine production (West et al., 1997). Moreover, macrophage reprogramming has been demonstrated *in vitro*, wherein endotoxin reexposure 1 day following its pretreatment, increased IL-1 β and nitric oxide release, but reduced that of TNF- α (Fahmi et al., 1996; West et al., 1997). Similar changes of macrophage activity may be responsible for the sensitization of neurochemical variations observed in the present investigation. Of course, the possibility cannot be excluded that the apparent sensitizing actions stem from effects of LPS on blood brain barrier permeability or variations of neutrophil and macrophage infiltration into brain (Andersson et al., 1992; Minami et al., 1998). While these explanations are attractive, they do not explain why the

sensitization was not evident in PVN or other extrahypothalamic sites. Interestingly, though, it was previously observed that the sensitizing effects of TNF- α were also less pronounced in the PVN than in regions such as the PFC and central amygdala, and the PVN sensitization was only apparent 28 days after initial cytokine challenge (Hayley et al., 1999).

Conclusions

Sufficient behavioral and neurochemical plasticity is necessary for an animal to cope with environmental demands. In this respect, stressful events not only elicit acute responses, but may also invoke mechanisms that influence the organism's responses to later challenges. Indeed, exposure to a variety of stressors, including immunological stimuli, prime biological systems so that an augmented response is elicited by later exposure to the same or somewhat different challenges (Tilders & Schmidt, 1998). The present investigation demonstrated that pretreatment with the bacterial endotoxin, LPS, sensitized the neuroendocrine responses of mice such that the response to subsequent challenges (e.g., endotoxin, TNF- α , IL-1 or a restraint stressor) was enhanced. Likewise, a cross-sensitization between LPS treatment and subsequent TNF- α exposure was evident with respect to sickness behavior and central monoamine activity. The fact that, in certain cases, these effects were blocked by administration of liposome-clondronate indicates that macrophages play a role in some of the HPA and central monoamine alterations induced by LPS and TNF- α . Although the LPS induced corticosterone sensitization was evident after 1 but not 28 days, unlike the progressive late developing neuroendocrine sensitization profiles previously reported for TNF- α and IL-1 β (Hayley

et al., 1999; Schmidt et al., 1995) the time course for the endotoxin sensitized central monoamine responses were reminiscent of those demonstrated previously for TNF- α . Thus, although the LPS and TNF- α induced behavioral and neuroendocrine sensitization profiles may be subserved by fundamentally different mechanisms, the central monoamine sensitization effects provoked by these inflammatory stimuli may share common mediators. These data may have implications for disorders that may involve sustained HPA axis activation and protracted central monoamine disturbances (e.g. depression). Indeed, LPS may induce a depressive-like condition that is attenuated by antidepressant administration (Yirmiya, 1996), a treatment which itself influences brain TNF- α levels (Ignatowski et al., 1997). Finally, the present results may also be applicable to models evaluating the role of sensitization in septic shock and the neurochemical consequences that may be associated with such conditions.

Chapter III

Sensitization to the Neuroendocrine, Central Monoamine and Behavioral Effects of murine Tumor Necrosis Factor- α : Peripheral and central mechanisms.

Abstract

Systemic administration of murine tumor necrosis factor- α (mTNF- α) (0.1-2.0 μ g; ip) dose-dependently increased plasma corticosterone and augmented monoamine utilization within the paraventricular nucleus of the hypothalamus (PVN), locus coeruleus, medial prefrontal cortex (PFC), central and medial amygdala. A time-dependent sensitization was induced in mice, wherein reexposure to mTNF- α 28 days (but not at 1 day) following the initial cytokine treatment provoked marked signs of illness (diminished activity, ptosis, piloerection) and increased plasma corticosterone levels. Within several brain regions, serotonin (5-HT) activity was augmented upon mTNF- α reexposure at the 1 or 28 day intervals (PFC, medial amygdala, respectively). Intracerebroventricular (icv; 1-500 ng) mTNF- α did not promote illness, but modestly increased plasma corticosterone levels. Neither of these effects was subject to a sensitization upon icv cytokine reexposure. Acute icv mTNF- α increased norepinephrine (NE), 5-HT and dopamine (DA) activity within the PVN and median eminence/arcuate nucleus complex (ME/ARC), and NE utilization within the central amygdala. Subsequent icv mTNF- α further enhanced the hypothalamic monoamine variations. Finally, systemic (ip) mTNF- α pretreatment did not proactively influence sickness or corticosterone responses to later icv cytokine challenge, but augmented locus coeruleus NE activity and 5-HT and DA utilization within the ME/ARC. It is suggested that the differential effects of central and systemic mTNF- α on sickness and corticosterone activity reflects the involvement of peripheral mechanisms. Moreover, it appears that mTNF- α promotes central neurochemical plasticity through independent mechanisms.

Introduction

Systemic administration of proinflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) provoke a constellation of behavioral and physiological changes that may reflect sickness (reduced locomotion, feeding, social exploration, anhedonia and increased fever, piloerection, ptosis) (Dantzer et al., 1998, 2000). As well, these cytokines increase hypothalamic and extrahypothalamic monoamine activity (Dunn and Wang, 1995; Lacosta et al., 1998) and stimulate release of hypothalamic corticotropin releasing hormone (CRH), hence promoting plasma ACTH and corticosterone secretion (Bernardini et al., 1990; Dunn and Wang, 1995; Whitmall et al., 1992). The mechanisms by which cytokines influence central processes remain to be identified. Cytokines may gain entry into brain through a saturable transport mechanism, or at circumventricular sites lacking an efficient blood-brain barrier (Banks et al., 1995). Alternatively, they may stimulate vagal afferents which then influence CNS functioning (Fleshner et al., 1998; Goehler et al., 1995; Kapcala et al., 1996) or activate circulating immune cells (e.g., macrophages or mast cells), resulting in the release of soluble inflammatory or vasoactive proteins such as histamine, prostaglandins or nitric oxide (Laflamme and Rivest, 1999; Song and Leonard, 1995; Purcell and Atterwill, 1995).

Cytokines may have direct actions on central neurotransmission as their central infusion increased *c-fos* expression within CRH neurons of the paraventricular nucleus of the hypothalamus (PVN) and augmented hypothalamic NE turnover (Ericsson et al., 1994; Terao et al., 1993; Vellucci et al., 1995). Central TNF- α also dose-dependently increased circulating ACTH levels, although inconsistent results were reported with

respect to corticosterone variations (cf. Pauli et al., 1998; van der Meer et al., 1996). Like “traditional” stressors (e.g. footshock), cytokines induce time-dependent, proactive effects on neurochemical functioning. For instance, systemic IL-1 β or TNF- α administration increased arginine vasopressin (AVP) and CRH within terminals of the external zone of the median eminence, peaking 1-2 weeks after treatment (Hayley et al., 2001a; Schmidt et al., 1995, 1996; Tilders & Schmidt, 1998). As well, upon cytokine reexposure a sensitization effect was evident wherein plasma ACTH and corticosterone levels were enhanced (Hayley et al., 1999; Schmidt et al., 1995). In addition, systemic TNF- α treatment also sensitized various sickness indices and central monoamine utilization, although the latter effects were amine- and brain-region specific (Hayley et al., 1999).

The sensitization effects induced by TNF- α may be related to either peripheral or to central factors. As this sensitization was only demonstrated with human TNF- α , and limited data have been reported concerning the neurochemical effects of the murine form of this cytokine, the present investigation assessed whether (a) murine TNF- α would induce sickness, increase plasma corticosterone and promote hypothalamic and extrahypothalamic monoamine variations, (b) these effects would be provoked by intracerebroventricular (icv) administration of this cytokine, and (c) challenge with central TNF- α would elicit the sensitized responses in mice that had previously received systemic cytokine administration. Given that cytokines are thought to induce stressor-like effects, particular attention was devoted to the analyses of monoamine activity in brain regions ordinarily affected by stressors.

Materials and Methods

Male, CD-1 mice, were obtained from Charles River Inc, Laprairie Quebec at 8-10 weeks of age. Mice were housed in groups of four in standard propylene cages, and acclimated to the laboratory for 2 weeks before serving as experimental subjects. Animals were maintained on a 12 hour light/dark cycle with lights on at 0800, room temperature was kept at 21° C, and mice were permitted an ad libitum diet of Ralston Purina mouse chow. All experimental procedures were approved by the Carleton University Animal Care Committee and met the Guidelines set by the Canadian Council on Animal Care.

Procedures

In order to minimize the contribution of diurnal variations associated with neurotransmitter or neuroendocrine processes, all experimental procedures were conducted between 0800 and 1200 hr. The recombinant murine mTNF- α (specific activity = 1.1×10^5 U/ μ g) was obtained from R&D systems in lyophilized form and subsequently dissolved in sterile PBS and stored in 20 μ l aliquots at -80°C.

Experiment 1 & 2: Systemic murine TNF- α influences sickness behavior, plasma corticosterone and central monoamine activity

Mice (n = 8-10/group) received an initial intraperitoneal (ip) injection of either sterile nonpyrogenic saline (0.9 %) or 2.0 μ g of recombinant murine TNF- α (mTNF- α) in a volume of 0.4 ml of sterile saline. Twenty-eight days following the initial treatment mice of each group were subdivided and given a second i.p. injection of either saline or 0.1, 0.5 or 1.0 μ g of mTNF- α . Behavioral indices of sickness were rated as described in

a proceeding section. At 60 min after the second injection mice were decapitated, brains collected and frozen in isopropyl alcohol, and then stored at -80°C until later sectioning and removal of nuclei for analysis of central biogenic amines and metabolites. Trunk blood was centrifuged and the supernatant stored at -80°C in tubes containing $10\ \mu\text{l}$ of EDTA for the determination of plasma corticosterone levels. The dissection procedure and assay methods are described later. The 60 min interval between mTNF- α administration and subsequent decapitation was chosen on the basis of earlier studies showing that behavioral and glucocorticoid variations were marked at this time (Brebner et al., 2000; Hayley et al., 1999).

In order to determine whether the sensitization effects would be apparent at brief times following cytokine treatment a second experiment was conducted to determine the behavioral and neurochemical changes associated with reexposure to mTNF- α 1 day after initial treatment. Mice ($n = 10/\text{group}$) received i.p. injection of either saline or mTNF- α ($2.0\ \mu\text{g}$), and then one day later half the animals of each group received saline treatment, while the remaining mice received reexposure to mTNF- α at a reduced dosage ($1.0\ \mu\text{g}$). The procedures were otherwise the same as in the preceding experiment.

As recombinant human TNF- α , rather than the murine form of the cytokine, was employed in earlier studies, the possibility exists that the observed effects were related to cross-species actions of the cytokine, particularly as human and murine TNF- α have functionally different effects in mice. Specifically, the murine form of the cytokine excites both the p55 and the p75 receptor, while human TNF- α excites only the p55 receptor (Brouckaert et al., 1989). Unless otherwise specified, the present experiments involved only mTNF- α .

Behavioral analyses

In addition to the neuroendocrine and neurotransmitter alterations, behavioral outputs related to sickness associated with mTNF- α were also assessed. Following the second i.p. injection mice were returned to their home cages, and their behavior was recorded up to the time at which the brain and blood were collected. Commencing 15 min after the injection, and at the 3 successive 15 min intervals, the following behaviors were rated, for a 10 s period, on 4 point scales: social interaction (1 = animal huddling with cagemates; 2 = occasional interaction with cagemates; 3 = predominately staying away from cagemates; 4 = isolated from cagemates, typically in corner); overall appearance (1 = normal looking; 2 = slightly lethargic, slow movement, slightly ruffled fur, eyes slightly drooping; 3 = lethargic, fur ragged, eyes drooping, breathing altered; 4 = very sick appearance, ptosis, ragged fur, curled body posture, difficulty breathing, and general nonresponsiveness). This procedure provided better than 90% agreement between raters blind as to the treatment mice received.

Experiments 3 & 4: Acute and reexposure effects of intracerebroventricular mTNF- α

An initial study assessed the effects of varying doses of icv mTNF- α administration on plasma corticosterone levels. A 1.0 μ l volume of sterile saline or mTNF- α (1, 25, 50 or 100ng) was slowly infused into the lateral cerebral ventricle of naïve CD-1 mice (n = 6-8/group) while animals were manually restrained. Infusions were administered over a 2-min period, after which the injector was left in place for 30 seconds to ensure adequate diffusion of the solution. The vehicle and mTNF- α were administered

through an injector comprising a 32 gauge needle (Small Parts Inc., FL; with internal and outer diameters of 0.004 and 0.009 inches, respectively) inserted into the implanted cannula (see section describing surgical procedures). Fluids were delivered to the cannula using polyethylene tubing (Cole-Parmer, IL; internal and outer diameters of 0.01 and 0.03 inches, respectively) which was connected to a 2.5 ml Hamilton syringe (Chromatographic Specialists Inc.).

Experiment 4 was undertaken to assess the effects of reexposure to icv mTNF- α administration on plasma corticosterone and on hypothalamic and extrahypothalamic monoamine activity. CD-1 mice ($n = 8/\text{group}$) had cannulae implanted in the lateral ventricle. To minimize the potential effects of handling observed in Experiment 3, these mice were prehandled on each of 7 days prior to treatment, and mice were allowed to move freely in their home cages while the icv infusions were delivered. Initially, mice received either icv administration mTNF- α (50 ng) followed 1, 7 or 28 days later by a second lower dose of icv mTNF- α (1.0 ng). Two additional groups that had initially been treated with mTNF- α (50 ng) or saline 28 days earlier now received icv saline, while a third group initially treated with saline received mTNF- α (1.0 ng) 28 days later. As already described, the saline or mTNF- α were administered in a volume of 1.0 μl over 2-min period and after 20 min mice were decapitated and trunk blood collected. In this experiment brains were also taken for determination of monoamine and metabolite levels. Cannula placements were verified in sliced frozen tissue, but otherwise brain regions were dissected as described earlier.

Experiment 5: Effects of systemic mTNF- α on the response to later intracerebroventricular TNF- α

The preceding experiments had indicated that icv administration did not provoke the sensitization with respect to either sickness behavior or plasma corticosterone levels upon later reexposure to mTNF- α via the same route of administration. Such an outcome suggests that the sickness and glucocorticoid variations were related to peripheral factors. That is, systemic mTNF- α may prime peripheral targets (e.g. gut, liver, vagal afferents, immune cells), which in turn may influence brain activity (e.g. receptor upregulation), such that reexposure to the cytokine re-excites these neuronal circuits. If central mechanisms were involved in the sensitization effects associated with systemic mTNF- α treatment, then initial intraperitoneal administration of the cytokine treatment should result in an augmented response upon later reexposure to icv administration of the cytokine. The possibility also exists that the initial mTNF- α treatment disrupted blood-brain barrier permeability, such that increased entry of the cytokine would be evident upon later reexposure. Indeed, it has been reported that cytokines compromise the integrity of the blood-brain-barrier and increase leukocyte infiltration into the brain (Quagliarello et al., 1991).

CD-1 mice with cannulae implanted in the lateral ventricle, as described in the preceding experiment, initially received ip administration of either vehicle or mTNF- α (2.0 μ g). Mice were returned to their home cages, and then one week later these groups were subdivided and treated icv with either mTNF- α (100 ng) or vehicle ($n = 10/\text{group}$). An additional group of mice was included to assess the effects of a longer interval between the two treatments. These mice ($n = 8/\text{group}$) initially received ip administration

of the cytokine (2.0 μ g) and then 28 days later they received icv mTNF- α (100 ng). Trunk blood and brain nuclei were taken 20 min after the second treatment, as described in the preceding experiments.

Surgery

Pre-operative analgesia was provided by adding a solution of acetaminophen (80mg/5ml; children's Tempra, Mead Johnson) to the drinking water (1ml of Tempra in 100ml H₂O) for 3 days prior the surgery. Prior to anesthesia (15 min before the surgery), mice were administered 0.01mg/kg subcutaneous (s.c.) glycopyrrolate (0.01 mg/mouse; Robinul, Wyeth-Ayerst, Montreal, Canada) to prevent the accumulation of salivary and bronchial secretions. Mice were secured in a Kopf stereotaxic instrument and anesthesia was maintained using Halothane (2.0-3.0 %), supplemented with oxygen (2.5 L/min), which was delivered through a custom made nose piece. A 26 gauge stainless steel guide cannula (0.8 cm in length, internal and outer diameters of 0.01 and 0.018 inches, respectively; Small Parts Inc., FL) was unilaterally implanted into one of the lateral cerebral ventricles using a Kopf micromanipulator. Cannula placement was varied between left and right hemispheres between animals to control for any effects of laterality. All cannulae were implanted according to the following stereotaxic coordinates: +0.14 mm anterior to bregma, 0.75mm lateral from the midsagittal suture and -2.35mm ventral from a flat skull surface. The guide cannula was fixed to the skull using dental cement and small jeweler screws to minimize damage to underlying cortical structures. Animals were given one week to convalesce before being subjected to testing procedures and cytokine administration.

In studies where cannulae were implanted, histological verification of cannula placements was conducted in a subset of animals. To this end, coronal microtome sections (30 μm) were cut throughout the extent of the cannula placement, approximately 0.02-0.26 mm relative to bregma, according to the atlas of Franklin and Paxinos (1997).

Brain dissection and HPLC procedure for analysis of brain amine and metabolite levels

The brain dissection was conducted in a cold chamber, with the brain resting on a stage containing dry ice. Frozen brains were placed on a stainless steel block with slots (spaced approximately 500 μm apart) that served as guides for razor blades, which were used to provide a series of coronal brain sections. Brain sections were mounted on glass slides and placed on a petri dish filled with powdered dry ice that served as a cold stage. Utilizing the mouse brain atlas of Franklin and Paxinos (1997), brain nuclei were removed by micropunch using hollow 16 or 20 gauge needles with a beveled tip. Tissue was taken from the locus coeruleus, PVN, median eminence/arcuate nucleus, medial prefrontal cortex and medial and central amygdaloid nuclei, placed in 0.3 M monochloroacetic acid containing 10% methanol and internal standards, for monoamine analyses using HPLC.

Levels of DA, NE and 5-HT, and their respective metabolites, MHPG, DOPAC and 5-HIAA were determined by HPLC using a modification of the method of Seegal et al., 1986. Tissue punches were sonicated in a homogenizing solution that comprised 14.17 g monochloroacetic acid, 0.0186g disodium ethylenediamine tetraacetate (EDTA), 5.0 ml methanol and 500 ml H_2O . Following centrifugation, the supernatants were used for the HPLC analysis. Using a waters M-6000 pump, guard column, radial compression

column (5m, C18 reverse phase, 8mm x 10cm), and a three cell coulometric electrochemical detector (ESA Model 5100,A) 20 ml of the supernatant was passed through the system at a flow rate of 1.5 ml/min (1400-1600 PSI). The mobile phase used was a modification of that used by Chiueh et al. (1983); each liter consisted of 1.3 g of heptane sulfonic acid, 0.1 g disodium EDTA, 6.5 ml triethylamine, 35 ml acetonitrile. The mobile phase was then filtered (0.22 mm filter paper) and degassed following which the pH was adjusted to 2.5 with phosphoric acid. The area and height of the peaks was determined using a Hewlett-Packard integrator. Protein content of each sample was determined using bicinchoninic acid with a protein analysis kit (Pierce Scientific, Brockville, Ontario) and a spectrophotometer (Brinkman, PC800 colorimeter). The lower limit of detection for the monoamines and metabolites was approximately 5.0 pg.

Plasma corticosterone assay

Plasma corticosterone was determined (in duplicate) using a commercially available radioimmunoassay (RIA) kit (ICN Biomedicals Inc., CA). To avoid interassay variability all samples were assayed within a single run. The intra-assay variability was less than 10%.

Statistical Analyses

The plasma corticosterone concentrations, and monoamine determinations were analyzed by either a single or a two-factor analysis of variance (ANOVA), as appropriate, followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) of the significant main effects or simple effects of significant interactions. Sickness behaviors animals displayed

(overall appearance and social interaction), were analyzed by a mixed factor ANOVA (Initial x Reexposure cytokine treatment x Sampling period). Newman-Keuls multiple comparisons ($\alpha = 0.05$) were again used to determine differences between means of the simple effects comprising any interactions. Over the course of the experiments several tissue samples were lost and consequently the degrees of freedom for the ANOVAs varied across brain regions.

Results

Overview: Owing to the large number of experiments, and the behavioral and neurochemical analyses performed in each, an overview of the results are depicted in Table 3.1. This table portrays the effects of acute ip or icv mTNF- α treatment, as well as the sensitizing effects of these treatments on plasma corticosterone levels, as well as NE, DA and 5-HT activity in several brain regions. As seen in this table, systemic and icv mTNF- α treatment affected plasma corticosterone and central monoamine activity, although the effects of the former treatment tended to be more pronounced, suggesting peripheral factors in the elicitation of these outcomes. Interestingly, upon systemic reexposure to the cytokine among mice that previously received ip mTNF- α treatment a time-dependent sensitization was apparent with respect to sickness, plasma corticosterone and 5-HT activity in medial amygdala and PFC. In contrast, when mice received the reexposure treatment through the icv route there was no indication of a sensitization with respect to either plasma corticosterone or sickness, irrespective of whether mice had initially received the treatment systemically or centrally. Yet, when the initial and the reexposure treatments were administered centrally a sensitization was apparent with

respect to monoamine functioning in hypothalamic sites (PVN and ME/ARC). Moreover, when the initial treatment was administered systemically and animals were reexposed to the cytokine through icv treatment, a sensitization was again apparent with respect to amine activity within the ME/ARC, and in addition locus coeruleus NE activity was augmented. Thus, it appears that mTNF- α elicits the sensitization of both peripheral and central factors, which may be independent of one another.

Insert Table 3.1 about here

Experiment 1: Behavioral changes associated with systemic mTNF- α exposure (28 days)

Following acute systemic mTNF- α administration plasma corticosterone was increased, as was the utilization of NE in several brain regions. Upon reexposure to the cytokine 28 days later, a sensitization effect was evident with respect to sickness behaviors, plasma corticosterone, as well as 5-HT activity within the medial amygdala.

The sickness profile of mice is shown in Figure 3.1 as a function of the initial and the reexposure cytokine treatments mice received. The ratings of the overall appearance of mice varied as a function of the interaction between the Initial mTNF- α treatment x Reexposure treatment x Sampling Time, $F(9, 198) = 2.69$, $p < 0.001$. The multiple comparisons indicated that among animals pretreated with saline, later re-exposure to mTNF- α did not affect the animal's appearance. Likewise, among mice pretreated with mTNF- α , later saline administration did not promote sickness. In contrast, when mTNF- α pretreated mice were reexposed to the cytokine, even at the lowest dosage (0.1 μg),

Table 3.1 Summary of changes of corticosterone and monoamine utilization induced by mTNF- α

	i.p TNF (acute)	icv TNF (acute)	ip $\rightarrow$ ip	icv $\rightarrow$ icv	ip $\rightarrow$ icv
Corticosterone	$\uparrow\uparrow$	$\uparrow$	$\uparrow\uparrow\uparrow$ (28)	$\uparrow$	$\uparrow$
PVN MHPG	$\uparrow$	--	$\uparrow$	$\uparrow\uparrow\uparrow$ (1 & 7)	$\uparrow$
PVN 5-HIAA	--	$\uparrow$	$\uparrow\uparrow$ (1, 28)	$\uparrow\uparrow$ (1 & 7)	$\uparrow\uparrow$ (7)
PVN DOPAC	--	--	--	$\uparrow\uparrow\uparrow$ (7)	--
ME/ARC MHPG	NA	$\uparrow^*$	NA	--	$\uparrow$
ME/ARC 5-HIAA	NA	$\uparrow$	NA	$\uparrow$	$\uparrow\uparrow\uparrow$ (28)
ME/ARC DOPAC	NA	-	NA	$\uparrow\uparrow\uparrow$ (7, 28)	$\uparrow\uparrow\uparrow$ (28)
LC MHPG	$\uparrow$	$\uparrow$	$\uparrow$	$\uparrow$	$\uparrow\uparrow\uparrow$ (28)
CeA MHPG	$\uparrow$	$\uparrow$	$\uparrow$	$\uparrow\uparrow$	--
CeA 5-HIAA	--	--	--	$\uparrow$ (7)	--
CeA DOPAC	--	--	--	--	$\uparrow$
PFC MHPG	$\uparrow\uparrow$	--	$\uparrow\uparrow$	--	--
PFC 5-HIAA	--	--	$\uparrow\uparrow\uparrow$ (1)	--	--
PFC DOPAC	$\uparrow$	--	$\uparrow$	--	--

$\uparrow$ = modest increase; $\uparrow\uparrow$ = large increase; $\uparrow\uparrow\uparrow$ = sensitization; -- = no effect; NA = not assessed

* increase evident only after relatively high dose

Number in bracket indicates time (days) at which sensitization effect was significant

ratings of illness were markedly increased. Social interaction was also found to vary as a function of the interaction between the Initial x Reexposure cytokine treatments, $F(3, 232) = 8.12$, $p < 0.01$. As in the case of overall appearance, the multiple comparisons indicated that a single administration of mTNF- α was entirely without effect on this behavior. However, among mice previously treated with the cytokine, social interaction was markedly impaired upon reexposure to either of the two higher mTNF- α doses (0.5 and 1.0 μg). The sensitization effects of mTNF- α , with respect to both of these indices of illness (appearance and social interaction), were not evident at 15 and 30 min, but were marked at 45 and 60 min after the cytokine reexposure. For the sake of clarity, Figure 3.1 only presents data obtained at the 60 min sampling time.

Insert Figure 3.1 about here

Corticosterone changes associated with mTNF- α reexposure (28 days)

As in the case of the behavioral changes, plasma corticosterone levels were subject to a sensitization effect. Specifically, levels of plasma corticosterone in each of the groups, shown in Figure 3.1, varied as a function of the Initial Cytokine treatment x Reexposure treatment interaction, $F(3, 57) = 4.88$, $p < 0.01$. The multiple comparisons indicated that among mice pretreated with saline, subsequent mTNF- α treatment dose-dependently increased plasma corticosterone levels, such that the 1.0 μg dose provoked a significant elevation relative to that of saline-treated mice. As in the case of the behavioral changes, the effect of the reexposure treatment was marked, in that a more

Figure 3.1 Mean ($\pm$ SEM) plasma corticosterone levels (top) and ratings of illness as reflected by social interaction (middle) and overall appearance (e.g. ptosis, piloerection, curled body posture) as determined using a 4-point scale. Mice were pretreated with saline (gray bars) or mTNF- α (2.0 μ g; i.p.; hatched bars) and 28 days later exposed to saline or mTNF- α (0.1, 0.5 or 1.0 μ g; i.p.). * $P < 0.05$ relative to mice treated with saline on two occasions, ° $P < 0.05$ relative to animals receiving a single injection of mTNF- α .

Initial Treatment

■ Veh
▨ TNF- α (2.0 μ g)

Reexposure TNF- α Treatment (μ g)

pronounced increase of corticosterone was provoked by mTNF- α in mice that had been treated with the cytokine 28 days earlier. This was the case in response to the 0.5 and 1.0 μg doses, while at the 0.1 μg dose the increase approached but did not reach statistical significance ($p < 0.10$).

Monoamine variations associated with TNF- α exposure (28 days)

Administration of mTNF- α influenced central monoamine activity in a dose- and region-specific fashion. As well, at least some of the effects of the cytokine were subject to a sensitization effect. Within the PVN the accumulation of the NE metabolite, MHPG, varied as a function of the Cytokine treatment mice received 1 hr prior to sacrifice, $F(3, 62) = 6.07$, $p < 0.01$, as did the level of NE, $F(3, 62) = 2.66$, $p = 0.05$. Each of the cytokine doses increased the accumulation of the metabolite, while the rise of NE only reached significance in response to the intermediate (0.5 μg) dose of mTNF- α . Interestingly, the increased utilization of NE, as reflected by the augmented metabolite accumulation, was even evident at the lowest mTNF- α dose (see Figure 3.2), although a further augmentation was not apparent with increasing doses.

The accumulation of 5-HIAA within the PVN varied as a function of the interaction between the Initial and the Reexposure mTNF- α treatments, $F(3, 62) = 2.66$, $p < 0.05$. The multiple comparisons indicated that acute mTNF- α administration did not affect the accumulation of 5-HIAA. However, as shown in Figure 3.2, in mice pretreated with the cytokine, reexposure to mTNF- α enhanced the metabolite accumulation. This effect was statistically significant at the 0.5 μg dose, but was entirely absent at the highest

dose. The level of 5-HT was affected by the second injection of mTNF- α , $F(3, 62) = 2.80$, $p < 0.05$, with Reexposure to the 1.0 μg dose (in mice previously treated with the cytokine) provoking a reduction of 5-HT levels relative to that of saline treated mice.

Insert Figure 3.2 about here

Within the locus coeruleus the levels of NE were unaltered by mTNF- α administration, while MHPG accumulation varied as a function of the treatment received on the day of sacrifice, $F(3, 64) = 3.01$, $p < .05$. Although each of the mTNF- α doses enhanced MHPG accumulation (mean $\pm$ SEM = 5.23 ± 0.55 , 5.99 ± 0.67 , 5.00 ± 0.34 ng/mg protein, for the 0.1, 0.5 and 1.0 μg doses, respectively) relative to saline treatment (4.06 ± 0.25 ng/mg protein), the multiple comparisons indicated that the increase was only significant in response to the 0.5 μg dose.

The amine variations within the prefrontal cortex were distinguishable from those evident within the PVN. Among animals that had initially received mTNF- α , the levels of NE were slightly, although significantly, reduced 28 days later (mean $\pm$ SEM = 6.22 ± 0.34 and 5.22 ± 0.20 ng/mg protein), as were the concentrations of MHPG (mean $\pm$ SEM = 3.25 ± 0.23 and 2.49 ± 0.20 ng/mg protein), $F(1, 67) = 5.95$ and 6.50 , p 's < 0.05 . The reexposure treatment influenced MHPG accumulation, $F(3, 67) = 4.39$, $p < 0.01$, and the multiple comparisons indicated that the 0.5 μg dose increased the metabolite level (mean $\pm$ SEM = 2.35 ± 0.23 , 2.60 ± 0.27 , 3.75 ± 0.35 , 2.77 ± 0.30 , for the saline, 0.1, 0.5 and 1.0 μg groups, respectively). Significant variations of 5-HT and 5-HIAA were not apparent

Figure 3.2 Mean (+SEM) concentrations of 5-HIAA (top) and MHPG (bottom) within the PVN as a function of the mTNF- α treatments. As in Figure 3.1, mice were pretreated with either saline (gray bars) or mTNF- α (2.0 μ g; i.p.; hatched bars) and 28 days later exposed to saline or mTNF- α (0.1, 0.5 or 1.0 μ g; i.p.). * $P < 0.05$ relative to mice receiving only saline injections, ° $P < 0.05$ relative to animals receiving a single injection of mTNF- α .

in this region, while DOPAC levels were moderately increased among mice initially treated with mTNF- α , but this effect did not reach statistical significance.

The effects of the cytokine on NE and MHPG within the medial amygdala were relatively modest. In particular, although mTNF- α provoked an increase of medial amygdala MHPG accumulation of ~30% at the higher doses, this effect did not reach statistical significance (mean $\pm$ SEM = 5.74 ± 0.86 , 6.86 ± 0.84 , 8.13 ± 1.20 , 7.41 ± 1.40 ng/mg protein, for saline and 0.1, 0.5 and 1.0 μ g mTNF- α treatments, respectively). While 5-HT levels were unaffected by mTNF- α , 5-HIAA accumulation varied as a function of the Initial treatment x Reexposure treatment interaction, $F(3, 56) = 4.92$, $P < 0.01$. As shown in Figure 3.3 and confirmed by the multiple comparisons, among mice initially treated with saline, later mTNF- α administration did not significantly increase the accumulation of 5-HIAA within the medial amygdala. In contrast, in mice pretreated with mTNF- α , subsequent re-exposure to the 0.5 or 1.0 μ g doses markedly increased 5-HIAA relative to that of mice that received only a single injection of the cytokine.

Within the central amygdala mTNF- α likewise induced a rise of MHPG (~25%), and as in the medial amygdala, this effect was not statistically significant (mean $\pm$ SEM = 5.55 ± 0.37 , 5.72 ± 0.45 , 7.18 ± 0.86 , 6.86 ± 0.82 ng/mg protein, for saline and 0.1, 0.5 and 1.0 μ g mTNF- α treatments, respectively). Furthermore, neither NE nor 5-HT variations in the central amygdala varied as a function of the cytokine treatment, and likewise, neither DA nor DOPAC was affected by the cytokine.

Experiment 2: Behavioral, corticosterone and monoamine changes associated with mTNF- α reexposure (1 day)

Unlike the effects seen upon reexposure to the cytokine 28 days after initial treatment, when mTNF- α was administered 1 day after initial treatment there was no indication of sickness behavior (Figure 3.4). The cytokine was again found to increase plasma corticosterone concentrations measured 1 hr treatment, $F(1, 36) = 45.17$, $p < 0.01$. However, as in the case of the behavioral changes there was no indication of a sensitization effect (Figure 3.4). That is, relative to mice treated with saline on both occasions and mice treated with mTNF- α followed by saline 1 day later, the level of corticosterone was elevated in mice treated with saline and then given acute mTNF- α . This effect, however, was not further increased in mice that received the cytokine on 2 occasions one day apart.

Insert Figures 3.3 and 3.4 about here

As in the preceding experiment, the acute 1.0 μg challenge dose of mTNF- α produced a small increase of PVN MHPG accumulation relative to that of saline treated mice (mean $\pm$ SEM = 6.03 ± 0.56 vs 5.02 ± 0.57 ng/mg protein), as well as a modest increase of 5-HT (mean $\pm$ SEM = 17.54 ± 1.76 vs 14.62 ± 2.05 ng/mg protein). However, neither of these effects were statistically significant. Commensurate with previous reports using the recombinant human form of TNF- α , there was no evidence of the initial mTNF- α treatment having any effect on PVN monoamine levels or utilization upon cytokine reexposure at the 1 day interval.

Figure 3.3 Concentrations of 5-HIAA (mean $\pm$ SEM) within the medial amygdala among mice that were treated with saline (gray bars) or mTNF- α (2.0 mg; i.p. hatched bars) and then 28 days later exposed to saline or mTNF- α (0.1, 0.5 or 1.0 μ g; i.p.). * $P < 0.05$ relative to mice treated with saline on two occasions.

Figure 3.4 Mean ($\pm$ SEM) plasma corticosterone levels (top) and ratings of illness (bottom) as reflected by overall appearance (e.g. ptosis, piloerection, curled body posture) as determined using a 4-point scale. Mice were pretreated with either saline or mTNF- α (2.0 μ g; i.p.) and subsequently reexposed to saline or mTNF- α (1.0 μ g; i.p.) 1 day later. Behavioral and hormonal measures were made at 60 minutes following the second injection. * $P < 0.05$ relative to mice treated with saline on two occasions.

Initial and Reexposure i.p. TNF- α Treatments (1 day)

Within the PFC the accumulation of MHPG was increased by exposure to the cytokine on the test day, $F(1, 36) = 6.13$, $p < 0.05$ (see Table 3.2), whereas the initial treatment was without effect. The concentrations of 5-HIAA, in contrast, varied as a function of the interaction between the initial cytokine treatment and the subsequent reexposure condition, $F(1, 33) = 5.41$, $p < 0.05$. As seen in Table 3.2, and confirmed by the multiple comparisons, mTNF- α increased 5-HIAA accumulation measured 1 hr later. In itself, treatment with mTNF- α 1 day earlier did not affect 5-HIAA accumulation, but a pronounced elevation of the metabolite was evident among mice initially treated with mTNF- α and then reexposed to the cytokine (i.e. sensitization effect).

As shown in Table 3.2, within the central amygdala, MHPG accumulation was increased by acute mTNF- α administered 1 hr earlier relative to that of mice treated with saline, $F(1, 33) = 7.91$, $p < 0.01$. Likewise, relative to saline-treated mice, the accumulation of 5-HIAA was significantly enhanced by mTNF- α administered 1 hr before sacrifice, $F(1, 33) = 6.30$, $p < 0.02$ (Table 3.2). In neither instance, however, was a sensitization effect apparent in that the metabolite accumulation elicited by mTNF- α was not affected by the animal's previous treatment.

Within the medial amygdala the utilization of NE, as reflected by MHPG accumulation, did not vary significantly with the cytokine treatment (Table 3.2). However, 5-HIAA accumulation varied as a function of the Initial treatment x Reexposure treatment interaction, $F(1, 30) = 8.65$, $p < 0.01$. As shown in Table 3.2, and confirmed by the multiple comparisons, acute mTNF- α did not increase the level of 5-HIAA, and in fact provoked a modest decline relative to that of saline treated mice. Upon

reexposure to the cytokine the level of 5-HIAA, while relatively variable, exceeded that of acutely treated mice or mice that had received the cytokine 1 day earlier.

Experiment 3: The acute effects of intracerebroventricular mTNF- α

The central administration of mTNF- α did not elicit any signs of sickness (data not shown). In contrast, the levels of corticosterone in Experiment 3 varied as function of mTNF- α administration, $F(4,30) = 2.64$, $p < 0.05$. The multiple comparisons indicated that relative to saline treated mice, corticosterone levels were elevated to a moderate, but significant extent in each of the mTNF- α groups. As indicated in Table 3.3, however, a progressive increase of corticosterone was not apparent with increasing dosage. It ought to be underscored that the corticosterone elevation associated with icv mTNF- α was proportionately smaller than that seen after i.p. administration of the cytokine (see Experiment 1), possibly owing to the high levels of corticosterone provoked by handling the animal when placing the injector into the cannula.

Insert Tables 3.2 and 3.3 about here

Experiments 4a and 4b: The sensitizing effects of intracerebroventricular mTNF- α

In Experiment 4a, as in the preceding experiment, icv mTNF- α did not elicit any signs of illness. This was the case for acute as well as reexposure to the mTNF- α (data not shown). Acute mTNF- α induced only a modest rise of plasma corticosterone levels, which failed to reach statistical significance. As seen in Table 3.4, corticosterone levels

Table 3.2. Monoamine variations (mean $\pm$ SEM ng/mg protein) within the Prefrontal Cortex, Central Amygdala and Medial Amygdala (1 day reexposure).

	Saline-Saline	Saline-TNF	TNF-Saline	TNF-TNF
<u>Prefrontal Cortex</u>				
MHPG	2.85 $\pm$ 0.19	3.61 $\pm$ 0.20	3.04 $\pm$ 0.27	4.10 $\pm$ 0.61*
5-HIAA	4.04 $\pm$ 0.61	7.87 $\pm$ 1.61*	3.77 $\pm$ 0.37	16.50 $\pm$ 3.39**
<u>Medial Amygdala</u>				
MHPG	6.30 $\pm$ 0.52	5.63 $\pm$ 0.48	4.19 $\pm$ 0.36	6.92 $\pm$ 1.07
5-HIAA	16.01 $\pm$ 2.56	12.31 $\pm$ 2.32*	11.95 $\pm$ 2.10*	21.26 $\pm$ 3.77**
<u>Central Amygdala</u>				
MHPG	4.77 $\pm$ 1.11	8.73 $\pm$ 0.80*	6.52 $\pm$ 1.07	8.42 $\pm$ 1.14*
5-HIAA	10.52 $\pm$ 1.61	18.58 $\pm$ 3.97*	10.65 $\pm$ 0.94	15.61 $\pm$ 3.73*

*p < 0.05 relative to Saline-Saline and **p < 0.05 relative to Saline-TNF treated mice (n = 8-10 mice per group).

Table 3.3 Effects of icv mTNF- α on plasma corticosterone levels (mean $\pm$ SEM).

Dose (ng)	Corticosterone (μ g/dl)
Saline	16.85 $\pm$ 3.2
1.0	27.23 $\pm$ 3.6*
25	28.12 $\pm$ 4.0*
50	23.70 $\pm$ 2.6*
100	24.72 $\pm$ 2.1*

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*p < 0.05 relative to Saline treated mice (n = 8-10 mice per group).

were generally lower than in Experiment 3, likely reflecting habituation to handling coupled with animals not being restrained during the entire infusion procedure. As the effects of the mTNF- α were also more pronounced in the former study, it is possible that the effects of the cytokine interacted with those of the handling stress, just as stressors were previously shown to synergistically influence the response to IL-1 β (Merali et al., 1997). Unlike the effects seen after systemic mTNF- α treatment, there was no indication of the cytokine resulting in a sensitization effect (Table 3.4) with respect to circulating corticosterone levels, irrespective of the time following initial treatment ($F < 1$). Since the i.p. induced sensitization of corticosterone was measured at 60 minutes following the final mTNF- α injection, but the icv studies involved decapitation after 20 minutes, it is possible that such sensitization effects may have been missed owing to the particular time at which measurements were made. However, additional studies in our laboratory, using identical icv procedures but employing a 30 minute post-injection decapitation interval, yielded virtually identical results (S. Hayley, K. Ethier, Z. Merali & H. Anisman, unpublished observations). Moreover, in this investigation we found that the i.p. induced corticosterone sensitization occurred as early as 15 minutes following the TNF- α reexposure treatment and lasted up to 120 minutes.

Table 3.5 depicts the corticosterone changes elicited by the higher initial and reexposure mTNF- α icv doses used in Experiment 4b. The mTNF- α treatments significantly altered plasma corticosterone levels, $F(3,30) = 5.70$, $p < 0.01$. The multiple comparisons revealed that the acute 100 ng dose of mTNF- α increased corticosterone relative to saline infused animals. However, icv reexposure to mTNF- α 7 days following

the initial 500 ng treatment further produced only a slight, non-significant increase of corticosterone levels (Table 3.5).

Insert Tables 3.4 and 3.5 about here

In Experiment 4a, the concentrations of NE within the PVN were not significantly influenced by icv mTNF- α infusion, although a modest decline of the amine was evident after the acute 1.0 and 50.0 ng doses relative to that elicited by saline treatment (mean $\pm$ SEM = 30.91 ± 4.88 , 28.7 ± 4.82 , 42.91 ± 7.21 ng/mg protein, respectively). In contrast, PVN MHPG levels were significantly altered by the cytokine treatments, $F(6, 53) = 3.56$, $p < 0.01$. As shown in Figure 3.5, a pronounced sensitization effect was apparent which peaked upon reexposure to mTNF- α 1 day following initial treatment with the cytokine. Indeed, the multiple comparisons indicated that MHPG accumulation among mice challenged with mTNF- α 1 or 7 days following the first injection exceeded that of animals treated only with saline or with a single injection of mTNF- α on test day. It is noteworthy that this pattern of MHPG utilization (i.e. maximal effect after 1-7 days, but normalization by 28 days) sharply contrasts with that observed when mTNF- α was systemically administered.

The concentration of 5-HT within the PVN was not significantly altered by the mTNF- α treatments; however, its metabolite, 5-HIAA, varied as a function of the cytokine regimen, $F(6, 51) = 2.79$, $p < 0.05$. Multiple comparisons revealed that relative to animals that received saline on test day, 5-HIAA accumulation was increased in

Table 3.4. Plasma corticosterone levels ($\mu\text{g/dl} \pm \text{SEM}$) as a function of the initial and reexposure (1, 7 and 28 days) icv mTNF- α treatments.

Initial/Reexposure treatment	Plasma corticosterone ($\mu\text{g/dl}$)
Saline / Saline	8.03 ± 1.69
Saline / TNF (1.0 ng)	10.11 ± 1.46
Saline / TNF (50.0 ng)	12.93 ± 1.61
TNF (50.0 ng) / Saline (28 d)	10.45 ± 2.21
TNF (50.0 ng) / TNF (1.0 ng; 1 d)	6.94 ± 0.88
TNF (50.0 ng) / TNF (1.0 ng; 7 d)	11.14 ± 1.76
TNF (50.0 ng) / TNF (1.0 ng; 28 d)	8.81 ± 1.64

Table 3.5. Plasma corticosterone levels ($\mu\text{g/dl} \pm \text{SEM}$) as a function of the initial and reexposure (7 days) icv mTNF- α treatments.

Initial treatment	Reexposure challenge	Plasma corticosterone ($\mu\text{g/dl}$)
Saline	Saline	4.98 ± 0.87
Saline	TNF (100 ng)	$8.97 \pm 1.46^*$
TNF (500 ng)	Saline	5.43 ± 1.36
TNF (500 ng)	TNF (100 ng)	$10.88 \pm 1.16^*$

* $p < 0.05$ relative to Saline treated mice ($n = 8-10$ mice per group).

response to the acute 1.0 ng mTNF- α dose and also in mice reexposed to the cytokine after 1 and 7 days (Figure 3.5). However, metabolite concentrations were at control levels upon cytokine reexposure 28 days after initial mTNF- α treatment. Curiously, 5-HIAA levels were not elevated by the 50 ng mTNF- α dose, suggesting that lower icv doses may be optimal in re-eliciting the monoamine changes.

Insert Figure 3.5 about here

Much like NE and 5-HT, PVN DA levels did not vary as a function of the mTNF- α treatments. However, concentrations of the DA metabolite, DOPAC, were significantly affected by the cytokine, $F(6, 45) = 7.38$, $p < 0.01$. Follow-up comparisons revealed that acute treatment with mTNF- α did not influence DOPAC accumulation. However, in mice initially treated with mTNF- α , a cytokine challenge 7 days later increased DOPAC levels, over and above that observed in mice treated with saline or a single mTNF- α injection on test day (Figure 3.5). A sensitization effect with respect to DOPAC levels was not evident at the 28 day interval, in that the accumulation of the metabolite following cytokine treatment was comparable to that of acutely treated mice.

Although NE concentrations within the median eminence/arcuate nucleus (ME/ARC) were not affected by icv mTNF- α , the accumulation of MHPG varied as a function of this treatment, $F(6, 50) = 2.70$, $p < 0.05$. The multiple comparisons revealed increased MHPG levels in response to the 50 ng mTNF- α relative to that of saline-treated

Figure 3.5. Concentrations of MHPG (top), 5-HIAA (middle) and DOPAC (bottom) within the PVN (mean $\pm$ SEM) among mice receiving the icv mTNF- α treatments. Mice were pretreated with either saline (gray bars) or mTNF- α (50 ng; icv; hatched bars). After a 28 day interval, saline pretreated animals received a second injection of the vehicle or mTNF- α (1.0 or 50 ng; icv) (left bars). Animals initially treated with mTNF- α were reexposed to the cytokine either 1, 7 or 28 days later (1.0 ng; icv), or received saline 28 days following the initial injection (right bars). * $P < 0.05$ relative to saline only treated mice, $^{\circ} P < 0.05$ relative to mice receiving saline pretreatment followed by acute TNF- α 20 minutes prior to decapitation.

mTNF- α icv Reexposure Treatment

mice. Treatment with 1.0 ng of the cytokine was without effect, irrespective of whether or not mice had previously been treated with mTNF- α . While 5-HT levels within the ME/ARC were not influenced by the cytokine treatments, 5-HIAA accumulation was affected, $F(6, 48) = 5.33$, $p < 0.05$. Specifically, both the 1.0 and 50 ng doses of mTNF- α increased 5-HIAA levels above those of saline-treated animals. Likewise, as shown in Figure 3.6, in mice pretreated with mTNF- α (50 ng) and then reexposed to the low 1.0 ng dose of the cytokine 7 or 28 days later, MHPG levels exceeded those of saline infused animals. Although there was a modest non-significant increase of 5-HIAA levels in mice reexposed to TNF- α after the 7 day interval, the effects of reexposure to the cytokine did not exceed that seen in animals that received the single 1.0 ng dose of the cytokine (i.e., a sensitization effect was not evident).

The effect of the mTNF- α treatments on ME/ARC DA levels was just shy of statistical significance, $F(6, 51) = 2.22$, $p = 0.06$. However, as can be seen in Figure 3.6, accumulation of DOPAC was significantly altered by icv mTNF- α , $F(6, 42) = 5.53$, $p < 0.01$. Indeed, levels of the metabolite within the ME/ARC were increased in mice that were reexposed to the cytokine 7 or 28 days following initial icv mTNF- α treatment. Moreover, the augmented DOPAC accumulation was not only greater than that of mice that received saline on test day, but also exceeded that of animals infused with a single 1.0 ng dose of mTNF- α at this time. Thus, it appears that a time-dependent sensitization of DA activity within this brain region is elicited by icv mTNF- α treatment.

Insert Figure 3.6 about here

Neither NE nor MHPG levels within the locus coeruleus were altered by the various mTNF- α regimens. Likewise, levels of NE, DA and 5-HT and their respective metabolites, MHPG, DOPAC and 5-HIAA, were not altered within the PFC in response to the cytokine treatments.

While NE levels within the central amygdala were unaffected by the mTNF- α treatments, MHPG accumulation varied as a function of Cytokine exposure, $F(6, 50) = 3.91$, $p < 0.01$. The multiple comparisons revealed that the acute 1.0 and 50 ng doses of mTNF- α provoked a modest (~ 40 %) but non-significant rise of MHPG relative to control levels. However, mice reexposed to mTNF- α 1 day after previous administration of the cytokine, displayed levels of the metabolite significantly greater than that of saline treated animals (Table 3.6). Curiously, mTNF- α pretreated mice administered saline 1 day later had slightly reduced MHPG concentrations relative to animals that received saline on both occasions. Unlike the changes of MHPG associated with the cytokine treatments, the ANOVAs indicated that amygdaloid 5-HT and 5-HIAA did not vary as a function of mTNF- α administration (Table 3.6). Nevertheless, it is noteworthy that 5-HIAA accumulation in mice challenged with mTNF- α 7 days following initial cytokine exposure was elevated by 130% and 87% relative to mice that had received saline or acute mTNF- α (1.0 ng), respectively.

Figure 3.6. Mean ($\pm$ SEM) concentrations of MHPG (top), 5-HIAA (middle) and DOPAC (bottom) within the median eminence/arcuate nucleus complex among mice receiving the icv TNF- α treatments. Mice were pretreated with either saline (gray bars) or mTNF- α (50 ng; icv; hatched bars). After a 28 day interval, saline pretreated animals received a second injection of the vehicle or mTNF- α (1.0 or 50 ng; icv) (left bars). Animals initially treated with mTNF- α were reexposed to the cytokine either 1, 7 or 28 days later (1.0 ng; icv), or received saline 28 days following the initial injection (right bars). * $P < 0.05$ relative to saline only treated mice, ° $P < 0.05$ relative to mice receiving saline pretreatment followed by acute TNF- α 20 minutes prior to decapitation.

mTNF- α icv Reexposure Treatment

Experiment 5: Plasma corticosterone and central monoamines following icv mTNF- α in mice initially treated with i.p. mTNF- α

As in the preceding experiments, icv mTNF- α did not influence ratings of sickness but significantly increased plasma corticosterone concentrations, $F(1, 34) = 20.44$, $p < 0.01$. Unlike the effects seen in mice treated systemically with the cytokine and then reexposed to the same treatment (Experiment 1), there was no indication of i.p. mTNF- α influencing the response to icv mTNF- α administered 7 or 28 days later (Table 3.7).

Insert Tables 3.6 and 3.7 about here

As in Experiments 3 and 4, where both treatments involved icv administration, when mice received i.p. followed by icv mTNF- α treatments, PVN NE levels were unaffected; however, accumulation of MHPG within this region was significantly altered by the mTNF- α treatment regimen, $F(4, 34) = 2.69$, $p < 0.05$. As shown in Figure 3.7, in mice that received the 100 ng mTNF- α infusion 7 days following either saline or i.p. mTNF- α (2.0 μ g) pretreatment, PVN MHPG levels exceeded those of animals treated with saline on the two occasions. Likewise, 5-HT levels within the PVN were unaffected by the mTNF- α infusions, but accumulation of 5-HIAA was influenced by the cytokine manipulations, $F(4, 34) = 2.85$, $p < 0.05$. Although a single acute icv mTNF- α injection provoked only a modest non-significant rise of 5-HIAA levels (~ 60%), reexposure to the

Table 3.6. Mean $\pm$ SEM MHPG and 5-HIAA concentrations (ng/mg protein) within the central amygdala as a function of the initial and reexposure (1, 7 and 28 days) icv mTNF- α treatments.

Initial treatment	Reexposure challenge	MHPG	5-HIAA
Saline	Saline	4.72 $\pm$ 0.57	12.87 $\pm$ 1.74
Saline	TNF (1.0 ng)	6.77 $\pm$ 1.25	16.19 $\pm$ 4.13
Saline	TNF (50.0 ng)	7.94 $\pm$ 1.81	20.75 $\pm$ 3.31
TNF (50.0 ng)	Saline	3.33 $\pm$ 0.34	16.09 $\pm$ 4.29
TNF	TNF (1 d)	8.82 $\pm$ 1.36*	24.07 $\pm$ 3.44
TNF	TNF (7 d)	4.37 $\pm$ 0.88	30.25 $\pm$ 5.44
TNF	TNF (28 d)	5.42 $\pm$ 0.81	23.69 $\pm$ 6.78

*p < 0.05 relative to Saline-Saline treated mice (n = 8-10 mice per group).

Table 3.7. Plasma corticosterone (mean $\pm$ SEM) following icv mTNF- α (100 ng) in mice pretreated 7 or 28 days earlier with i.p. mTNF- α (2.0 μ g).

Injection 1 & 2	Interval (days)	Corticosterone (μ g/dl)
Saline - Saline	7	5.46 $\pm$ 1.00
Saline - TNF	7	8.09 $\pm$ 0.84*
TNF - Saline	7	5.28 $\pm$ 0.80
TNF - TNF	7	10.05 $\pm$ 0.64*
TNF - TNF	28	9.79 $\pm$ 1.16*

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*p < 0.05 relative to Saline-Saline treated mice (n = 8-10 mice per group).

cytokine 7 days following systemic mTNF- α administration significantly increased metabolite concentrations above those of saline treated mice (Figure 3.7).

Insert Figure 3.7 about here

As in the case of the PVN, mTNF- α influenced monoamine activity within the ME/ARC complex. The accumulation of MHPG was not significantly affected by 100 ng of icv mTNF- α , although acute cytokine administration provoked a modest (33%) increase of the metabolite. The levels of NE, however, were found to vary significantly with the cytokine administration, $F(4, 36) = 2.64$, $p < 0.05$. The multiple comparisons indicated that relative to saline treated mice, NE levels were reduced in animals that initially received i.p. administration of mTNF- α , and then reexposed to the cytokine (icv) either 7 or 28 days later. In contrast to the NE changes, 5-HT levels were not significantly affected by the cytokine treatments, whereas the accumulation of 5-HIAA was markedly altered, $F(4, 33) = 6.88$, $p < 0.01$. The multiple comparisons confirmed that relative to saline treatment on test day, acute icv administration of mTNF- α significantly increased 5-HIAA accumulation. In mice that had received i.p. mTNF- α followed 7 days later by icv mTNF- α , levels of 5-HIAA exceeded those of saline treated mice, but were only moderately higher than that of animals that received acute mTNF- α on the test day. However, when the two cytokine treatments were spaced 28 days apart, the accumulation of 5-HIAA significantly exceeded that of mice that received the cytokine only on the test

Figure 3.7. Mean ($\pm$ SEM) concentrations of MHPG (top) and 5-HIAA (bottom) within the PVN among mice receiving systemic followed by central TNF- α treatments. Mice were systemically pretreated with either saline or mTNF- α (2.0 μ g ng; i.p.) and subsequently received central infusion of saline or the cytokine. Reexposure to mTNF- α (100 ng; icv) occurred either 7 or 28 days after the initial treatment later (right bars). * $P < 0.05$ relative to saline only treated mice.

day. Thus, a sensitization was induced by the initial systemic treatment and this effect became more pronounced with the passage of time (see Figure 3.8).

Insert Figure 3.8 about here

Within the ME/ARC both DA and DOPAC were significantly altered by the cytokine treatment regimen, $F(4,39) = 3.38$ and 5.12 , P 's < 0.05 . The multiple comparisons indicated that acute icv mTNF- α did not affect DOPAC and elicited a small non-significant rise of DA levels. In mice that were treated with i.p. mTNF- α and then reexposed to icv mTNF- α 7 days later, DA levels were significantly increased, while the rise of DOPAC approached significance ($p < 0.07$). However, as shown in Figure 3.8, when these treatments were applied 28 days apart the elevations of DOPAC and DA were both statistically significant, and exceeded that of mice that had received i.p. saline followed by icv mTNF- α on the test day. Curiously, among mice that received i.p. mTNF- α followed 7 days later by icv saline, the levels of DA and DOPAC were both elevated, as well (Figure 3.8). Whether this reflected a carry-over effect of the initial treatment or amine changes elicited by the stress of injection is not known.

It will be recalled that in Experiment 4, where the two mTNF- α treatments were administered icv, a sensitization effect was not apparent within the locus coeruleus. In contrast, NE and MHPG within this brain region were significantly altered when the initial mTNF- α treatment was administered i.p. followed by icv reexposure treatment, F 's $(4, 38) = 3.35$, $p < 0.05$ and $(4,38) = 9.95$, $p < 0.01$, respectively. The multiple

Figure 3.8. Mean ($\pm$ SEM) concentrations of MHPG (top), 5-HIAA (middle) and DOPAC (bottom) within the median eminence/arcuate nucleus complex among mice that received systemic followed by central TNF- α treatments. As in Figure 7, mice were systemically pretreated with either saline or mTNF- α (2.0 μ g; i.p.) and subsequently treated with central infusion of either saline or mTNF- α (100 ng; icv). The latter treatments were administered either 7 or 28 days after initial cytokine treatment (right bars). * $P < 0.05$ relative to saline only treated mice, $^{\circ}P < 0.05$ relative to mice that received saline pretreatment followed by acute TNF- α 20 minutes to decapitation.

comparisons revealed that acute icv mTNF- α administered on the test day did not affect NE or MHPG levels. However, upon icv reexposure to mTNF- α 28 days following i.p. pretreatment with the cytokine, NE levels were reduced relative to that observed in mice that received icv saline infusion on the test day. Furthermore, when icv mTNF- α was administered either 7 or 28 days following an initial i.p. dose of the cytokine, MHPG accumulation within the locus coeruleus was increased beyond that observed in mice that received saline or acute mTNF- α , indicating a sensitization of NE activity (see Figure 3.9).

Insert Figure 3.9 about here

Within the PFC, neither NE, DA nor their respective metabolites, MHPG and DOPAC, varied as a function of the mTNF- α treatments. Likewise, while there was some evidence of mTNF- α induced alterations of 5-HT and 5-HIAA levels, these results were highly variable. Finally, within the central amygdala the cytokine treatment had limited effects on monoamine activity and levels, with the exception of DOPAC which was altered by the mTNF- α regimen, $F(4, 39) = 3.22$, $p < 0.05$. The multiple comparisons indicated that acute icv mTNF- α increased DOPAC accumulation. This effect was not further enhanced among mice that had previously received i.p. treatment with the cytokine, and was in fact slightly reduced when the icv treatment followed i.p. mTNF- α by 7 days.

Figure 3.9. Mean ($\pm$ SEM) concentrations of MHPG within the locus coeruleus among mice receiving systemic followed by central TNF- α treatments. Mice were systemically pretreated with either saline or mTNF- α (2.0 μ g; i.p.) and subsequently treated with central infusion of either saline or mTNF- α (100 ng; icv). The latter treatments were administered either 7 or 28 days after initial cytokine treatment (right bars). * $P < 0.05$ relative to saline only treated mice, $^{\circ}P < 0.05$ relative to mice that received saline pretreatment followed by acute TNF- α 20 minutes to decapitation.

Discussion

Behavioral effects of mTNF- α

Commensurate with the effects of human TNF- α (Hayley et al., 1999), systemic treatment with the murine form of this cytokine provoked dose-dependent signs of sickness upon reexposure to the cytokine 28-days later, but not at a 1-day interval. When administered icv, illness was not evident, even after supramaximal doses. Thus, it appears that TNF- α activates peripheral targets to produce sickness, possibly reflecting a shock reaction. As will be discussed shortly, mTNF- α markedly influences neuroendocrine and central neurotransmitter processes even in the absence of sickness, indicating that these effects were not secondary to malaise.

Effects of mTNF- α on Plasma Corticosterone

Like hTNF- α (Ando and Dunn, 1999; Bernardini et al., 1990; Hayley et al., 1999), corticosterone levels were increased following systemic mTNF- α administration. Paralleling the sickness behaviors, the corticosterone increase induced by systemic mTNF- α was subject to a time-dependent sensitization, being enhanced upon reexposure after the 28, but not the 1-day interval. In earlier studies this profile was apparent using human TNF- α ; however, a desensitization was evident (i.e. corticosterone levels were reduced compared to acute treatment) upon reexposure at the 1-day interval (Hayley et al., 1999). As only the murine form of the cytokine activates both the p55 and p75 receptors (the human form activates only the p55 receptor) (Beutler, 1999), it is possible

that the desensitization involves the differential activation of these two receptor subtypes. Although the p55 receptor may play a more prominent role than p75 in provoking the central effects of TNF- α (Benigni et al., 1996; Sipe et al., 1996), co-activation of these two receptors may be important for certain processes, including systemic toxicity (Brouckaert et al., 1989).

Like systemic administration, icv mTNF- α also increased plasma corticosterone levels, but the increase was considerably less pronounced, even at pathophysiological doses. Indeed, as the effects of the cytokine did not increase in a dose dependent fashion, it is possible that the optimal effects of the cytokine would have been realized at still lower doses. Moreover, in contrast to systemic mTNF- α , icv treatment did not induce the sensitization of HPA activity, suggesting that this effect does not involve direct actions of mTNF- α within the brain.

In assessing the expression of a sensitization in response to mTNF- α , it needs to be considered that two distinct phases (or components) are necessarily involved. Specifically, the sensitization of neuronal processes must be established upon initial cytokine treatment, and once established, an appropriate challenge is required for the elicitation of the effect. Given that augmented plasma corticosterone release is readily evident in mice that received mTNF- α on two occasions 28 days apart indicates that the initial intraperitoneal treatment effectively established a sensitized system. However, in mice that received systemic mTNF- α , the augmented corticosterone response was not subsequently elicited by central cytokine administration, suggesting that the manifestation of the enhanced corticosterone release 28 days later involves peripheral factors. Indeed, TNF- α may directly influence the pituitary (Kobayashi et al., 1997) and

the adrenal (Ritchie et al., 1996), and it should be considered that the sensitized corticosterone response may involve direct effects at these sites.

At this juncture it is important to introduce a caveat concerning conclusions regarding the site of action of the cytokine sensitization. It is likely that following icv administration the distribution of TNF- α within the brain, as well as its binding with specific receptors, would be different from that induced by i.p. treatment. In particular, typically only around 0.1% of systemically administered cytokines reach the brain parenchyma (Guitierrez et al., 1993) and would be concentrated to a greater extent in regions accessible through the vascular system (e.g., circumventricular organs). It has been demonstrated (Laflamme & Rivest, 1999; Nadeau & Rivest, 1999) that TNF- α receptors are particularly dense at such vascular sites and stimulation of these receptors was reported to induce prostaglandin synthesis. As well, stimulation of vagal afferents by systemically administered TNF- α may activate synthesis of cytokines or other neuroactive factors within the nucleus of the solitary tract or other brain regions, while icv infusion of TNF- α may have limited effects in this respect. In light of these considerations, it is exceedingly difficult to equilibrate the effective dose of TNF- α that reaches the various brain regions via the central and peripheral injection sites and hence, one cannot readily draw definitive conclusions regarding the site of action for the cytokine-induced sensitization of corticosterone activity. For the moment, however, it is clear that despite the fact that icv TNF- α elicits an increase of corticosterone, this effect is not subject to a sensitization like that seen after ip treatment, despite the fact that the challenge doses (0.1 μ g by ip administration and 1-100 ng by icv) elicited comparable effects. At this juncture, one cannot rule out a central site of action for the sensitization

elicited by TNF- α , particularly as reexposure to the cytokine provoked enhanced monoamine functioning within the PVN and ME/ARC.

Central neurotransmitter variations

Systemic mTNF- α provoked increased NE utilization within the PVN, locus coeruleus, PFC, central amygdala and medial amygdala, as well as 5-HIAA accumulation within the PVN and medial amygdala. These data extend the previous findings concerning hTNF- α (Ando and Dunn, 1999; Hayley et al., 1999), and indicate that mTNF- α influences both hypothalamic and extrahypothalamic neuronal activity. Furthermore, central mTNF- α administration increased 5-HT utilization in PVN, ME/ARC, central amygdala and to some degree in hippocampus, while NE and DA utilization were increased within the PVN and ME/ARC. Clearly, the latter effects were not entirely congruent with those induced by systemic cytokine administration. Whether this stemmed from differences of mTNF- α entry into brain following systemic vs icv treatment (Gutierrez et al., 1993), differences in dispersion across brain tissues, or even toxicity factors, remains to be established. However, it is possible that stimulation of vagal and/or spinal projections (Dantzer et al., 2000; Hosoi et al., 2000; Maier et al., 1998) or enzymatic alterations at peripheral target sites (e.g liver) (Libert et al., 1991), contribute to the effects of peripheral but not central cytokine administration.

As in the case of the human TNF- α (Hayley et al., 1999), amine utilization was augmented upon subsequent reexposure to mTNF- α . The sensitization, which varied across brain regions, was time-dependent, and was most notable with respect to 5-HT utilization. Within the PFC and central amygdala, reexposure to mTNF- α 1 day

following initial treatment enhanced 5-HIAA accumulation, while such an effect was evident within the medial amygdala at both the 1 and 28 day reexposure intervals. The temporal profile for these sensitization effects corresponded with those previously observed using the human form of the cytokine. Further, commensurate with the corticosterone variations, the sensitization effects within the PVN were most prominent at the 28 day interval. The finding that the sensitization was limited to 5-HT neuronal processes, whereas use of recombinant human TNF- α was previously shown to sensitize NE activity, may reflect differential potency and dosage effects, or species-typical cytokine actions.

While the central administration of mTNF- α did not elicit sensitization with respect to sickness or HPA changes, under these conditions NE and DA utilization were markedly enhanced within the PVN, as was DA utilization within the ME/ARC. However, there was no indication of a sensitization with respect to locus coeruleus NE activity. Interestingly, however, if mice initially received the cytokine systemically and then were reexposed via an icv route, a sensitization was apparent with respect to 5-HT and DA activity within the ME/ARC and NE within the locus coeruleus. Thus, it seems that the sensitizing effects of systemic and central mTNF- α are readily distinguishable between hypothalamic and extra-hypothalamic structures. Although either route of administration may prime ME/ARC neurons, the PVN sensitization appears to be more closely aligned with central cytokine infusion. It may be that circulating mTNF- α sensitizes ME/ARC but not PVN aminergic neurons because of easier access to this site. Interestingly, sensitization of locus coeruleus NE activity was apparent in mice that initially received ip treatment with mTNF- α and then challenged by icv cytokine

treatment. It is not known why the sensitization within this region required an initial peripheral challenge with mTNF- α , but the effect may involve activation of sympathetic fibers innervating brainstem sites, including the locus coeruleus and nucleus of the solitary tract (Van Bockstaele et al., 1999). As mentioned earlier with respect to the immediate effects of mTNF- α (Dantzer et al., 2000; Maier et al., 1998) it is certainly possible that vagal afferents also contribute to the sensitization effect.

Stressor-like effects of mTNF- α

As cytokines, such as IL-1 β , may elicit “stressor-like” effects (Anisman and Merali, 1999; Dunn, 1995), it was of interest to establish whether mTNF- α would also promote such an outcome. It will be recalled that with the passage of time following IL-1 β or stressor exposure, the HPA response to subsequent challenges are augmented, possibly owing to increased CRH and AVP co-expression within median eminence terminals (Schmidt et al., 1995, 1996; Tilders and Schmidt, 1998). Similarly, hTNF- α enhanced median eminence CRH and AVP immunoreactivity and co-localization, peaking 7-14 days after hTNF- α exposure. In effect, it appeared that hTNF- α provoked effects akin to those of IL-1 β and footshock, but the time course for the effects differed somewhat. Furthermore, given that the corticosterone sensitization was most prominent upon cytokine reexposure 28 days after initial treatment, peptide co-expression likely did not underlie the sensitization of the corticosterone response (Hayley et al., 2001a).

It is known that stressors trigger DA alterations within the arcuate nucleus (Kobayashi et al., 1976), hence stimulating prolactin release (Freeman et al., 2000; Gunnet and Moore, 1988; Lookingland et al., 1987). In the present investigation icv

mTNF- α likewise affected DA neuronal functioning within the ME/ARC complex, although this effect was pronounced only upon cytokine reexposure. These findings, coupled with the sensitization of PVN neuronal activity in response to icv mTNF- α treatment, points to the fact that neuronal plasticity is elicited by direct cytokine challenges and provisionally implicates hypothalamic functioning as important for cytokine-elicited behavioral changes. It remains to be established whether cross-sensitization occurs between TNF- α and psychogenic stressors, as observed with respect to IL-1 β (Schmidt et al., 1995).

In addition to the hypothalamus, the medial amygdala appears to be particularly stressor reactive (Martinez et al., 1998; McIntyre et al., 1999; Palkovits, 2000). However, the effects of footshock on amygdaloid *c-fos* expression were distinguishable from those of IL-1 β , in that the cytokine effects were restricted to the central amygdala, whereas footshock increased *c-fos* immunoreactivity within the basolateral and medial nuclei (Sawchenko et al., 1996). In the present study acute mTNF- α influenced central amygdala monoamine functioning, while having limited effects within the medial amygdala. Interestingly, upon cytokine reexposure 5-HT activity was increased within the medial amygdala, provoking a stressor-like effect. Of course, it is premature to dismiss the possibility that the initial cytokine and reexposure treatments promote qualitatively different neuronal changes. Nevertheless, given that cytokine challenges promote outcomes reminiscent of those associated with stressors and with depressive illness, particularly with respect to neuroendocrine and central monoamine activity, the present findings are consistent with the view that cytokines may contribute to psychological disturbances (Griffiths et al., 2000; Maes, 1999).

Chapter IV

Murine tumor necrosis factor- α sensitizes plasma corticosterone activity and the manifestation of shock: Modulation by histamine.

Abstract

Murine tumor necrosis factor-alpha (mTNF- α) results in the sensitization of mechanisms underlying plasma corticosterone activity and sickness behavior, the latter being reminiscent of septic or anaphylactic shock. The mTNF- α induced sensitization of sickness and corticosterone are attenuated by pretreatment with the combinations of histamine H₁ (diphenhydramine, mepyramine) and H₂ (cimetidine) antagonists. Likewise, co-administration of diphenhydramine and cimetidine prevented the mTNF- α provoked rise of monoamine activity within the posterior hypothalamus. Although dexamethasone ameliorated the mTNF- α induced sensitization of corticosterone, illness behavior was unaffected. It is suggested that mTNF- α induced illness and the neuroendocrine sensitization are mediated by endogenous histamine.

Introduction

Proinflammatory cytokines, such as IL-1 β and mTNF- α , promote increased central monoamine functioning at hypothalamic and extrahypothalamic sites, including the prefrontal cortex and central nucleus of the amygdala (Dunn, 1992; Lacosta et al., 1998; Hayley et al., 1999). As well, these cytokines enhance the expression and release of the hypothalamic neuropeptides, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), hence promoting release of pituitary ACTH and adrenal corticosteroids (Hayley et al., 2001a; Bernardini et al., 1990; Dunn, 1992; Linthorst et al., 1995; Tilders & Schmidt, 1998). These neurochemical effects are accompanied by signs of illness, including lethargy, ptosis, piloerection, fever and curled body posture (Bluthe et al., 1994; Dantzer et al., 1998; Hayley et al., 1999, 2001b).

In addition to eliciting acute effects, cytokines may proactively influence neurochemical and behavioral responses elicited upon their subsequent reexposure (sensitization). Interestingly, the development of the sensitization appears to be dependent upon the passage of time. In particular, upon re-exposure to the TNF- α at relatively brief intervals (1-14 days) the neuroendocrine and sickness response elicited by the cytokine is not enhanced. However, when reexposure occurred 28 days following initial TNF- α treatment the levels of plasma corticosterone were markedly increased and sickness elicited by the cytokine was exaggerated (Anisman et al., 2001; Hayley et al., 1999, 2001b).

Although the precise mechanism through which circulating cytokines, such as TNF- α , affect behavioral and neurochemical processes is unclear, it has been suggested that activation of peripheral nerve afferents or the induction of secondary chemical messengers may serve in this capacity. In particular, induction of vasoactive inflammatory factors, including prostaglandins or histamine, may be important in modulating bidirectional immune - brain interactions (Igaz et al., 2001). Indeed, receptors for TNF- α are found on mast cells (primary producers of histamine) and stimulation with the cytokine provokes histamine release (Brzezinska-Blaszczyk et al., 2000). Further, TNF- α is produced by mast cells under basal conditions and secretion of the cytokine from these cells is increased in response to immune activation or stimulation by neuropeptides (Cocchiara et al., 1997; Iuvone et al., 1999).

In addition to being localized in peripheral and central mast cells, histamine is also produced by neurons located within the tuberomammillary nucleus of the posterior portion of the hypothalamus (Brown et al., 2001; Schwartz et al., 1991). Like other monoamines, histamine acts as a neurotransmitter, thereby affecting behavioral and hormonal outputs. For instance, hypothalamic H₁ histamine receptors may regulate feeding (Doi et al., 1994; Masaki et al., 2001), while central H₃ receptors influence performance in cognitive tasks (object recognition, passive avoidance), and elicit behaviors that may reflect a depressive-like state (e.g., diminished active responding in a forced swim test) (Giovannini et al., 1999; Perez-Garcia et al., 1999). In addition, both H₁ and H₂ receptors have been implicated in stimulation of the HPA axis, as agonists for these receptors increase CRH mRNA within the paraventricular nucleus of the

hypothalamus (PVN), coupled with elevated levels of plasma adrenocorticotrophic hormone (ACTH) and corticosterone (Kjaer et al., 1998; Tsujimoto et al., 1993).

In addition to these neuroendocrine variations, mediators of shock, including platelet activating factor, acute phase proteins, eicosanoids, as well as histamine, are increased by TNF- α (Kuna & Kaplan, 1996; Quin & Slotman, 1999). Further, levels of TNF- α and histamine may be concomitantly elevated in cases of extreme immune challenge or shock, and antihistamine treatments often improve clinical outcomes under such conditions (Dimmeler et al., 1995). In addition to its regulatory actions in peripheral inflammation, histamine may play a prominent role in neuroinflammatory responses (Bebo et al., 1996), and has been implicated in brain inflammation associated with neurological disease (e.g. multiple sclerosis, Parkinson's disease), viral or bacterial infections, and traumatic head injury (Mogi et al., 1994; Pan et al., 1999; Issazadeh et al., 1995).

In light of the similarity that exists between the sickness symptom profile evident in TNF- α sensitized mice and that apparent in clinical shock, it was of interest to determine if the sensitization elicited by this cytokine would be attenuated by antihistamine treatment, just as such treatment affects shock-like reactions. As well, it was predicted that treatment with H₁ and H₂ histamine antagonists would attenuate neurochemical effects manifested in mTNF- α reexposed animals. In addition, owing to the potent anti-inflammatory and neurochemical consequences of corticoids, the impact of dexamethasone on the mTNF- α elicited sensitization effects were also determined.

Materials and Methods

Male, CD-1 mice were obtained from Charles River Inc, Laprairie Quebec at 8-10 weeks of age, and were housed in groups of four in standard polypropylene cages for 2 weeks before serving as experimental subjects. Animals were maintained on a 12 hour light/dark cycle with lights on at 0800, room temperature was kept at 21° C, and mice were permitted an ad libitum diet of Ralston Purina mouse chow. All experimental procedures were approved by the Carleton University Committee for Animal Care and met the Guidelines set out by the Canadian Council on Animal Care.

Experiment 1: Effects of antihistamine treatment on mTNF- α induced sensitization of illness, plasma corticosterone and hypothalamic monoamine levels

To avoid confounding associated with diurnal variations of corticosterone, all procedures were conducted between 0800 and 1100 hr. The initial experiment investigated the possibility that histamine mediates some of the sensitizing actions of mTNF- α on sickness behavior, plasma corticosterone levels and central monoamine activity. The mTNF- α obtained from R & D Systems had a specific activity of specific activity = 1.1×10^5 U/ μ g. The cytokine was obtained in lyophilized form and subsequently dissolved in sterile PBS and stored in 20 μ l aliquots at -80°C. Mice were assigned to one of four conditions (n = 7-8/group) and treated on two occasions 28 days apart. Two of these groups initially received ip injection of vehicle followed 28 days later by either vehicle or mTNF- α (1.0 μ g). Two further groups initially received pretreatment with mTNF- α (2.0 μ g) and then reexposed 28 days later to a second ip injection of the cytokine (1.0 μ g). For mice of one of the latter two groups the mTNF- α was administered

one hr after ip treatment with vehicle, while the second group received a cocktail of the H₁ and H₂ antagonists (Cimetidine, 50 mg/kg) and Diphenhydramine (5 mg/kg). All injections were administered in a volume of 0.40 ml of fluid.

One hour following the final injection sickness behavior was monitored in the animal's home cage using a 4-point rating scale. Overall appearance was recorded as follows; 1 = normal looking; 2 = slightly lethargic, ptosis (droopy eyes) *or* piloerection (puffy fur); 3 = very lethargic, ptosis *and* piloerection, curled body posture; 4 = very sick appearance, ptosis, piloerection, curled body posture, difficulty breathing, and general nonresponsiveness. As well, the scratching that animals displayed was recorded as this may have been related to increased release of histamine from mast cells. In earlier studies (Hayley et al., 1999) the inter-rater reliability for these behavioral assessments was found to exceed 90%.

Immediately following behavioral ratings, mice were decapitated and trunk blood was collected in chilled tubes containing 10 µl EDTA. Blood was centrifuged (3500 rpm for 15 minutes), and the supernatant stored at -80°C for subsequent determination of plasma corticosterone concentrations. Additionally, brains were rapidly removed, dissected to remove the posterior hypothalamus, which was then frozen at -80°C until processing for norepinephrine (NE) and serotonin (5-HT), and their respective metabolites, MHPG and 5-HIAA. This brain region was of particular interest because it contains the brain's histamine producing neurons and previous reports indicated that histaminergic activity in this region is particularly sensitive to stressor or immunological challenges (Brown, 2001).

Experiment 2: Histamine receptor involvement in the behavioral and neuroendocrine sensitization elicited by mTNF- α

The second experiment represents a more detailed profile of the effects of several antihistamines on reexposure to mTNF- α and was designed to investigate which specific histamine receptors may be involved in the sensitizing effects of this cytokine. Mice were assigned to one of seven conditions ($n = 7$ -8/group); two groups received ip injection of vehicle, while the remaining five groups were treated with mTNF- α (2.0 μ g, ip) in a volume of 0.40 ml. At 28 days following the initial injection, animals of one of the vehicle pretreated groups was again treated with the vehicle, while the mice of the second group received ip injection of the mTNF- α (1.0 μ g). Mice initially pretreated with mTNF- α (28 days earlier) were reexposed to a lower dose of the cytokine (1.0 μ g), which is ordinarily sufficient to elicit a sensitized response (Hayley et al., 2001a). In an attempt to ascertain the involvement of histamine in the effects of mTNF- α reexposure, various antihistamine treatments were administered before reexposure to the second dose of the cytokine. Specifically, 1 hr prior to mTNF- α reexposure, mice received ip treatment with either vehicle, the H₂ antagonist (Cimetidine, 50 mg/kg), the H₁ antagonist (Mepyramine, 10 mg/kg), an alternate H₁ antagonist (Diphenhydramine, 5 mg/kg) or a cocktail of the H₁ and H₂ antagonists (Mepyramine, 10 mg/kg + Cimetidine, 50 mg/kg).

As the preceding study assessed the effects of the histamine antagonists only on sensitized responses, an additional study assessed the effects of this treatment on the acute actions of mTNF- α . However, as acute treatment with mTNF- α increases plasma corticosterone, without engendering sickness (Hayley et al., 1999, 2001a) the effects of the H₁ and H₂ antagonists were restricted to evaluation of cytokine action on plasma

corticosterone. Mice of Experiment 2b were assigned to one of 10 treatment conditions ($n = 8/\text{group}$). These mice received a 0.40 ml volume of either (1) vehicle, (2) acute mTNF- α (1.0 μg), (3) the H_2 antagonist, Cimetidine (50 mg/kg), (4) the H_1 receptor antagonist, Diphenhydramine (5 mg/kg), (5) the alternate H_1 receptor antagonists (Mepyramine, 10 mg/kg) or (6) a cocktail of Cimetidine (50 mg/kg) plus Diphenhydramine (5 mg/kg). The remaining 4 groups of mice received acute mTNF- α (1.0 μg , ip), but were pretreated 1 hr earlier with either the H_2 receptor antagonist, Cimetidine (50 mg/kg), the H_1 receptor antagonist, Diphenhydramine (5 mg/kg) or that of Mepyramine (10 mg/kg), or a cocktail of the antihistamines, Cimetidine (50 mg/kg) plus Diphenhydramine (5 mg/kg). As in the preceding experiments, 1 hr following the final injection, mice were decapitated and trunk blood collected for subsequent determination of plasma corticosterone concentrations.

Experiment 3: Dexamethasone effects on mTNF- α induced shock and corticosterone sensitization

Through its negative feedback effects, dexamethasone elicits an reduction of adrenal glucocorticoid release, and diminished liberation of cytokines, including IL-1 β and mTNF- α , and other inflammatory factors, from peripheral immune cells and brain astrocytes (Gow et al., 2001; Brenner et al., 1993). Given that the sensitized behavioral response to mTNF- α reexposure may reflect a shock reaction involving HPA and immune activity, it was of interest to establish whether pretreatment with the synthetic corticoid, dexamethasone, would attenuate the behavioral sickness elicited by cytokine reexposure. Mice were pretreated i.p. with either vehicle or mTNF- α (2.0 μg), and then

28 days later subdivided ($n = 10/\text{group}$) and treated with either sterile vehicle or a reduced dosage of mTNF- α (1.0 μg , i.p.). Each of these groups received i.p. saline 6 hr before the latter treatment. A separate set of animals also received the two mTNF- α treatments 28 days apart, but 6 hr prior to the second mTNF- α injection mice received dexamethasone HCl (1.0 mg/kg, i.p.). The dosage of dexamethasone was based on reports showing it to be effective in attenuating septic shock (Hattori et al., 1998). At 1 hr following the second injection mice were decapitated and trunk blood was collected and centrifuged (3500 rpm for 15 minutes) for subsequent corticosterone determinations.

As in the preceding studies the sickness behavior displayed by mice was recorded. Additionally, a crude index of reduced blood volume (hypovolemia) and pressure (hypotension), both of which are characteristic of shock, were also obtained. In particular, immediately after decapitation trunk blood was drawn in a graded 1-ml syringe and the volume obtained recorded and taken as a general index of hypovolemia/hypotension. In addition, the distribution of blood may be altered during acute phase responses, with a preferential pooling of blood in certain visceral or limb regions (Hardaway, 2000; Kettelhut et al., 1987). Thus, the sickness ratings also included assessment of the reddening of the tail, ears and nose, reflecting increased accumulation of blood cells and inflammation (cyanosis).

Plasma corticosterone assay

Plasma corticosterone levels were determined (in duplicate) using radioimmunoassay (RIA) kits (ICN Biomedicals Inc., CA). The intra-assay variability

was less than 10%, and in order to preclude inter-assay variability, analyses of all samples were conducted in a single run.

Brain dissection and HPLC procedure for analysis of brain amine and metabolite levels

Freshly dissected brains were placed on a stainless steel block with slots (spaced approximately 500 μm apart) that served as guides for razor blades, which were used to provide a series of coronal brain sections. Brain sections were placed in ice cold PBS and utilizing the mouse brain atlas of Franklin and Paxinos (1997), the posterior hypothalamus was excised from the tissue (Bregma -2.18 to -2.70 mm). Hypothalami were then immediately placed in 0.3 M monochloroacetic acid containing 10% methanol plus internal standards and frozen on dry ice for later HPLC determination of monoamines.

Levels of NE and 5-HT, and their respective metabolites, MHPG and 5-HIAA were determined by HPLC using a modification of the method of Seegal et al., 1986. Tissue punches were sonicated in a homogenizing solution comprising 14.17 g monochloroacetic acid, 0.0186g disodium ethylenediamine tetraacetate (EDTA), 5.0 ml methanol and 500 ml H_2O . Following centrifugation, the supernatants were used for the HPLC analysis. Using a waters M-6000 pump, guard column, radial compression column (5m, C18 reverse phase, 8mm x 10cm), and a three cell coulometric electrochemical detector (ESA Model 5100,A) 20 ml of the supernatant was passed through the system at a flow rate of 1.5 ml/min (1400-1600 PSI). The mobile phase used was a modification of that used by Chiueh et al. (1983); each liter consisted of 1.3g of heptane sulfonic acid, 0.1g disodium EDTA, 6.5ml triethylamine, 35ml acetonitrile. The mobile phase was then

filtered (0.22mm filter paper) and degassed following which the pH was adjusted to 2.5 with phosphoric acid. The area and height of the peaks was determined using a Hewlett-Packard integrator. Protein content of each sample was determined using bicinchoninic acid with a protein analysis kit (Pierce Scientific, Brockville, Ontario) and a spectrophotometer (Brinkman, PC800 colorimeter). The lower limit of detection for the monoamines and metabolites was approximately 5.0 pg.

Statistics

All dependent measures (sickness behaviors, plasma corticosterone, brain monoamine and metabolites) were analyzed by either a single or multiple factor analysis of variance (ANOVA), as appropriate. Pairwise Bonferonni corrected t tests were used to evaluate the differences of the means of significant main effects or of the means of simple effects comprising significant interactions. Sensitization was considered statistically present when the mean for mice reexposed to mTNF- α following initial administration of the cytokine, significantly exceeded that of animals challenged with mTNF- α after saline pretreatment.

Results

Experiment 1: Effect of antihistamine treatments on the sensitization of sickness, corticosterone and central monoamines elicited by mTNF- α

The treatments mice received influenced ratings of illness, $F(3, 23) = 17.71$, $p < 0.01$. As seen in Figure 4.1 (upper panel), relative to vehicle treated mice, acute mTNF- α did not affect overall appearance. However, a sensitization effect was apparent, such that

reexposure to the cytokine 28 days after mTNF- α treatment provoked pronounced ptosis, piloerection, curled body posture, and general immobility. Interestingly, these symptoms were greatly attenuated in mice that had been pretreated with the combination of H₁ and H₂ antagonists (diphenhydramine + cimetidine) prior to cytokine reexposure. Indeed, the sickness profile of these mice was indistinguishable from that elicited by acute exposure to the cytokine or by saline treatment.

Paralleling the sickness symptoms, plasma corticosterone levels (Figure 4.1, lower panel) were also influenced by the mTNF- α and antihistamine treatments, $F(3, 24) = 15.45$, $p < 0.01$. The multiple comparisons confirmed that plasma corticosterone levels increased in response to acute mTNF- α , and that this effect was appreciably more pronounced in mice that were reexposed to the cytokine 28 days later. Thus, reexposure to the cytokine elicited a sensitization effect with respect to the steroid release, just as it produced such an outcome with respect to sickness. As in the case of illness, the antihistamine cocktail significantly reduced this sensitization effect, such that levels of corticosterone were not significantly different from those of animals that received a single injection of the cytokine. However, corticosterone levels still exceeded that of vehicle treated mice.

Insert Figure 4.1 about here

The levels of NE and 5-HT within the posterior hypothalamus, and that of their metabolites, MHPG and 5-HIAA, are shown in Figure 4.2 as a function of the cytokine

Figure 4.1. Mean ($\pm$ SEM) ratings of illness (e.g. ptosis, piloerection, curled body posture) (top) and levels of plasma corticosterone (bottom) in response to the mTNF- α treatments. From left to right, mice received saline on two occasions, saline followed by mTNF- α (1.0 μ g) or mTNF- α (2.0 μ g) with subsequent reexposure to mTNF- α (1.0 μ g) with or without antihistamine (diphenhydramine + cimetidine, 5 and 50 mg/kg, respectively) pretreatment 1 hour earlier. All injections were administered i.p with a 28-day interval between the two treatments and measures were made 60 minutes following the second injection. * $P < 0.05$ relative to mice treated with saline on two occasions, ϕ $P < 0.05$ relative to animals pretreated with saline that subsequently received a single acute injection of mTNF- α .

and antihistamine treatments. Neither the NE nor the 5-HT levels were significantly affected by the treatments, whereas the utilization of NE, as reflected by MHPG accumulation, varied as a function of the Treatment condition, $F(3, 49) = 3.92$, $p = 0.05$. The multiple comparisons confirmed that acute mTNF- α , as well as reexposure to the cytokine, increased the metabolite accumulation relative to that seen in vehicle treated mice. However, these groups did not differ from one another indicating that the sensitization evident with respect to neuroendocrine activity was not paralleled by a similar outcome with respect to hypothalamic NE or 5-HT functioning. Among mice pretreated with the antihistamine cocktail the MHPG levels in mice reexposed to mTNF- α were reduced, so that the metabolite levels did not differ from that of mice that had not received the cytokine. The accumulation of 5-HIAA was also elevated somewhat in the posterior hypothalamus (Figure 4.2), but the variability was relatively large, precluding the detection of a significant effect.

Insert Figure 4.2 about here

Figure 4.2. Mean ($\pm$ SEM) levels of MHPG within the posterior hypothalamus in response to the mTNF- α treatments. From left to right, mice received saline on two occasions, saline followed by mTNF- α (1.0 μ g) or mTNF- α (2.0 μ g) with subsequent reexposure to mTNF- α (1.0 μ g) with or without antihistamine (diphenhydramine + cimetidine, 5 and 50 mg/kg, respectively) pretreatment 1 hour earlier. All injections were administered i.p with a 28-day interval between the two treatments and measures were made 60 minutes following the second injection. * $P < 0.05$ relative to mice treated with saline on two occasions.

Experiment 2: Modulation of mTNF- α sensitized sickness and corticosterone effects by specific histamine receptor antagonists

In Experiment 2, ratings of sickness, as reflected by the animal's overall appearance, were influenced by the mTNF- α and antihistamine treatments, $F(6, 54) = 23.95$, $p < 0.01$. As depicted in Figure 4.3, and confirmed by the post hoc tests, acute mTNF- α did not affect ratings of illness; however, reexposure to the cytokine induced a sensitization effect, such that sickness ratings were increased relative to that of mice that received either saline or a single dose of mTNF- α . Although pretreatment with the selective H_1 antagonist, mepyramine, did not affect mTNF- α induced sickness, the alternate H_1 antagonist, diphenhydramine, as well as the H_2 antagonist, cimetidine, both produced modest (~ 35%), but non-significant, reductions in the sickness symptoms provoked by the cytokine (Figure 4.3). The co-administration of mepyramine and cimetidine prior to mTNF- α reexposure engendered a more profound, and statistically significant reduction of the sickness symptoms, such that sickness in these mice was indistinguishable from that evident in saline treated animals.

Insert Figure 4.3 about here

As in the case of sickness, plasma corticosterone concentrations varied as a function of the treatments received, $F(6, 39) = 4.76$, $p < 0.01$. As shown in Figure 4.3,

Figure 4.3. Mean ($\pm$ SEM) ratings of illness (e.g. ptosis, piloerection, curled body posture) (top) and levels of plasma corticosterone (bottom) in response to the mTNF- α treatments. Mice pretreated with saline subsequently received an injection of the vehicle or mTNF- α (1.0 μ g; i.p.) (left), while those pretreated with mTNF- α (2.0 μ g; i.p.) were reexposed to the cytokine (1.0 μ g; i.p.) 28 days later (right). One hour prior to the mTNF- α reexposure, mice received either saline, mepyramine (Mep; 10 mg/kg), diphenhydramine (Dip; 5 mg/kg), cimetidine (Cim; 50 mg/kg) or a cocktail of mepyramine and cimetidine (Mep + Cim; 10 and 50 mg/kg, respectively). All behavioral and hormonal measures were made 60 minutes following the second injection. * $P < 0.05$ relative to mice treated with saline on two occasions, ϕ $P < 0.05$ relative to animals pretreated with saline that subsequently received a single acute injection of mTNF- α .

acute mTNF- α increased plasma corticosterone levels above that of saline treated mice. Moreover, this effect was further augmented in animals reexposed to mTNF- α 28 days following initial treatment, so that the hormone levels significantly exceeded that of mice acutely treated with the cytokine (i.e. sensitization effect). The post hoc tests revealed that in mTNF- α reexposed animals, both diphenhydramine and cimetidine, reduced corticosterone levels to a degree comparable to that observed in mice acutely treated with mTNF- α . Likewise, co-administration of mepyramine plus cimetidine prior to mTNF- α reexposure, also prevented the sensitization of corticosterone. However, mepyramine pretreatment by itself was without effect in this respect.

In Experiment 2b, which assessed the effects of the antihistamine treatments on plasma corticosterone increases elicited by acute mTNF- α it was found that the hormone levels varied with the treatment mice received, $F(9,69) = 10.86$, $p < 0.01$. As in the preceding experiment, acute mTNF- α significantly increased corticosterone levels relative to vehicle treated mice. A single administration of either mepyramine or cimetidine did not affect plasma corticosterone levels. Curiously, however, diphenhydramine or the combination of diphenhydramine plus cimetidine increased levels of the hormone relative to that seen in the saline treated mice. In contrast to the antagonism of the sensitizing effects of mTNF- α (Experiments 1-2), the antihistamine pretreatments did not influence corticosterone levels in mice that received only a single injection of mTNF- α . Indeed, as shown in Figure 4.4, levels of the hormone were virtually identical in mTNF- α treated mice that had been pretreated with vehicle, cimetidine or mepyramine. Also, in mice that received mTNF- α , pretreatment with

either diphenhydramine or a cocktail containing this H_1 antagonist plus cimetidine actually provoked a modest (~ 15 and 25%, respectively) non-significant increase of corticosterone levels (Figure 4.4). Thus, unlike the considerable attenuation of corticosterone release associated with reexposure to the cytokine, the histamine treatments did not reduce the impact of acute mTNF- α .

Insert Figure 4.4 about here

Experiment 4: Dexamethasone effects on mTNF- α sensitization of illness and corticosterone

Immediately prior to decapitation the majority of animals that were pretreated with mTNF- α and then reexposed to the cytokine were cyanotic and cold, irrespective of whether they received dexamethasone 6 hr earlier. Moreover, these animals did not bleed as profusely as the saline-treated mice and blood clotted immediately upon extravasation. In contrast, animals that received only a single injection of mTNF- α were indistinguishable from saline treated mice.

Ratings of the overall appearance of mice indicated that the development of sickness (as well as the development of cyanosis) appeared at the 30 min sampling time, and increased equally in all conditions thereafter. As the data collected at the 60 min post-treatment time, which represents the peak effect, are likely a better reflection of the actions of the various treatments, these data are presented here. The ANOVA indicated that the sickness profile was influenced by the Treatment condition, $F(3, 16) = 3.45$, $p <$

Figure 4.4. Mean ($\pm$ SEM) levels of plasma corticosterone in response to acute mTNF- α and antihistamine treatments. Mice were administered either saline, mTNF- α (1.0 μ g; i.p.), diphenhydramine (Dip), mepyramine (Mep), cimetidine (Cim) or a cocktail of diphenhydramine and cimetidine (Dip + Cim). The remaining four groups received the same antihistamine treatments in addition to receiving mTNF- α (1.0 μ g; i.p.) 1 hour later. In all instances the i.p. administered doses for the antihistamines were 5, 10 and 50 mg/kg for diphenhydramine, mepyramine and cimetidine, respectively. * $P < 0.05$ relative to mice treated with saline on two occasions.

0.05. The pairwise comparisons revealed that although acute mTNF- α had no effect on appearance at the peak time relative to that elicited by saline, mice reexposed to the cytokine after the 28 day interval displayed profound signs of illness (Figure 4.5). However, this effect was not further modified by dexamethasone administration.

The treatments altered plasma corticosterone concentrations, $F(3, 34) = 7.86$, $p < 0.01$. Although a single mTNF- α injection provoked a modest (25%) increase of corticosterone, reexposure to the cytokine 28 days following initial mTNF- α treatment resulted in markedly augmented levels of the hormone, once again indicating a sensitization effect (Figure 4.5). Unlike the effects observed with respect to sickness, in animals that received the cytokine on two occasions, dexamethasone ameliorated the corticosterone sensitization so that the levels of the hormone were comparable to that of saline treated animals.

Insert Figure 4.5 about here

Discussion

As previously observed, upon reexposure to mTNF- α a marked sensitization was apparent with respect to the appearance of sickness behavior and levels of plasma corticosterone (Hayley et al., 1999, 2001a). It was again noted that the illness elicited by mTNF- α closely resembled that typically associated with septic or anaphylactic shock.

Figure 4.5. Mean ($\pm$ SEM) plasma corticosterone levels (top) and ratings of illness (e.g. ptosis, piloerection, curled body posture) (bottom) in response to the mTNF- α treatments. Mice received saline on two occasions, saline followed by mTNF- α (1.0 μ g) or mTNF- α (2.0 μ g) with subsequent reexposure to mTNF- α (1.0 μ g) with or without dexamethasone (Dex) (1.0 mg/kg; i.p) pretreatment 6 hours earlier. All injections were administered i.p with a 28-day interval between the two treatments and measures were made 60 minutes following the second injection. * $P < 0.05$ relative to mice treated with saline on two occasions.

Consistent with the view that histamine contributes to the sensitization of sickness behavior, administration of a cocktail comprising the H_1 and H_2 antagonists, diphenhydramine and cimetidine, or, mepyramine plus cimetidine, attenuated the illness sensitization normally engendered by mTNF- α . However, administration of either of these antihistamines alone was not sufficient to block the induction of the sickness sensitization, suggesting the possibility that that mTNF- α elicited illness may involve the co-activation of these two receptors. Of course, complete dose response curves were not undertaken with these antagonists, and hence it is possible that higher doses of each antagonist, administered individually, would have attenuated the effects of the cytokine. Nevertheless, as the antihistamines provoked a diminution of the illness normally evident in mTNF- α sensitized mice, it is suggested that vasoregulatory (or other effects) associated with histamine may underlie the sickness engendered by the cytokine. Given that H_1 and H_2 receptors may synergistically modulate CNS cAMP activity (Garbarg & Schwartz, 1988), the possibility should also be entertained that the antihistamines may have interacted in a synergistic fashion in modulating sickness behavior.

Consistent with previous studies demonstrating potent CRH and corticosterone inducing effects of histamine, as well as reports that this monoamine may be involved in the stimulation of HPA activity in response to certain stressful or pharmacological challenges (Bugajski et al., 1993; Kjaer et al., 1998), either H_1 and H_2 histamine antagonism blocked the sensitization of plasma corticosterone. Unlike the sensitization of the sickness response, the elevated corticosterone levels could be diminished by the administration of the H_2 antagonist, cimetidine, or the H_1 antagonist, diphenhydramine,

alone. While diphenhydramine was more effective than mepyramine in this respect, it is uncertain whether this is related to characteristics of these compounds or simply differential dose effects. Indeed, co-administration of either of the H_1 antagonists together with cimetidine further reduced the corticosterone response to mTNF- α reexposure, attesting to the efficacy of both H_1 antagonists. Yet, it is essential to underscore that although the antihistamine treatments attenuated the corticosterone sensitization, levels of the hormone did not fall below that evident after an acute injection of mTNF- α , and in no instance were levels of the hormone found to approach that of saline treated controls. In effect, these antagonists seemed not to be impacting on the basic effects of the mTNF- α , which may involve mechanisms unrelated to histaminergic action (e.g., binding with receptors sensitive to this cytokine), but acted on the downstream processes (e.g., inflammatory immunological factors) related to the provocation of a sensitized response.

Given that hormone changes are elicited by mTNF- α even in the absence of behavioral alterations (i.e. acute mTNF- α treatment) supports the view that the sickness-inducing effects of mTNF- α are independent of the altered corticosterone levels (Hayley et al., 1999). Yet, the fact that the H_1 plus H_2 antagonist pretreatment abolished both the sickness and plasma corticosterone sensitization effects raises the possibility that histamine may be a common mediator of the HPA and behavioral sensitization elicited by mTNF- α . This does not imply, however, that these two effects are causally related. Indeed, dexamethasone treatment completely blocked the corticosterone sensitization without affecting the sickness, suggesting that independent processes subserve these sensitization effects. It should be noted, nevertheless, that the sickness associated with a

sensitization to mTNF- α , just like that induced by endotoxin (Johnson et al., 1996), may be related to the actions of corticosterone. Accordingly, although dexamethasone would have prevented the *de novo* release of adrenal corticosterone, the dexamethasone occupancy of the corticosterone receptors may still have influenced the development of sickness.

Supporting a role for mTNF- α in affecting central histaminergic activity, the cytokine augmented NE utilization within the posterior region of the hypothalamus. This region was of interest, as indicated earlier, because it contains the tuberomammillary nucleus within which the histamine producing neurons are located (Brown, 2001). Moreover, these histamine-containing neurons send projections to tyrosine hydroxylase-positive, catecholamine rich brainstem regions (Iwase et al., 1998), allowing for interactions between these aminergic systems. Although mTNF- α had an acute effect on NE activity within the posterior hypothalamus, no sensitization was evident, nor was 5-HT activity affected. Yet, in mice pretreated with an H₁ plus H₂ antihistamine cocktail, MHPG was reduced to levels comparable to those of vehicle injected animals, raising the possibility that the acute effects of mTNF- α on hypothalamic NE activity may be related to local histaminergic functioning.

Although the mechanism through which the antihistamine treatment prevented the sensitization of corticosterone and illness elicited by mTNF- α is unclear, previous reports indicated that histamine may act either within the CNS or periphery to modulate shock-like syndromes. For instance, elevated histamine levels are evident in shock-like pathological states, such as those associated with severe trauma (Barron, 1993), and intravenous histamine administration may provoke shock-like symptoms (e.g.

cardiopulmonary depression) (Silverman et al., 1988). As well, antihistamine treatments ordinarily ameliorate shock-like symptomatology, although the efficacy of these treatments vary with the particular compound administered (e.g. H_1 vs H_2 antagonists), the nature of the inducing insult, and the pathological profile displayed (Ferrario & Gomer, 1990; Silverman et al., 1988). The findings of the present investigation are consistent with these previous studies, and suggest that mTNF- α reexposure induces a histamine reversible induction of a shock-like reaction. Of course, this does not imply that histamine alone subserve these effects, as both histamine and serotonin have been shown to contribute to the vasoactive effects elicited by endotoxins (Balsa et al., 1997).

Regardless of whether the pathology observed in TNF- α sensitized mice represents anaphylactic shock or some other related syndrome, it is clear that the cytokine provokes a sensitized state comprising pronounced illness and dysfunction of multiple physiological systems. In this respect, the illness profile is consistent with the multi-system organ failure recently reported to occur in rats pretreated with TNF- α and later exposed to LPS (Goto et al., 2001). Animal exposed to LPS 48 hr following TNF- α pretreatment (at a time when multi-system organ failure occurs after trauma) also displayed markedly enhanced circulating interferon- γ and lactate levels, coupled with increased mortality (Goto et al., 2001). Moreover, viral or TNF- α challenged animals were more susceptible to the IL-6 inducing and lethal effects of subsequent high doses of IL-1 and/or TNF- α (Wallach et al., 1988; Brouckaert et al., 1993). The precise role of histamine in such "immune/cytokine-sensitized" states is not entirely clear, but may involve the effects of the amine on vascular or neuroendocrine processes. Indeed, histamine may act to magnify the effects of allergic inflammation or stressors by

provoking the release of proinflammatory cytokines and subsequent HPA activation (Hurwitz & Morgenstern, 2001).

General Discusison of the Sensitizing effects of Tumor Necrosis Factor- α

Behavioral and physiological effects of TNF- α : Illness profile

It will be recalled that proinflammatory cytokines, such as IL-1 β and TNF- α , induce a behavioral profile comprising various symptoms reflecting sickness. These behaviors have been thought to minimize energy expenditure, conserve body temperature, and generally allow the organism to maximize its ability to contend with viral and bacterial insults (Maier & Watkins, 1998; Dantzer et al., 1998). For instance, the fever response provoked by proinflammatory cytokine treatment is thought to act in an adaptive capacity by making the internal milieu unfavorable for microorganism growth (Bluthe et al., 1994). In addition to metabolic gains, reduced locomotion and social interaction in the face of illness may help keep the animal away from predators and “out of harms way”.

It is important to note that under normal, physiological conditions TNF- α has important hemodynamic functions and during infection the cytokine helps contain the pathogen as well as instigate tissue repair (Janeway, 1999). In particular, elevated TNF- α induced by infectious challenge increases endothelial permeability, enhancing leukocyte extravasation, which in turn isolates the pathogen from general circulation for subsequent removal by the lymphatics (Janeway, 1999). However, in response to pronounced systemic infections (e.g. high circulating concentrations of LPS) that provoke sepsis, the very same mechanisms that normally allow TNF- α to provide protection now exacerbate the septic event. Indeed, the increased endothelial permeability and widespread inflammation of organs precipitated by TNF- α often results in multiple organ failure characterized by respiratory collapse, hypotension and tissue hypoxia (Burdon et al., 2000).

In the present investigation, as reported in chapters I & IV, acute TNF- α did not overtly affect sickness however, upon reexposure to the cytokine after several weeks profound illness was elicited (sensitization), characterized by ptosis, piloerection, cyanosis of the extremities as well as reduced locomotion and social interaction. It is suggested that the initial dose of TNF- α serves to prime physiological mechanisms (likely both at a peripheral and central level) responsible for various illness-related symptoms (e.g. fever, reduced locomotion), such that reexposure to the cytokine after some protracted period engenders a heightened response. The precise mechanism(s) for this effect remains to be fully elucidated, but may involve receptor upregulation or activation of secondary inflammatory mediators (e.g. histamine, prostaglandins).

Role of Histamine in the sickness provoked by TNF- α (Chapter IV)

Peripheral histamine is liberated from mast cells in response to antigenic challenge resulting in immediate type allergic reactions reflected by vasodilatation, edema, mucus secretion, itching and sneezing as well as respiratory distress (Durham, 1998). As antihistamines (e.g. the H₁ and H₂ antagonists, dimethindene and ranitidine, respectively) can reduce the mortality as well as the elevation of circulating TNF- α induced by high doses of endotoxin (Dimmeler et al., 1996; Stewart et al. 1995), it is likely that interactions between TNF- α and histamine play a fundamental role in the modulation of shock reactions. Indeed, as TNF- α binding to cell surface receptors triggers mast cell degranulation (release of intracellular histamine), high circulating levels of TNF- α may induce a shock reaction, characterized by hypovolemia and reduced core temperature, by eliciting enhanced histamine release (Brzezinska-Blaszczyk et al., 2000). Interestingly,

mast cells have been reported to co-release TNF- α together with histamine in response to ischemia (often present during shock reactions) (Frangogiannis et al., 1998), raising the possibility that circulating TNF- α may act in a feedforward manner to increase its own expression as well as that of histamine, thereby potentially exacerbating signs of illness. The cytokine may also alter H₁ and H₂ histamine receptor functioning (Igaz et al., 2001), suggesting that TNF- α may influence cell sensitivity to the monoamine over protracted time intervals following exposure to the cytokine.

In the present investigation, reexposure to TNF- α 28 days following initial exposure to the cytokine induced signs of illness reminiscent of those evident in the case of septic or anaphylactic shock. Consistent with the proposition that this behavioral sensitization elicited by TNF- α reflects a shock reaction, administration of the H₁ and H₂ histamine antagonists, diphenhydramine and cimetidine, ameliorated the TNF- α sensitization of illness. It is suggested that the initial TNF- α exposure may sensitize histaminergic activity at mast cells, such that reexposure to the cytokine results in an augmentation of release of the monoamine, hence affecting multiple organ systems. In fact, histamine, as well as TNF- α , has been implicated in the multiple organ failure evident in clinical shock (Goto et al., 2001).

In addition to its systemic effects, histamine acts as a brain neurotransmitter and hence may influence various behavioral processes. Indeed, the amine has important effects on learning and memory, and is also believed to play a fundamental role in the regulation of sleep (Brown, 2001). It is possible that some of the effects of the antihistamine treatments used in the present investigation derived from actions within the brain. As already mentioned, the H₁ antihistamine, diphenhydramine, readily penetrates

the BBB and induces somnogenic effects through actions at histaminergic neurons within the hypothalamus (Brown, 2001). However, in the present investigation, diphenhydramine and cimetidine did not produce such an effect, possibly suggesting that the concentrations used may not have been sufficient to elicit somnogenic consequences. Nevertheless, given the important role that the hypothalamus plays in sickness behaviors, coupled with the high density of histaminergic neurons present in this region, the possibility should not be dismissed that the antihistamines affected aspects of this brain region important for the modulation of the TNF- α provoked sensitization of illness.

Cross-sensitization of sickness behavior: TNF- α , LPS, IL-1 β (Chapter II)

The behavioral sensitization induced by TNF- α was specific for the cytokine. Specifically, in recent experiments we observed that mice exposed to LPS or IL-1 β 28 days following TNF- α pretreatment did not display the shock-like illness evident in animals reexposed to TNF- α at this time (S. Hayley, C. Dodd, T. Borowski & H. Anisman, Unpublished observations). This finding is important since the LPS reexposure treatment would be expected to induce endogenous TNF- α , which may act in a manner similar to that of exogenously administered TNF- α . The fact that LPS (and presumably the provocation of endogenous TNF- α) did not elicit a behavioral sensitization in animals previously treated with the cytokine supports the proposition that immunological events specific to the recombinant cytokine are required for the sensitization. Yet, it is possible that the levels of endogenous TNF- α , provoked by the LPS exposure, were not sufficient to elicit such an effect. Indeed, typically circulating TNF- α levels in the order of only picogram-nanograms are evident after severe inflammatory challenges, as opposed to the

0.1-1.0 ug doses of exogenous TNF- α used in the present investigation. As well, the timing and rate of release of LPS-provoked endogenous TNF- α may not have been optimal for the manifestation of the sensitization.

The fact that TNF- α did not sensitize illness in response to subsequent LPS or IL-1 β treatment may indicate that the recombinant TNF- α molecule is acting as a potent antigenic stimulus. Specifically, the immune system may be recognizing variant glycosylation or other products, resulting from the recombinant processing of the molecule, as being foreign. As a consequence, an immunological memory response may be mounted against the epitope structure of the recombinant TNF- α molecule initially applied, thereby resulting in a more vigorous immune response to the reexposure challenge with the cytokine. Such an overactive inflammatory/immune response may represent an anaphylactoid reaction that may progress to full-blown anaphylactic shock. In this instance the sensitization is akin to the priming response provoked by clinical immunization against various pathogens (e.g. measles, smallpox, tuberculosis), in which a robust immunological response to subsequent exposure to the pathogen results in a rapid clearance of the infectious agent. As well, the time-course for such immunization reactions is similar to that required for the TNF- α sensitization of sickness. It is well known that the production of antibodies and priming of helper T cells requires at least a week and that the maximal immune response occurs between 14-28 days following immunization. This may explain why the behavioral sensitization of TNF- α was evident after 28 days but not after only 1 day.

In contrast to the absence of a behavioral cross-sensitization in TNF- α primed animals, LPS pretreatment sensitized behavioral manifestations of illness in response to

subsequent exposure to either LPS, TNF- α or IL-1 β (Chapter II). Indeed, signs of illness including, ptosis, piloerection and reduced locomotion were evident in these mice. Interestingly, however, the time course for the LPS provoked sensitization of sickness differed greatly from that elicited by TNF- α priming. It will be recalled from Chapters I and IV that reexposure to TNF- α after the lengthy interval of 28 days (but not earlier times of 1 or 7 days) resulted in the sickness sensitization. However, the opposite was true for LPS, such that reexposure to the endotoxin, TNF- α or IL-1 β 1 day following the initial endotoxin injection resulted in a behavioral sensitization, but no such effect was evident when a 28-day interval was interposed between the two injections. Thus, it appears that the temporal pattern for the sensitizing of central responses depends upon the particular immune/inflammatory challenge presented. It is likely that differential signaling pathways related to receptor activation (p55/p75 for TNF- α vs. CD14 for LPS) are upregulated across different time intervals following these insults.

TNF- α Elicited Neuroendocrine Alterations: Systemic administration

Much like stressors, peripheral and central cytokine administration effectively activate HPA axis activity. Systemic injection of IL-1 β potently stimulates pituitary ACTH and adrenal corticosterone release. This appears to stem, at least in part, from secretion of CRH from the PVN, as the pituitary and adrenal hormonal effects elicited by the cytokine could be prevented by passive immunization with antisera to CRH and by CRH receptor antagonists (see Dunn, 1995). As in the case of IL-1 β , systemic TNF- α administration induces elevated circulating ACTH and corticosterone levels (Bernardini et al., 1990; Brebner et al., 2000; Hayley et al., 1999). However, TNF- α may also have

direct actions on the pituitary and adrenal glands, thereby provoking hormonal release (Kobayashi et al., 1997; Ritchie et al., 1996).

In the present investigation acute systemic TNF- α increased circulating corticosterone levels in a dose-dependent fashion. As reported in Chapter III, a 1.0 μ g dose of TNF- α provoked an increase of plasma corticosterone, and this effect was further augmented by higher doses (2.0 and 4.0 μ g). The cytokine induced corticosterone variations also varied over time, such that levels of the hormone were elevated by 15 minutes, peaked after 30 minutes and were still above control levels 120 minutes following injection (Hayley, Merali & Anisman, Unpublished observations).

In addition to CRH, the related hypothalamic ACTH secretagogue, AVP, may also be involved in the HPA activating effects of cytokines (Tilders & Schmidt, 1998). However, such effects may be related to the chronicity or timing of the cytokine treatment, as well as the specific cytokine in question. For example, although acute IL-1 β challenge did not alter hypothalamic AVP levels or activity, repeated injection of the cytokine for several days effectively augmented AVP concentrations within hypothalamic CRH containing neurons (Tilders & Schmidt, 1998). Less information is available concerning the impact of TNF- α on AVP within the PVN. However, the existing data suggests that AVP may not play an important role in the acute effects of TNF- α on corticosterone release (Kageyama et al., 1995). Although AVP acts as a relatively weak ACTH secretagogue by itself, the peptide strongly enhances the ACTH inducing effects of CRH. Specifically, co-secretion of AVP and CRH from the PVN results in a greatly augmented release of pituitary ACTH, suggesting that these two neuropeptides act in a synergistic fashion (Torpy et al., 1994).

Sensitization of Neuroendocrine Activity: Systemic TNF- α administration

As hypothalamic derived AVP and CRH may synergistically drive pituitary-adrenal steroid release, it follows that chronic stressor or cytokine treatments, both of which increase both AVP and CRH activity, may induce pronounced HPA variations through the co-release of these peptides. In fact, such a situation has been suggested to underlie the hormonal sensitization effects associated with repeated social stress or immobilization treatments (De Goeij et al., 1991,1992). Indeed, repeatedly exposing rats to social defeat for 19-28 or immobilization for 9-16 days, resulted in increased synthesis of AVP and CRH in the parvocellular PVN neurons, coupled with increased co-storage of these peptides in the terminals of the median eminence (De Goeij et al., 1991,1992). It will be recalled that these PVN neurons project to the median eminence, where they release AVP and CRH into the hypophyseal portal system and the peptides subsequently bind to receptors located on the adjacent pituitary. The increased AVP and CRH expression was associated with a decidedly augmented release of ACTH and corticosterone upon subsequent exposure to a novel challenge. Interestingly, while repeated presentation of the same stressor has been demonstrated to result in a diminution of the corticosterone eliciting capacity of the challenge, exposure to a novel stressor results in a sensitization of hormonal release. Thus, although chronic stressor exposure may elicit time-dependent increased AVP/CRH expression, the increased peptide activity does not inevitably result in augmented hormonal release. Evidently the sensitization of ACTH and corticosterone activity may depend, in part, upon the nature of the "trigger" stimulus.

Several reports indicated that the passage of time following stressor or cytokine exposure, rather than the chronicity per se, may determine the impact of such treatments on hypothalamic AVP and CRH activity. In this respect, augmented co-storage of AVP and CRH within the median eminence was observed with the passage of time following the stressor exposure, being evident 7-11 days following footshock or brain surgery (Schmidt et al., 1996). Much like the effects of stressors, cytokine treatment can also sensitize HPA activity. In particular, after a delay of four days following a single injection of IL-1 β , increased co-localization of AVP and CRH was evident within the median eminence (Schmidt et al., 1995). The augmented peptide co-localization was still observed one month following the single IL-1 β injection, suggesting that the cytokine can elicit protracted alterations of the expression of HPA regulatory factors (Schmidt et al., 1995). Functionally, the augmented hypothalamic peptide expression was associated with increased ACTH and corticosterone release in response to a second injection of the cytokine 11 days following the initial treatment (Schmidt et al., 1995).

The present investigation indicated that TNF- α could also sensitize HPA activity in a time-dependent fashion. In particular, as demonstrated in Chapters I, III, IV & V, reexposure to TNF- α 28 days following initial treatment with the cytokine resulted in a pronounced elevation of plasma corticosterone, indicating a sensitization of HPA activity. Following the second TNF- α injection, the sensitization of corticosterone was apparent 60 and 120 minutes, but not at earlier times following the reexposure treatment (Hayley, Merali & Anisman, Unpublished observations). The HPA sensitization was so robust that reexposure to doses of TNF- α as low as 1/20th (0.1 μ g) the size of the initial injection (2.0 μ g) still provoked a reliable corticosterone sensitization (Chapter III). The passage

of time appears to be critical to the establishment of the sensitization effect, as reexposure to TNF- α after earlier time intervals (1, 7 and 14 days) did not promote the augmented corticosterone activity.

As in the case of IL-1 β , the hormonal sensitization elicited by TNF- α was associated with variations of hypothalamic CRH and AVP expression. As reported in Chapter I, at 7 and 14 days following initial TNF- α challenge, immunoreactivity for both AVP and CRH was increased within the external zone of the median eminence. Moreover, these peptides were predominately co-localized together within terminals of the external zone of the median eminence, allowing for the augmentation of their co-release. Curiously, this effect peaked 7-14 days following cytokine treatment, but normalized after 28 days. This contrasts with the sensitization of the adrenal corticosterone response evident in mice reexposed to the TNF- α 28 days after pretreatment, but not after earlier intervals (Hayley et al., 1999). Thus, it appears likely that the augmented median eminence AVP/CRH-ir does not play a primary role in this HPA sensitization effect. Rather, the possibility ought to be considered that the sensitization with respect to corticosterone involved direct actions at the pituitary or adrenal glands, particularly as TNF- α may have direct actions at these sites (Kobayashi et al., 1997; Ritchie et al., 1996). In this respect, it was reported that IL-6 can stimulate ACTH secretion independent of hypothalamic CRH contribution (Bethin et al., 2000), and there is no reason to believe that other cytokines might not act in a similar fashion. Alternatively, as reexposure to the cytokine may induce sensitization of sickness behavior (Hayley et al., 1999), it is possible that the elevated corticosterone was secondary to the

stress associated with illness or related factors (e.g. activation of acute phase proteins, histamine), rather than effects directly attributable to altered AVP/CRH expression.

The CRH changes associated with TNF- α were not limited to the hypothalamus. Within the central amygdala, the sensitizing effects of TNF- α on CRH expression were readily distinguishable from those observed in the median eminence. In particular, as already indicated, the CRH/AVP changes at the median eminence evolved simply with the passage of time following cytokine treatment, and were independent of the reexposure conditions. In contrast, changes of CRH-ir at the central amygdala were only evident upon reexposure to the cytokine, and were maximal 1-7 days following initial treatment. In effect, the potential for increased CRH-ir within the central amygdala could be elicited by TNF- α , but an actual increase of immunoreactivity was only realized upon reexposure to the cytokine. Since augmented CRH activity within the amygdala has been implicated in the provocation of fear and anxiety behaviors (Davis, 1992), it is suggested that this increased expression of CRH positive amygdaloid fibers may have implications for cytokine induced anxiogenic-like behaviors. Indeed, central administration of TNF- α has been reported to engender anxiogenic-like responses in mice subjected to an elevated plus maze (Connor et al., 1998).

TNF- α induced Sensitization of Hypothalamic c-fos expression (Chapter I)

The increased median eminence AVP/CRH accumulation observed 7-14 days after TNF- α may stem from enhanced PVN neuropeptide synthesis. Indeed, at the 14-day interval, TNF- α provoked augmented PVN c-fos-ir, which occurred largely within AVP positive neurons. Although these AVP-ir neurons are presumed to be predominately

magnocellular, the possibility exists that some of these cells may co-express other peptides, including CRH. Moreover, parvocellular CRH and magnocellular AVP neurons in mice do not display the well-defined patterns of distribution observed in rats (Dijkstra et al., 1998). Thus, although the external zone of the median eminence largely comprises terminals originating from parvocellular PVN, some of the *c-fos* responsive PVN AVP-ir neurons observed in the present investigation may project to this region, accounting for the concomitant increase of AVP together with CRH staining. It is also possible that a leaky blood brain barrier at the median eminence makes this region a target for circulating TNF- α , hence potentially leading to direct actions of TNF- α on these terminals. Indeed, in the case of IL-1 β , direct infusion of the cytokine into the median eminence increased local release of both AVP and CRH (McCoy et al., 1994; Watanobe & Takebe, 1992).

Within the supraoptic nucleus of the hypothalamus, a large number of AVP producing magnocellular neurons send terminals to the posterior pituitary (Holmes et al., 1986). Together with projections from similar neurons within the PVN, this AVP signal has important osmoregulatory functions (Szczepanska-Sadowska, 1996). As reported after systemic IL-1 β administration (Konsman et al., 2000), TNF- α provoked the expression of *c-fos* immunoreactivity within the supraoptic nucleus of the hypothalamus. Moreover, reexposure to TNF- α 7 or 14 days following pretreatment with the cytokine further augmented this response, indicating a sensitization effect. As reported in chapter I, the time-course for this effect largely followed that of the PVN, peaking 3 hours after administration of the reexposure dose of TNF- α (14 days following initial treatment with the cytokine). It remains to be determined whether this effect represents direct binding of

TNF- α to receptors at this site. In this respect, there are data demonstrating low levels of expression of the p55 TNF- α receptor within the supraoptic nucleus (Laflamme & Rivest, 1999). Other studies have shown co-localization of IL-1 as well as its type 1 receptor in AVP immunopositive neurons within the supraoptic nucleus (Diana et al., 1999). Moreover, LPS challenge (which provokes IL-1 β and TNF- α release) has been reported to increase c-fos immunoreactivity within the supraoptic nucleus and provokes the secretion of AVP (Wan et al., 1994; Knigge et al., 1999). These data suggest that cytokines or other immunogenic challenges may augment the activity of magnocellular hypothalamic AVP neurons, which may impact upon peripheral water balance and other functions necessary for homeostasis. It is interesting, in this respect, that the sensitization of illness provoked by TNF- α (Chapters I, III, V) comprised symptoms that likely reflected a disruption of water balance and blood flow implicating the possible disruption of the AVP magnocellular system. It will be recalled that upon TNF- α reexposure 28 days following initial cytokine treatment, mice appeared to be hypovolemic and cyanosis of the extremities (Anisman et al., 2000; Hayley et al., 1999, 2002b). These sickness symptoms are consistent with disturbed blood flow and/or pressure associated with systemic or anaphylactic shock (Hardaway, 2000; Kettelhut et al., 1987). The possibility exists that over protracted periods TNF- α may influence AVP transport to the posterior pituitary.

Finally, the immunohistochemical sensitization effects of TNF- α were not restricted to brain processes, as we recently observed that the time-dependent AVP/CRH alterations were paralleled by elevations of immunoreactivity for c-fos, thymidylate synthase, IL-1 α and heme oxygenase-1 within the ileum and colon of mice reexposed to

TNF- α (Peaire et al., 2001). Although speculative, the temporal correspondence between the gut and the brain AVP/CRH-ir variations may be related to activation of peripheral vagal afferents, given that visceral projections to brain stem nuclei influence hypothalamic neurotransmission (Laye et al., 1995; Maier et al., 1998).

TNF- α Elicited Neuroendocrine Alterations: icv administration (Chapter III)

Previous studies concerning the immediate HPA effects of central TNF- α infusion are controversial. Some reports indicated that icv administration of the cytokine increased plasma ACTH and corticosterone (Pauli et al., 1998), while others failed to detect such an effect (van der Meer et al., 1996b). In the present investigation, icv mTNF- α increased plasma corticosterone levels, however, to a much lesser degree than that observed in response to systemic injection. In fact, icv administration of a 0.50 μ g dose of the cytokine elicited a smaller rise of corticosterone than did ip injection of the same dose. Although a wide range of doses were employed (1-500 ng), the fact that the effects of icv TNF- α did not increase in a dose dependent fashion raises the possibility that the optimal effects of the cytokine would have been realized at still lower doses. As previous studies assessing the effects of icv murine TNF- α are unavailable, and protein levels of this cytokine in mouse brain have not been established, the dose-response relationship concerning this cytokine's effects on neurochemical processes is relatively vague.

In contrast to systemic mTNF- α , icv treatment did not induce the sensitization of HPA activity, suggesting that this effect does not involve direct actions of mTNF- α within the brain. Not only was the HPA sensitization absent in mice receiving both the

initial and reexposure TNF- α treatments by the icv route, but those animals that were ip pretreated and then subsequently reexposed to the cytokine by icv administration, also failed to show the sensitization effect. It is clear that initial ip TNF- α (2.0 μ g) treatment effectively primes HPA activity to permit the expression of a sensitization given an appropriate challenge. Evidently, cytokine reexposure by the icv route was not effective in triggering the manifestation of the sensitization. These results, as reported in chapter III, suggest that both the acute and sensitization neuroendocrine effects of TNF- α involve activation of peripheral targets (e.g. liver, spleen or vagal afferents) that may then stimulate HPA activity, or as already suggested, the cytokine may directly stimulate pituitary-adrenal activity. This last proposition is in accord with our data from Chapter I demonstrating that the increased hypothalamic AVP/CRH does not necessarily result in augmented corticosterone release.

Cross-sensitization of plasma corticosterone: TNF- α , LPS, IL-1 β , and restraint (Chapter II)

Paralleling the effects on sickness behavior, a sensitization effect was provoked by ip LPS, such that plasma corticosterone levels were augmented in animals subsequently treated with TNF- α . As well, LPS treatment sensitized corticosterone responses to subsequent restraint, suggesting that the endotoxin can prime physiological mechanisms involved in the HPA activating effects of both immune and non-immune stressors. However, while LPS stimulates IL-1 β release, and IL-1 β potently increases corticosterone levels (Dunn, 1988, 1990; van der Meer et al., 1996a) the endotoxin did not further modify plasma corticosterone responses to IL-1 β administered 1 day later.

The absence of a sensitization with respect to plasma corticosterone levels was not a result of a ceiling effect, as only a modest increase of the hormone level was elicited by the particularly low dose of IL-1 β used (0.05 μ g). However, as only a single dose of IL-1 β was used and corticosterone was only sampled at a single post-treatment interval, a sensitization effect might have been detected at other doses or times following treatment. Yet, the possibility should not be dismissed that TNF- α plays a more prominent role than IL-1 β in the neuroendocrine sensitizing effects of the endotoxin, just as TNF- α may be the primary cytokine involved in endotoxic shock induced by LPS (Galanos & Freudenberg, 1993).

Central monoamine alterations: Systemic TNF- α (Chapter III)

Much like the effect of recombinant human TNF- α (Hayley et al., 1999), systemic administration of the murine form of the cytokine provoked increased NE utilization within the PVN, locus coeruleus, PFC, central amygdala and medial amygdala, as well as 5-HIAA accumulation within the PVN and medial amygdala. Even at very low reexposure doses (0.1 - 1.0 μ g), TNF- α sensitized some aspects of brain monoamine activity. Furthermore, this sensitization effect was brain region-specific, time-dependent and was most notable with respect to 5-HT utilization. Within the PFC, reexposure to mTNF- α 1 day following initial treatment enhanced 5-HIAA accumulation, while such an effect was evident within the medial amygdala at both the 1 and 28-day reexposure intervals. Although the temporal profile for these sensitization effects corresponded with those observed using the human form of the cytokine, the murine TNF- α form favored changes of 5-HT turnover while human TNF- α was equally potent in provoking NE

sensitization effects. Such variation among the recombinant forms of TNF- α may reflect differential potency and dosage effects, or species-typical cytokine actions.

Given that nuclei within the amygdala appear to have distinct anxiogenic or affective roles, it was of particular interest to investigate the TNF- α -elicited monoamine effects across several amygdaloid brain regions. Previous characterizations of the sensitization effects attributed to TNF- α have been confined to the central nucleus of the amygdala. However, traditional stressors (e.g. restraint, footshock) often have more profound effects on c-fos expression within the medial amygdala (Rosen et al., 1998). In the present investigation, the acute effects of TNF- α were, in fact, more pronounced within the medial rather than central amygdala. Moreover, a pronounced sensitization of 5-HT activity was evident within the medial amygdala, as indicated by increased 5-HIAA accumulation upon reexposure to the cytokine 28 days following its pretreatment.

In accord with the differential behavioral effects mediated by the various amygdaloid nuclei, it appears that the acute and sensitizing monoamine effects elicited by TNF- α vary between the different sub-nuclei of the amygdala. Given the possibility that different aspects of the amygdala play different roles in subserving mood-related disorders, it is tempting to speculate that TNF- α may be important for the provocation of depressive-like symptomology. Indeed, 5-HT disturbances within these amygdaloid regions is suggested to be related to depressive illness (Ressler & Nemeroff, 2000). Further to this point, recent studies suggest that major affective disorders likely involve alterations in pathways typically activated by cytokines (Chen et al., 2001). In particular, the mitogen activated protein (MAP) kinase pathway, which is involved in cytokine

signaling (e.g. IL-6, BDNF), as well as pathways involving the cAMP response binding element (CREB) factor, which has important neuroplastic effects, are both proposed to play a role in the long term neurochemical changes evident in depression (Manji et al., 2001; Chen et al., 2001). Interestingly, antidepressants can induce the expression of the anti-apoptotic and neurotrophic factor, bcl-2, which has a potent neuroprotective influence in neurodegenerative states (Manji et al., 2001). In fact, it was proposed that alternate treatment strategies that target neurotrophic factors involved in promoting cellular resiliency may prove beneficial for the treatment of mood disorders (Manji et al., 2001). Like antidepressants, TNF- α has also been shown to provoke bcl-2 expression, as well as the pro-apoptotic factor, Bax, suggesting that a delicate balance between the expression of these two death regulatory signals may ultimately determine the consequences of the cytokine with respect to cellular survival and resiliency. It may be the case that TNF- α priming induces changes in these intracellular pathways (e.g. phosphorylation states) that "set the stage" for the development of aberrant neurochemical responses to subsequent challenges. From this perspective, cytokine activation (and particularly sensitized responses elicited by cytokines) may contribute to apoptotic processes that contribute to the provocation of depressive illness (Manji et al., 2001; Chen et al., 2001).

Central monoamine alterations: icv TNF- α (Chapter III).

The present investigation represents the first attempt to characterize the brain region specific alterations of monoamines in response to icv infusion of TNF- α . Commensurate with a central site of action for the neurochemical effects of TNF- α , icv

administration of the cytokine increased 5-HT utilization in PVN, ME/ARC, and central amygdala, while NE and DA utilization were increased within the PVN and ME/ARC. It is clear that the profile of brain monoamine changes elicited by icv TNF- α differs from that evident in response to systemic treatment with the cytokine. Whether this stemmed from differences of mTNF- α entry into brain following systemic vs. icv treatment (Gutierrez et al., 1993), differences in dispersion across brain tissues, or even toxicity factors, remains to be established. However, it is possible that stimulation of vagal and/or spinal projections (Dantzer et al., 2000; Hosoi et al., 2000; Maier et al., 1998) or enzymatic alterations at peripheral target sites (e.g. liver) (Libert et al., 1991), contribute to the effects of peripheral but not central cytokine administration.

Although central administration of mTNF- α did not sensitize sickness or HPA activity, icv infusion of the cytokine effectively engendered a sensitization of central monoamine activity. Interestingly, this sensitization was evident in hypothalamic brain regions but not in cortical or amygdaloid areas, as observed after systemic treatment with the cytokine. As in the case of icv pretreatment, Chapter III reports that comparable sensitization effects were evident within the ME/ARC of mice pretreated *systemically* (ip) with TNF- α and subsequently reexposed to the cytokine via an *icv* route of administration. However, icv reexposure to TNF- α following systemic pretreatment with the cytokine also sensitized NE utilization within the locus coeruleus. This is contrary to the lack of an effect within the locus coeruleus when both the initial and reexposure treatments were applied by an icv route of administration. Thus, it seems that the sensitizing effects of systemic and central mTNF- α are distinguishable from one another at hypothalamic and extra-hypothalamic structures.

Although either route of administration may prime ME/ARC neurons, the PVN sensitization appears to be more closely aligned with central cytokine infusion. It may be that circulating TNF- α sensitizes ME/ARC but not PVN aminergic neurons because of easier access to this site. It will be recalled that the ME, a circumventricular organ, lacks an efficient BBB, hence allowing peripherally administered cytokines relatively easy access. Moreover, previous studies reported that IL-1 β could directly alter ME neural activity (Watanobe & Takebe, 1993). The source for the sensitization of locus coeruleus NE activity following ip treatment with TNF- α is not immediately apparent. It is possible that this effect may involve activation of sympathetic fibers innervating brainstem sites, including the locus coeruleus and nucleus of the solitary tract (Van Bockstaele et al., 1999). As mentioned earlier with respect to the immediate effects of mTNF- α (Dantzer et al., 2000; Maier et al., 1998), it is certainly conceivable that vagal afferents contribute to the sensitization effect.

TNF- α Elicited Central effects: p55 receptor involvement (Appendix A)

It will be recalled that the effects of TNF- α are subserved by two receptor subtypes, p55 and p75, which modulate different aspects of the actions of this cytokine. Generally, the p55 receptor has been implicated in the induction of apoptotic cell death as well as generation of inflammatory factors, while the p75 receptor regulates cellular proliferation and other immunological functions (Beutler, 1999). The p55 receptor is believed to be most important for the central effects of TNF- α , as the receptor has been shown to mediate the behavioral (e.g. sleep, fever), neuroendocrine and

neuroprotective/neurodestructive actions of the cytokine (Fang et al., 1997; Benigni et al., 1996; Gary et al., 1998; Carlson et al., 1999).

The involvement of the p55 receptor in regulating neuroendocrine functioning comes from studies showing that central application of p55 agonists increase plasma corticosterone levels (Benigni et al., 1996), possibly reflecting suggesting the TNF- α influence on hypothalamic AVP and CRH activity. Consistent with the proposition that p55 mediates the HPA effects of TNF- α , we found that systemic administration of recombinant murine or human TNF- α to mice produced comparable increases of plasma corticosterone. In the mouse, murine TNF- α stimulates both the p55 and p75 receptors, while the human form of the cytokine selectively activates p55. Thus, in normal mice, activation of p55 is sufficient to provoke HPA variations and p75 appears to add little to such effects. As well, in the present investigation the usual elevation of plasma corticosterone elicited by acute TNF- α was prevented in knockout mice lacking the p55 receptor. However, the wild-type strain, C57BL/6, displayed elevated corticosterone levels, much like that evident in the CD1 strain employed in our other studies. Despite the clear involvement of the p55 receptor in the present investigation, this does not suggest that p75 is not be involved in other situations, such as in the case of organisms exposed to immunological or other traumatic insults. In the case of HIV infection, it appears that the p55 and p75 receptors may interact in a cooperative or even synergistic fashion to influence manifestation of disease, a phenomenon termed ligand-passing (Butera et al., 1996).

TNF- α Elicited Central Effects: Histamine as a neuroimmune mediator (Chapter IV)

Although it is widely believed that cytokines act as signaling molecules between the immune system and CNS, it is not clear whether these immunotransmitters affect central processes through their infiltration of the brain parenchyma or if they first activate peripheral targets which then promote the liberation of downstream effectors. In the case of the latter proposition, inflammatory factors, such as prostaglandins or histamine, are released by immune cells in response to numerous challenges (e.g. cytokines, stressors, bacterial/viral insults) and have well documented central effects. Indeed, a recent report indicated that bi-directional communication occurs between histamine and cytokines, such that these factors modulate each other's production (Igaz et al., 2001). Histamine has numerous central effects as the amine influenced wakefulness, fever, learning, as well as activity of hypothalamic ACTH secretagogues and other neuropeptides (Brown, 2001). Given that the HPA stimulatory effects of LPS have been ascribed to the release of inflammatory cytokines (Dantzer et al., 1996), it is certainly possible that interactions between histaminergic and cytokine systems mediate some of the hormonal effects of LPS. In fact, some of the HPA activating effects of LPS are abrogated by administration of H_1 antihistamines (diphenhydramine or mepyramine) (Wan et al., 1994; Knigge et al., 1999).

The fact that our antihistamine treatments influenced the behavioral, neuroendocrine and central monoamine variations elicited by TNF- α (Chapter IV) suggests that endogenous histamine is involved in the central consequences of this cytokine. Based on the well-documented role of histamine in anaphylactic shock, together with the shock-like appearance of TNF- α sensitized mice, we were prompted to

attempt to antagonize the sickness effects provoked by TNF- α reexposure through the use of antihistamine treatments.

Supporting the suggestion that the sensitization of illness reflects a shock reaction, co-administration of either of the selective H₁ antagonists, diphenhydramine or mepyramine, together with the H₂ antagonist, cimetidine, 1 hour prior to the TNF- α reexposure in mice pretreated with the cytokine 28 days earlier, blocked the onset of illness symptoms normally manifested by the reexposure challenge. Moreover, the sensitization of plasma corticosterone in these mice was also ameliorated by the antihistamine treatment. In particular, the rise of corticosterone was prevented by either diphenhydramine or cimetidine but not mepyramine, administered prior to the TNF- α reexposure. In effect, the sensitization of sickness was only affected when both H₁ and H₂ receptors were antagonized, but the corticosterone sensitization was attenuated by blocking either of these receptors (although this effect was dependent upon the specific antihistamine used). It may be the case that the antihistamines interacted in a synergistic fashion to block the illness. Indeed, it has been reported that H₁ and H₂ receptors synergistically modulate CNS cAMP activity (Garbarg & Schwartz, 1988). However, the antihistamines had no effect on the acute rise of plasma corticosterone provoked by TNF- α ; this was the case when the histamine antagonists were administered either singly or together. Thus, it appears that histamine may be important for the sensitizing, but not the acute effects of TNF- α on HPA activity.

In the present investigation (Chapter IV), acute TNF- α treatment increased the accumulation of MHPG within the posterior portion of the hypothalamus. However, a sensitization was not evident in this respect, nor was 5-HT activity affected by the

cytokine. Consistent with the proposition that $\text{TNF-}\alpha$ may be affecting local histaminergic activity, the elevated MHPG levels induced by $\text{TNF-}\alpha$ were reduced by antihistamine treatment. Specifically, co-administration of the H_1 and H_2 antihistamines (diphenhydramine and cimetidine, respectively) 1 hour prior to acute $\text{TNF-}\alpha$ injection reduced MHPG levels to a level comparable to that observed in saline treated mice. Importantly, this hypothalamic brain region contains the tuberomammillary nucleus (TM), which is the source of brain histamine, raising the possibility that $\text{TNF-}\alpha$ may directly influence activity of histaminergic neurons. However, the lack of a sensitization effect in this brain region may reflect the participation of peripheral mast cells in such effects.

It might be noted at this juncture that the effects of cytokines and stressors may interact in affecting histamine involvement in the central amine changes, and hence behavioral outputs. Specifically, like cytokines, stressors have been found to alter brain mast cell release of histamine and to stimulate peripheral mast cell infiltration into CNS parenchyma (Esposito et al., 2001). Such treatments may also increase BBB permeability (Esposito et al., 2001), raising the possibility that individuals exposed to high levels of chronic stress may be more susceptible to the central effects of cytokines. Ordinarily, as indicated earlier, cytokines do not readily penetrate the BBB, owing to their large size and charge. However, protracted stressor exposure may compromise the BBB and hence, thereby increasing brain infiltration of cytokines and augmenting the neurotransmitter variations engendered by these immunotransmitters. The increased neurotransmitter alterations may reflect, in part, augmented neuronal or mast cell histaminergic activity, which may impact upon other monoamine systems (e.g. DA, NE, 5-HT). Thus, in

extreme cases, the superimposition of a stressor onto a common immunological activating condition (e.g. influenza infection), which stimulates cytokine and histamine release, may have profound central consequences.

Neurochemical cross-sensitization between LPS and TNF- α : Macrophage involvement (Chapter II)

Several immune cells that participate in inflammatory reactions may play an important role in neuro-immune communication. For instance, inflammatory or traumatic challenges provoke the release of histamine, from both peripheral and central mast cells, which has numerous central actions (Brown, 2001). As well, circulating macrophages can also influence central processes through their release of IL-1, IL-6, TNF- α and other inflammatory factors. Indeed, cerebrovascular insults (e.g., cerebral artery occlusion) enhanced TNF- α brain mRNA likely originating from microglia or infiltrating blood-borne macrophages (Gregersen et al., 2000). As well, TNF- α immunoreactive macrophages detected within the pituitary were upregulated in response to LPS challenge (Arras et al., 1996). Commensurate with the view that macrophages influence central functions, selective depletion of macrophages using a liposome-clondronate suspension reduced the neurodegenerative effects of LPS as well as that of experimental allergic encephalomyelitis (Zito et al., 2001; Huitinga et al., 1990).

As LPS is believed to affect the CNS primarily through the induction of macrophage-derived cytokines, it was of interest to investigate their role in the neurochemical cross-sensitization engendered by LPS and TNF- α treatments. In the present investigation, liposome-clondronate administration blocked the corticosterone

rise induced by acute TNF- α . However, this treatment did not attenuate the cross-sensitization of corticosterone apparent in mice initially administered LPS and subsequently exposed to TNF- α . Thus, it is possible that the effects of macrophage depletion were unique to the immediate actions of LPS or TNF- α , and mechanisms independent of macrophages, were responsible for the sensitization. Alternatively, the effectiveness of the liposome treatment may depend upon the magnitude of the corticosterone changes elicited by the challenge, being absent in response to treatments that ordinarily promote particularly marked hormonal increases. In fact, liposome-clondronate pretreatment attenuated the rise of ACTH induced by subpyrogenic, but not by pyrogenic LPS dosages (Derijk et al., 1991). Likewise, *in vivo* macrophage depletion failed to affect the corticosterone elevation induced by the bacterial superantigen, staphylococcal enterotoxin B (Shurin et al., 1997).

The finding that macrophage depletion limited the corticosterone rise elicited by acute TNF- α , implicates macrophage functioning in the HPA activation elicited by the cytokine. Although macrophages are primary producers of TNF- α under basal conditions, and production is increased in the face of many immunological and metabolic challenges (Chancellor-Freeland et al., 1995; Liu et al., 2000), scant data exist concerning the direct signal transducing actions of TNF- α on macrophages. Nevertheless, receptors for the cytokine have been demonstrated on macrophages and it was postulated that the cytokine may act in an autocrine fashion regulating its own production (Beutler, 1999; Welborn et al., 1993; Witsell et al., 1992).

Paralleling the reduction of the corticosterone response, macrophage depletion also blocked the acute effects of TNF- α on MHPG accumulation within the PVN,

suggesting involvement of peripheral macrophages (e.g., release of cytokines, nitric oxide or other proteins) in some of the central actions of the TNF- α . Unlike the effects within the PVN, the liposome clondronate suspension did not attenuate the TNF- α -elicited increases of MHPG accumulation within the PFC, central amygdala, hippocampus and locus coeruleus, nor that of 5-HIAA within the PFC and hippocampus. However, the liposome-clondronate treatment did attenuate the monoamine cross-sensitization effects between LPS and TNF- α . In particular, macrophage depletion prevented the sensitized 5-HT and DA responses within the central amygdala and PFC, respectively. Indeed, non-liposome treated animals displayed markedly augmented accumulation of amygdaloid 5-HIAA and that of DOPAC within the PFC in response to TNF- α 1 day after LPS administration. In other regions, including the locus coeruleus and PVN, where acute TNF- α increased either NE and/or 5-HT activity, there was no evidence of a sensitization effect.

The possibility exists that the central sensitization effects observed were secondary to sensitization of immune cells. For instance, it is possible that the initial LPS treatment induced macrophage hyper-responsivity, such that subsequent challenges provoked more robust cellular responses, which then affected CNS processes. Indeed, LPS increased subsequent macrophage functions, including tumor cytotoxicity, bactericidal activity and cytokine production (West et al., 1997). Moreover, macrophage reprogramming has been demonstrated *in vitro*, wherein endotoxin reexposure 1 day following its pretreatment, increased IL-1 β and nitric oxide release, but reduced that of TNF- α (Fahmi et al., 1996; West et al., 1997). Similar changes of macrophage activity may be responsible for the sensitization of neurochemical variations observed in the

present investigation. Of course, the possibility cannot be excluded that the apparent sensitizing actions stem from effects of LPS on blood brain barrier permeability or variations of neutrophil and macrophage infiltration into brain (Andersson et al., 1992; Minami et al., 1998). In this respect, cytokines, LPS and even stressors have all been reported to increase blood brain barrier permeability (Banks et al., 1999; Quagliarello et al., 1991). Finally, it is important to underscore that the LPS-TNF- α cross-sensitization of monoamine activity was reminiscent of the neurochemical profile apparent using the TNF- α sensitization model. Specifically, in both cases (i.e. LPS or TNF- α primed mice) augmented monoamine activity was evident within the PFC and central amygdala upon subsequent exposure to TNF- α 1 day after the priming challenge. This pattern contrasts starkly with the later occurring sensitization (after a 28 day interval) provoked by TNF- α with respect to HPA activity and noradrenergic turnover within the PVN (Hayley et al., 1999). Taken together, these data raise the possibility that LPS may sensitize central monoamine utilization through the liberation of TNF- α from peripheral macrophages.

Potential mechanisms through which cytokines affect central processes

The process by which peripherally released cytokines influence CNS functioning has yet to be fully elucidated, and several potential hypotheses have been offered. According to one view, cytokines released from activated immune cells may serve to stimulate cytokine receptors present on the dendrites of the vagal nerve. Indeed, both IL- 1β and LPS induce *c-fos* expression at the nodose ganglion and provoke electrical activity of neurons within the gastric branch of the vagus (Dantzer et al., 1998; Maier & Watkins, 1998). After peripheral activation, the vagus may transmit neural information,

via the nodose ganglion, to brainstem regions such as the NTS, which in turn stimulates the hypothalamus. An alternative explanation (although not necessarily a mutually exclusive one) is that macrophage-derived cytokines, such as IL-1 β , IL-6, and TNF- α , as well as IL-2 secreted from T-helper cells, may act as mediators (immunotransmitters) in this respect. Despite the fact that peripheral cytokines comprise fairly large molecules, entry into brain may occur where the blood-brain barrier is less restrictive (e.g., the organum vasculosum laminae terminalis), or by a saturable transport system (Banks et al., 1991; Gutierrez et al., 1993). As well, blood brain barrier permeability may be compromised under some pathological conditions (e.g., seizure) culminating in greater accessibility to brain. In addition, it is possible that the cytokines may interact with receptors at circumventricular sites, leading to either central cytokine production or activation of prostaglandins which serve to excite parenchymal cells (Quan, et al., 1994; Zang & Rivest, 2000).

Although circulating immune cells represent a source of cytokines that can potentially influence CNS processes, several cytokines and their receptors have also been documented within the brain (Breder, 1993; Schobitz et al., 1994). The cytokine levels or synthesis can be elicited by a number of factors, including systemic bacterial endotoxins (Pitossi et al., 1997), central endotoxin application (Quan et al., 1994), as well as by brain injury, cerebral ischemia, and seizure (Rothwell, 1999; Yabuuchi et al., 1993). The functional significance of central cytokine alterations remains to be elucidated. Proinflammatory cytokines, such as interleukin-1 β (IL-1 β), may function in a reparative capacity in neurological diseases or brain trauma, or alternatively may actually promote neuronal damage following central insults (Rothwell, 1999). In any case, acute events

(e.g. stroke), chronic degenerative disease (e.g. Alzheimer's) or central autoimmune neuropathology (e.g. multiple sclerosis) all have common elements of inflammation characterized by abnormal accumulation of immune components (e.g. complement and infiltrating leukocytes) coupled with an upregulation of central cytokines such as IL-1 and TNF- α (Rothwell, 1999). Of course, it is possible that cytokines may simply represent bystanders, not involved in neurodegenerative processes at all. However this is unlikely given that cytokines may orchestrate the infiltration of immune cells into brain, as well as modulating their actions within the brain parenchyma, much like their well-documented effects in periphery. Moreover, de novo synthesized brain cytokines may activate apoptotic pathways, as well as inducing the production of potentially toxic free radicals or arachidonic acid metabolites (Chao et al., 1995; Zang & Rivest, 2000).

Role of TNF- α in psychiatric disorders

In addition to their illness-inducing effects, cytokines have also been suggested to have psychological sequelae reminiscent to those associated with stressors and psychiatric illness. Indeed, depressive illness is associated with elevated levels of IL-1 β , IL-2, IL-6, TNF- α , and soluble cytokine receptors (Maes, 1999). Furthermore, the effects of endotoxin on consumption of palatable food substances could be attenuated by chronic antidepressant administration (Yirmiya, 1996), possibly indicating that the treatment acts to temper the anhedonia, a fundamental characteristic of depressive illness. It might be noted at this juncture that humans undergoing immunotherapy (e.g., with IL-2 or with interferon- α) display numerous adverse neuropsychological, neurologic and psychiatric disturbances, including depression, which may be sufficiently severe to necessitate

discontinuation of treatment (Capuron et al., 2001; Denicoff et al., 1987). Interestingly, it has been reported that the selective serotonin reuptake inhibitor, paroxetine, attenuated the depressive-like effects associated with interferon- α treatment of malignant melanoma (Musselman et al., 2001).

Although TNF- α has been used in cancer treatment, its extreme toxicity and shock inducing properties limit the systemic application of this cytokine. However, recent reports indicate that it may be possible to isolate the toxic effects of TNF- α from the anti-tumor actions of the cytokine (Barabara et al., 1996). There have also been suggestions that elevated circulating TNF- α or its p55 receptor may be associated with psychiatric illness (Maes, 1999) and such an effect could come about by virtue of the cytokine's effects on central monoamine turnover (Ignatowski et al., 1994). Indeed, antidepressant medication has been reported to alter levels of TNF- α in brain regions such as the hippocampus and locus coeruleus (Ignatowski et al., 1994). Given the behavioral and neurochemical effects of TNF- α already mentioned, the present results provide further support for the suggestion that TNF- α has psychoactive actions. As well, the potent sensitization of illness provoked by the cytokine suggests caution be taken in clinical trials using the cytokine in an immunotherapeutic fashion, particularly with respect to the schedule of cytokine administration.

Proposed model for central effects of TNF- α

The findings presented in this thesis prompted us to propose the following scheme of events as one possible model to explain how TNF- α may have acute and sensitizing

actions on behavioral and neurochemical functioning. In brief, peripheral TNF- α may stimulate circulating macrophages and mast cells to release other cytokines, free radicals, prostaglandins and histamine. These, in turn, enter the brain through circumventricular organs, compromise the BBB and may also act to induce increased brain synthesis and local activity of cytokines, histamine and other inflammatory factors. It is suggested that a single injection of TNF- α primes this sequence of events, such that reexposure to the cytokine results in an augmented or possibly even a fundamentally altered response. Since the nature of this sensitization depends upon the passage of time following the initial challenge, it is suggested that slowly developing changes in intracellular signaling pathways (e.g. involving MAP and JNK kinases, NF κ B or Jak/Stat) or immunological reactivity (e.g. priming of humoral immunity) underlie the different sensitizing responses of TNF- α . Such intracellular changes may ultimately be manifested in altered central monoamine (NE, 5-HT, DA) and neuropeptide activity (CRH, AVP), which may in turn, impact upon behavioral processes. However, as the time of TNF- α reexposure and route of administration of the cytokine (i.p. vs icv) differentially affect the sensitization of sickness and monoamine activity, it is suggested that different mechanisms likely underlie these two distinct phenomenon. In particular, it is suggested that the sickness syndrome provoked by TNF- α (after a 28 day interval) represents a shock reaction that may be mediated by histamine, among other soluble factors. Finally, it appears that the p55 receptor may be the predominate receptor subtype responsible for many of the acute and sensitization effects of TNF- α described in this paper.

Conclusions

Numerous challenges, including stressful and immunological stimuli, may prime biological systems so that augmented responses are elicited upon later challenges with either the same or somewhat different stimuli. In addition to having immediate effects, cytokines appear to exert long lasting behavioral and neurochemical consequences. Indeed, like stressors, a single exposure to TNF- α primes CNS activity, such that later reexposure to the cytokine provoked profound alterations of sickness, HPA activation and brain monoamine utilization. Such effects may stem from increased receptor sensitivity or the augmented release of various endogenous factors, such as other cytokines, prostaglandins or acute phase proteins. In this respect, enhanced co-activity of these factors would be expected to result in synergistic actions on central processes.

The processes responsible for the development of the sensitization remain to be established. However, it is clear that multiple sensitizing effects are elicited by various treatments. In the case of TNF- α , it seems that the altered sickness and corticosterone variations are independent of one another, and both of these are unrelated to the central monoamine changes. Indeed, icv treatment with TNF- α induces the sensitization of monoamine functioning, but not that of illness or corticosterone alterations. Moreover, the central sensitizing effects of TNF- α are modifiable by variations of macrophage functioning, and histamine appears to contribute to the peripheral sensitization associated with the cytokine. Finally, although LPS induces macrophage release of TNF- α , it is clear that these two treatments induce different types of effects. The LPS treatment engenders a sensitization that is marked at short intervals, while TNF- α induces a sensitization that progresses with the passage of time.

It appears that cytokines may play some role under conditions of impaired CNS functioning. Several studies have demonstrated the presence of IL-1, IL-6 and TNF- α and their receptors in discrete brain regions under normal conditions, and these cytokines may even play a role in brain development. Brain levels of these cytokines are markedly upregulated in a number of neurological conditions (e.g. multiple sclerosis and cerebral infections), as well as in response to traumatic events (e.g. head injury or stroke). Furthermore, IL-1 β and TNF- α provoke HPA and central monoamine variations reminiscent of those observed in response to stressful stimuli. Moreover, increased circulating levels of IL-1 β and TNF- α , as well as IL-2, are often associated with depressive or schizophrenic conditions, attesting to their potential involvement in psychiatric illnesses (Maes, 1999; Griffiths et al., 2000). It is unclear, however, whether the emergence of the depression is secondary to variations of cytokine functioning, or whether cytokine alterations simply represent an epiphenomenon (bystander effects) associated with the mood disorders. As some of the depressive effects attributable to LPS treatment are modifiable by antidepressant medication (Yirmiya et al., 1999), as are the depressionogenic actions associated with interferon- α immunotherapy (Musselman et al., 2001), it is tempting to argue that cytokines play a pivotal role in the emergence of the affective condition. Yet, it has been demonstrated that the elevated cytokine levels associated with depressive illness do not necessarily normalize with the alleviation of symptoms following successful pharmacotherapy (Anisman & Merali, 2001; Maes, 1999). Thus, the precise relationship between cytokines and depressive illness remains to be elucidated. This notwithstanding, the fact that cytokines may increase the neurochemical responses to later stressors, and stressors may act as a provocative agent

for the induction of depression, the possibility needs to be considered that cytokine indirectly increase vulnerability to affective illness. Indeed, it has even been suggested that protracted alterations of HPA functioning stemming from early-life stressor or immunological challenges may contribute to the organism's subsequent ability to contend with environmental challenges, and hence may contribute to the development of stressor-related psychopathology (Heim et al., 1997; Meaney et al., 1996; Shanks et al., 2000).

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Appendix A.

Effects of TNF- α , LPS and stressors on plasma corticosterone levels in p55 knockout mice.

Abstract

Central or systemic administration of the proinflammatory cytokine, tumor necrosis factor- α (TNF- α), provokes sickness behaviors coupled with elevated neuroendocrine and monoamine activity. We have shown that many of these effects are subject to a time-dependent sensitization, such that their expression is greatly exaggerated when mice are pretreated with TNF- α and subsequently reexposed to the cytokine. As many of the central consequences of TNF- α are mediated by its p55 receptor, the present investigation sought to determine the role of this receptor in some of the acute and protracted effects of TNF- α . Likewise, there is reason to believe that stressors and immune challenges affect central functioning by stimulating the release of cytokines. Thus, plasma corticosterone levels were assessed in p55 knockout mice subject to (1) acute or reexposure TNF- α treatment, (2) novelty or restraint stressors, or (3) acute or reexposure LPS treatment. Mice deficient for p55 displayed a greatly attenuated plasma corticosterone response to TNF- α irrespective of whether they had been previously exposed to the cytokine. However, plasma corticosterone elevations were not prevented by p55 deletion in animals exposed to either of the stressor or LPS treatments. Curiously, the restraint induced corticosterone rise was modestly greater in the p55 knockout mice compared to their wild type counterparts. It appears that although p55 modulates the corticosterone inducing effects of TNF- α , the receptor does not play a critical role in the activation of the HPA axis by "traditional" psychogenic stressors (novelty, restraint) or systemic endotoxin challenge. These data may be relevant for clinical situations, involving the use of p55 agonists or antagonists (e.g. cancer, multiple sclerosis), in which circulating corticosterone levels may influence the course of the illness.

Introduction

The proinflammatory cytokines, interleukin (IL)-1 β and tumor necrosis factor- α (TNF- α), have been implicated in the instigation of behavioral disturbances associated with stressors, as well as bacterial and viral insults (Anisman et al., 2001; Hayley et al., 1999, 2001a; Griffiths et al., 2000; Dunn & Wang 1995). Indeed, the levels of these cytokine are elevated in response to a variety of nonimmunogenic challenges, including cerebrovascular insult, neurodegenerative diseases and even psychological stressors (Boutin et al., 2001; Mogi et al., 1994; Barone et al., 1997; Nguyen et al., 1998). Moreover, it has repeatedly been demonstrated that systemic or central administration of these cytokines provoke behavioral alterations, increased brain monoamine utilization and hypothalamo-pituitary-adrenal (HPA) activity (plasma ACTH and corticosterone release) as well as febrile and other metabolic responses (Bernardini et al., 1990; Hayley et al., 1999, 2001b; Dunn & Wang 1995; Schmidt et al., 2001).

Based on the congruence between the neurochemical effects of stressors and cytokine or endotoxin treatments (Anisman et al., 2001; Lenczowski et al., 1998), the view was offered that the brain interpreted cytokine activation as if it were a stressor (Anisman & Merali, 1999; Dunn, 2000). In this respect, cytokines have reliably been shown not only to affect HPA neuroendocrine activity and to increase hypothalamic monoamine release, but also to increase norepinephrine and serotonin release at several extrahypothalamic sites (Anisman & Merali, 1999; Dunn, 2000). Moreover, administration of the endogenous IL-1 receptor antagonist attenuated the hypothalamic

neurochemical alterations ordinarily engendered by a restraint stressor (Shintani et al., 1995).

In addition to their acute effects, IL-1 β and TNF- α provoke the sensitization of central neurochemical functioning, such that a single application of either of these cytokines influences later corticosterone, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) responses to the same cytokine (Schmidt et al., 1995; Hayley et al., 1999, 2001b). Paralleling the time course for the corticosterone sensitization provoked by TNF- α , which develops with the passage of time, reexposure to this cytokine 28 days following initial TNF- α treatment provoked marked signs of illness reminiscent of a shock reaction (hypovolemia, ptosis, piloerection, cyanosis of the extremities) (Hayley et al., 2001b). As well, a neurochemical sensitization was evident in mice reexposed to TNF- α after a 1-day interval, as indicated by enhanced norepinephrine (NE) activity within the central amygdala and medial prefrontal cortex (Hayley et al., 1999).

Although cellular signaling by TNF- α is mediated by two receptor subtypes, p55 and p75, it seems likely that p55 may play a more dominant role in the central effects of the cytokine (Beutler, 1999; Nadeau & Rivest, 1999). Indeed, activation of p55 by exogenous TNF- α has inflammatory as well as apoptotic consequences through stimulation of receptor-associated death domains and intracellular caspases (Beutler, 1999; Akassoglou et al., 1998); however, endogenous TNF- α may have neuroprotective effects since p55 deficient mice have been reported to display increased neuronal damage in response to ischemic insults (Gary et al., 1998). Such protective effects may stem from selective activation of the branches of the TNF- α signaling pathway which recruit

TNF-receptor associated factors 1 and 2 (TRAF-1, -2), which can induce NF κ B or AP-1 dependent production of various anti-oxidant and anti-apoptotic factors (Natoli et al., 1998).

The p55 receptor also appears to be the major mediator of the behavioral (e.g. somnolence) and neuroendocrine effects of TNF- α (Fang et al., 1997; Benigni et al., 1996). In fact, unlike wild type mice, p55-deficient mice failed to show a corticosterone rise in response to central infusion of TNF- α (Benigni et al., 1996). Moreover, icv administration of a selective p55 agonist increased circulating corticosterone levels to a greater extent than did selective stimulation of central p75 receptors (Benigni et al., 1996). However, p75 stimulation, through TRAF involvement, can promote inflammation as well as cellular proliferation (Baud & Karin, 2001).

Considering the importance of p55 in the actions of TNF- α , it was of interest to determine whether this receptor contributes to the acute and sensitizing behavioral and HPA effects of TNF- α . Owing to the proposition that TNF- α may contribute to the neuroendocrine effects of lipopolysaccharide (LPS) challenge (Ando & Dunn, 1995), the role of p55 was also investigated in this respect. Finally, since psychogenic stressors have been reported to increase TNF- α levels (Weber et al., 2002; Hale et al., 2001), coupled with the finding that IL-1 receptor antagonist prevented the immobilization induced ACTH rise in rats (Shintani et al., 1995), it was of interest to determine if p55 contributes to HPA activation by a restraint or auditory stressor (both of which have a strong psychological component). To this end, corticosterone alterations were determined in knockout mice deficient in the gene responsible for the production of p55, as well as the appropriate background strain (C57BL/6J).

Materials and Methods

Male and female C57BL/6J p55 knockout mice (homozygous for a targeted mutation of the TNF- α p55 receptor, located on chromosome 6) and stock C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, MA). The p55 deficient mice are largely normal in immune cell development, but have impaired TNF- α signaling. They are resistant to the toxic effects of LPS and *Staphylococcus aureus* but are more susceptible to *Listeria monocytogenes* (Pfeffer et al., 1993). Mice were maintained in a breeding colony room with one male housed with three females; pregnant females were housed singly until pups were weaned (approximately 21 days). Following weaning, pups were housed by litter (culled to only males), while the dam was allowed to rest for three weeks before being reintroduced to the breeding colony.

Animals were maintained on a 12 hour light/dark cycle with lights on at 0800, room temperature was kept at 21° C, and were permitted an ad libitum diet of Ralston Purina mouse chow. All experimental procedures were approved by the Carleton University Committee for Animal Care and met the Guidelines set out by the Canadian Council on Animal Care.

Experiment 1: Acute and sensitizing effects of TNF- α

Mice from each of the C57BL/6J and C57BL/6J-p55 strains received an initial intraperitoneal (ip) injection of either sterile nonpyrogenic saline (0.9 NaCl %) or 2.0 μ g of recombinant murine TNF- α (TNF- α) in a volume of 0.4 ml of sterile saline. Twenty-eight days following the initial treatment mice were further subdivided, so that animals

from each group were given a second i.p. injection of either saline or 1.0 μg of mTNF- α ($n = 8-10/\text{group}$). At 60 min after the second injection, mice were decapitated trunk blood collected and placed in chilled tubes containing 10 μl of EDTA. Blood was centrifuged and the supernatant extracted and stored at -80°C until determination of plasma corticosterone levels. The 60 min interval between TNF- α administration and subsequent decapitation was chosen on the basis of earlier studies showing that behavioral and glucocorticoid variations were marked at this time (Brebner et al., 2000; Hayley et al., 1999).

Experiment 2: Acute and sensitizing effects of LPS

The stimulatory effects of bacterial and viral challenges on HPA activity has been well established, and macrophage-derived cytokines, particularly IL-1 β and TNF- α , are believed to play an important role. Moreover, LPS was shown to provoke a sensitized HPA response, as reflected by elevated plasma corticosterone upon exposure to TNF- α 1 day following treatment with the endotoxin. However, unlike the sensitization elicited by initial TNF- α treatment, the peak sensitization effect elicited by LPS occurred one day after treatment (Hayley et al., 2001a). To investigate the role of the p55 TNF- α receptor in acute LPS induced neuroendocrine effects mice from each of the C57BL/6J and C57BL/6J-p55 strains ($n=8/\text{group}$) received an ip injection of either sterile nonpyrogenic saline or LPS (5 μg) and trunk blood collected 2 hrs later, the time of the peak LPS effects. In a second study, aimed at assessing the sensitized corticosterone response, mice of each strain (C57BL/6J and C57BL/6J-p55) were treated with LPS (5 μg ; ip) and then reexposed to a lower dose of the endotoxin (1.0 μg ; ip) 1 day later. Separate groups of

animals from each strain received either saline followed by the low dose of LPS (1.0 µg; ip) or saline only on two occasions spaced 1 day apart. At 60 min following the final injection, trunk blood was collected for corticosterone determination.

Experiment 3: Differential stressor effects

Given that the behavioral and neuroendocrine alterations provoked by stressors may, in part, be related to the cytokines induced by such treatments, it was of interest to determine the impact of p55 receptor deletion on stressor induced corticosterone levels. To this end, mice from both strains, C57BL/6J and C57BL/6J-p55, were subjected to restraint or an auditory stressor, both of which have been shown to elicit marked elevations of plasma corticosterone in C57BL/6J mice (Anisman et al., 2001). Restraint, which lasted 10 min, involved placing the mouse in an acrylic tube (inside diameter of 4.5 cm, and an overall adjustable length of 4.5 cm to 5.7 cm), securing the barriers such that they provided a snug fit. The mouse's tail, which protruded from the tube, was taped down to prevent the animal from moving. Consequently lateral, as well as forward movement of the animal, was prevented, and only limited paw and head movement was possible. To apply the auditory stressor, mice were placed in a sound attenuated chamber (30 cm x 55 cm x 50 cm) that contained a wideband speaker (1 kHz - 16 kHz). Mice were individually placed in a clear, cylindrical acrylic holder (4.5 cm x 5.7 cm), approximately 20 cm from the audio source. Following a 5 min acclimatization period mice received 30 stimulus presentations (50 ms pulse of white noise at 115 dB) at an interstimulus interval of 30 seconds. Following the restraint or noise stressors, mice were placed in a holding

cage for 5 min after which they were decapitated and trunk blood collected. Control mice from each strain remained undisturbed in their home cages until decapitation.

Plasma corticosterone assay

Plasma corticosterone was determined (in duplicate) using a commercially available radioimmunoassay (RIA) kit (ICN Biomedicals Inc., CA). To avoid interassay variability all samples were assayed within a single run. The intra-assay variability was less than 10%.

Statistical analyses

Plasma corticosterone concentrations were analyzed by analysis of variance (ANOVA) with Strain x Cytokine or Stress treatments (and reexposure treatment where appropriate) as between group factors. Post hoc tests were conducted using Bonferonni corrected t tests.

Results

Acute and sensitizing effects of TNF- α

In contrast to the marked sickness behavior previously observed in CD-1 mice upon reexposure to TNF- α , no such effect was evident in the C57BL/6J animals used in the present investigation. Treatment with TNF- α , however, markedly increased the levels of plasma corticosterone, $F(1, 55) = 11.37$, $p < 0.01$, and the effect of the cytokine interacted with the strain of mouse, $F(1, 55) = 7.99$, $p < 0.01$. As shown in Figure 1 and

confirmed by the *t* tests, although TNF- α increased plasma corticosterone levels in wild type mice, it had no such effect in the p55 knockouts. Although the Strain x Initial injection x Reexposure injection interaction failed to reach significance $F(1, 55) = 2.14$, $p = 0.14$, specific *a priori* predictions had been made in this respect, and hence the Bonferroni corrected *t* tests were conducted of the means comprising the simple effects for this interaction. As previously observed in CD1 mice, the C57BL/6J mice that received acute TNF- α displayed increased corticosterone levels relative to saline treated animals. Moreover, relative to the effects of the acute TNF- α treatment, corticosterone levels were increased still further upon reexposure to TNF- α 28 days later, indicating sensitization of HPA activity in C57BL/6J mice as observed previously in the non-inbred CD1 strain. The basal levels of corticosterone in the p55 knockouts were comparable to those seen in C57BL/6J mice. However, plasma corticosterone levels in C57BL/6-p55 knockout mice were neither affected by the initial nor reexposure TNF- α treatments.

Insert Figure 1 about here

Acute and sensitizing effects of LPS

As in the case of TNF- α , the C57BL/6J mice failed to show appreciable illness in response to the relatively low doses of LPS used in the present investigation. However, plasma corticosterone levels were increased by the LPS treatments, $F(1, 24) = 40.67$, $p < 0.01$. In contrast to the results of the preceding experiment, where the effects of TNF- α were eliminated in the knockout mice, the effects of LPS on plasma corticosterone levels

Figure 1. Involvement of the p55 TNF- α receptor in the sensitization of plasma corticosterone elicited by the cytokine. Wild type C57BL/6J (left) and p55 knockout (right) mice were pretreated with either saline or mTNF- α (2.0 μ g) and subsequently 28 days later reexposed to either saline or the cytokine (1.0 μ g). Thus, mice from each strain were divided into the following groups: saline saline (black bars), saline mTNF- α (gray bars), mTNF- α saline (hatched bars) or mTNF- α mTNF- α (white bars). Corticosterone determinations were made 60 minutes following the final injection. *p < 0.05 relative to saline saline treatment, ° P < 0.05 relative to saline mTNF- α treatment.

Initial-Reexposure treatments (28 days)

- Saline-Saline
- ▨ Saline-TNF
- ▧ TNF-Saline
- TNF-TNF

were comparable in the knockout and wild type mice (Figure 2). In the second LPS experiment, where mice that initially been treated with LPS and then reexposed to the endotoxin, plasma corticosterone levels were again affected by the treatment condition, $F(2, 28) = 5.02$, $p = 0.01$. However, the strains of mice were found not to differ from one another, and in both strains LPS increased corticosterone measured 2 hr later. This was the case irrespective of the treatment mice received 1 day earlier.

Insert Figure 2 about here

Effects of stressor treatments

Plasma corticosterone levels varied as a function of stressor treatment mice received, $F(2, 37) = 55.36$, $p < 0.01$. As shown in Figure 3 and confirmed by the multiple comparisons, the startle and restraint stressors increased plasma corticosterone levels in both C57BL/6J and p55 knockout mice. However, corticosterone levels did not differ between the two strains, although the effect of the restraint stressor was somewhat less pronounced in the C57BL/6J than in the p55 knockout ($p < 0.07$).

Insert Figure 3 about here

Figure 2. Involvement of the p55 TNF- α receptor in the effects of LPS on plasma corticosterone levels. Wild type C57BL/6J (left) and p55 knockout (right) mice received either saline (black bars) or LPS (white bars; 5.0 μ g; i.p.). Corticosterone determinations were made 120 minutes following injection. *p < 0.05 relative to saline treatment.

Figure 3. Involvement of the p55 TNF- α receptor in the plasma corticosterone responses to psychogenic stressors. Wild type C57BL/6J (left) and p55 knockout (right) mice received either no treatment (black bars), 10 minutes of restraint (gray bars) or 30 presentations of a 115 dB pulse of white noise (white bars). Corticosterone determinations were made 5 minutes following stressor termination. * $p < 0.05$ relative to untreated controls.

Discussion

The effects of TNF- α in C57BL/6J and in the p55 knockout mice were clearly distinguishable from that previously seen in CD-1 mice. Ordinarily, acute administration of TNF- α within this dosage range does not elicit signs of illness in CD-1 mice. However, upon reexposure to the cytokine marked signs of illness are elicited, characterized by ptosis, piloerection, curled body posture, reduced social interaction and exploration (Hayley et al., 1999, 2001a). In the present investigation, using the same procedures, neither acute nor reexposure to TNF- α elicited even a hint of illness. Given the profound effects seen in CD1 mice upon TNF- α reexposure, the lack of behavioral changes in C57BL/6J mice was unexpected. Yet, the absence of illness in mice with the C57BL/6 background is in keeping with reports indicating that this particular strain is resistant to a number of challenges, including kainic acid induced seizure generation (Kurschner et al., 1998), or mortality and proinflammatory cytokine production associated with viral and bacterial infections (Schauwecker & Steward, 1997; Uzonna et al., 1999). As well, C57BL/6 mice have been shown to be resistant to various immunological challenges, such as the sleeping sickness inducing pathogen, *Trypanosoma rhodesiense* (Greenblatt et al., 1985), and exhibit less pronounced corticosterone elevations in response to IL-1 β , relative to that seen CD-1 or BALB/cByJ mice (Anisman et al., 2001). Yet, C57BL/6 mice have been reported to display illness in response to challenges, including IL-1 β (Bluthe et al., 2000) or the gram-negative bacteria, *Legionella pneumophila* (Gibertini et al., 1995).

Consistent with our previous findings in the CD1 strain (Hayley et al., 1999), acute TNF- α increased plasma corticosterone levels in C57BL/6J mice. Likewise, reexposure to the cytokine 28 days later augmented the corticosterone rise still further, indicating a sensitization effect. In p55 deficient mice, in contrast, neither acute nor reexposure to TNF- α affected plasma corticosterone levels, suggesting that the cytokine must bind to this receptor subtype in order to stimulate HPA activity. The present findings do not speak directly to whether this effect was related to TNF- α binding to peripheral or central p55 receptors. It is noteworthy, however, that p55 receptors, which are found in abundance within peripheral organs (e.g. liver, spleen, intestines), have also been localized in relatively high concentrations in the hypothalamus, particularly within the PVN as well as in the brainstem and circumventricular organs (Nadeau & Rivest, 1999). Yet, when administered icv, TNF- α induces only a moderate rise of plasma corticosterone, far below that elicited by systemic administration of the cytokine (Hayley et al., 2002a), implicating a peripheral site of action for the cytokine.

As previously reported (Benigni et al., 1996), deletion of the p55 receptor did not affect the plasma corticosterone response to LPS. Evidently, binding of TNF- α to the p55 receptor does not play a prominent role in LPS induced corticosterone secretion. Although it has been suggested that soluble TNF- α may contribute to the neuroendocrine stimulatory effects of LPS challenge (Ando & Dunn, 1999), immunoneutralization of the cytokine has been reported to have little effect on LPS provoked plasma corticosterone activity (Dunn, 1992). However, the evidence does exist to support a role for TNF- α in the hypophagic consequences of LPS administration (Swiergiel & Dunn, 1999). In this respect, blocking either TNF- α , IL-1 or IL-6 alone had only modest effects on LPS

induced hypophagia, however, concomitant antagonism of all three cytokines markedly attenuated the reduction of food intake (Swiergiel & Dunn, 1999), suggesting that the interactive cytokine actions are critical.

Although the possibility exists that $\text{TNF-}\alpha$ like $\text{IL-1}\beta$, contributes to the neuroendocrine response to stressors, the present findings indicated that the p55 null mice displayed a pronounced corticosterone rise in response to the auditory stressor that was comparable to that evident in the C57BL/6J strain. Restraint also increased corticosterone concentrations in both strains of mice, although the extent of this increase was less pronounced in wild type mice. The latter finding was unexpected, and certainly is inconsistent with the proposition that the p55 receptor is activated by stressors, thereby leading to a rise of corticosterone. However, it should be considered that although p55 may play a role in the HPA effects of restraint or other stressors, alternate factors (e.g. other cytokines, prostaglandins) might compensate for the loss of this receptor. Furthermore, since $\text{IL-1}\beta$ itself has potent HPA activating effects, coupled with the fact that stressors and immune insults readily increase expression of $\text{IL-1}\beta$, raises the possibility that this cytokine may be responsible for some of the observed neuroendocrine effects. Indeed, it was previously reported that the endogenous IL-1 antagonist, IL-1ra , attenuated the ACTH inducing effects of an immobilization stressor (Shintani et al., 1995). There is also reason to believe that IL-6 may subserve some of the neuroendocrine effects of LPS, since immunoneutralization of this cytokine was reported to reduce the plasma ACTH and corticosterone elevations engendered by the endotoxin (Wang & Dunn, 1999). However, this effect was apparent at 3 but not 1 h following LPS

administration (Wang & Dunn, 1999), suggesting that factors other than IL-6 contribute to the early HPA stimulatory effects of the immune challenge.

Taken together the present results indicate that activation of the p55 TNF- α pathway is not necessary for stressor or LPS induced corticosterone elevation. However, as the rise of corticosterone elicited by acute and reexposure TNF- α treatments was prevented in p55 knockout mice, it appears that the cytokine must bind to this receptor subtype to activate the stressor-sensitive HPA axis. Since substantial evidence exists to indicate that elevated glucocorticoids can have damaging effects on neuronal survival, the possibility should be considered that clinical treatments involving chronic stimulation of the p55 receptor might have deleterious central consequences through the release of excessive levels of corticosterone. Moreover, it was reported that while p55 activation was associated with a high rate of mortality and toxic side effects (chills, fever, delirium), specific activation of the p75 TNF- α receptor in mice, using a recombinant adenovirus vector, promoted tumor regression in the absence of toxicity (Marr et al., 1999). It is possible that some of these toxic systemic effects of p55 activation may be related to enhanced circulating corticosterone, which may come influence central and peripheral metabolic processes. As such, treatments aimed at minimizing activation of the p55 receptor, while maximizing p75 stimulation, may prove efficient for the treatment of cancers. As well, recent clinical trials using p55 receptor antagonists have showed some success in the treatment of autoimmune diseases (e.g. rheumatoid arthritis, Chron's disease). It is conceivable that such beneficial effects may stem, in part, from blunting the HPA response to high levels of circulating TNF- α associated with these inflammatory states. In effect, the hormonal sequelae (interactions with central and peripheral

processes) arising from alterations of p55 receptor activity needs to be considered within the context of the aforementioned clinical situations.

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