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**Time Dependent Sensitization of Behavioral, Central
Neurochemical and Neuroendocrine activity in Response to
Tumor Necrosis Factor- α**

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

Master of Science in Psychology
Specialization in Neuroscience

Shawn Hayley
Department of Psychology
Carleton University (1998)

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The undersigned hereby recommend to the Faculty of Graduate Studies and Research
acceptance of the thesis submitted by

Shawn Hayley

in partial fulfilment for the degree of
Master of Science

.....
Chair, Department of Psychology

.....
Thesis Supervisor

Carleton University

July, 1998

Abstract

Consistent with the proposition that cytokines act as immunotransmitters between the immune system and the brain, systemic administration of the proinflammatory cytokine, tumor necrosis factor- α (TNF- α) (1.0 - 4.0 μ g) enhanced hypothalamic-pituitary-adrenal (HPA) functioning, as reflected by elevated plasma corticosterone levels, and altered levels and/or activity of norepinephrine (NE), dopamine (DA) and serotonin (5-HT) in several brain regions. These effects were subject to a time-dependent sensitization effect. Upon reexposure to a sub-effective dose of the cytokine (1.0 μ g) 2-4 weeks following initial treatment, marked illness was provoked accompanied by activation of hypothalamic-pituitary-adrenal functioning and enhanced NE turnover within the paraventricular nucleus of the hypothalamus (PVN). In contrast, reexposure to the cytokine after 24 hr resulted in a desensitization of plasma corticosterone levels in the absence of any overt sickness symptoms. In addition, at the shorter reexposure interval substantial increases of amine utilization were evident in the medial prefrontal cortex and to a lesser extent in the locus coeruleus and PVN. Interestingly, both an early and a late sensitization pattern was observed for 5-HT in the prefrontal cortex and PVN. While the later sensitization may be related to sickness, the mechanisms responsible for the two sensitization effects have yet to be identified. Nevertheless, these findings may have important implications concerning brain-immune interactions, alterations of emotional and cognitive functioning associated with cytokine treatment, and procedures appropriate in immunotherapy.

Acknowledgments

I would like to express my sincere thanks to my supervisor, Dr. Hymie Anisman, who made this thesis possible through his many creative insights and the excellent guidance he has given me during the last two years. I am also indebted to Dr. Jerzy Kulczycki for his unremitting expert technical assistance. Further thanks apply to my family, friends and lab mates, all of whom had faith in my abilities and the patience to put up with me. This research was supported by a grant from the Medical Research Council of Canada to Hymie Anisman and Zul Merali.

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Introduction

It has been proposed that multidirectional communication exists between the immune, autonomic, hormonal and central nervous systems (Anisman, Zalcman & Zacharko, 1993; Blalock, 1994; Cunningham & De Souza, 1993; Neta, Sayers & Oppenheim, 1992). Immune activation promotes alterations in central neurotransmitter activity (Besedovsky et al., 1981, 1983; Dunn, 1993; Dunn & Welch, 1991; Lacosta, Merali, Zalcman & Anisman, 1994; Zalcman, Shanks & Anisman, 1989b), as well as peripheral neurochemical and hormonal changes (e.g. plasma corticosterone or ACTH) (Besedovsky, Sorkin, Keller & Muller, 1975), and conversely, alterations in CNS neurotransmitters have been shown to provoke immune alterations (e.g. lymphocyte proliferation) (Lysle et al., 1995; Pezzone, Dohanics & Rabin, 1994).

It is believed that, among other things, immune activation stimulates the hypothalamic-pituitary-adrenal (HPA) axis (Anisman et al., 1993; Besedovsky & Del Rey, 1991; Blalock, 1989, 1994; Dantzer & Kelley, 1989; Dunn, 1990; Felten et al., 1985; Ottaway & Husband, 1992; Rabin et al., 1989), and in several respects has actions similar to those elicited by stressors (Anisman et al., 1993; Dunn, 1992). Thus, it was proposed that hormonal variations involving the HPA axis may function as one possible biological substrate through which information is channeled between the immune system and CNS. Additionally, it has been suggested that the proinflammatory cytokines, including interleukin-1 β (IL-1 β), IL-6 and tumor necrosis factor alpha (TNF- α), which are released primarily from activated macrophages, may serve in such a signaling capacity (i.e. they may act as immunotransmitters) (Dunn, 1992; Hopkins & Rothwell, 1995).

Cytokines not only have immediate effects on HPA activity and on some behaviors (e.g. sickness behavior), but may result in sensitization of neuroendocrine and neurotransmitter processes, such that later responses to cytokine challenge (as well as stressors) may be enhanced. For instance, it was demonstrated that IL-1 β engenders an increase in the colocalization of arginine vasopressin (AVP) and corticotropin releasing

hormone (CRH) in CRH neurons with terminals in the external zone of the median eminence (ZEME) (de Goeij, Kvetnansky, Whitnall, Jezova, Berkenbosch & Tilders, 1991; Schmidt, Janszen, Wouterlood & Tilders, 1995). It was suggested that since AVP and CRH act synergistically to provoke pituitary ACTH release, such a process may account for some of the protracted effects associated with cytokine or stressor exposure (Tilders, Schmidt, & de Goeij, 1993). Interestingly, it has been suggested that protracted alterations of HPA functioning stemming from early-life stressor or immunological challenges may contribute to the subsequent development of pathological conditions (Heim, Owens, Plotsky, Nemeroff, 1997; Meaney, Diorio, Francis, Widdowson, LaPlante, Caldji, Sharma, Seckl, Plotsky, 1996).

Despite the impressive evidence suggesting that IL-1 β , like stressors may induce the sensitization of neurochemical processes, there is scant information available concerning the potential proactive effects of TNF- α . This is particularly surprising since both cytokines are derived from macrophages, and it is thought that many immune and behavioral consequences may reflect the conjoint actions of these cytokines. The purpose of the present investigation was to ascertain (a) the immediate effects of systemic TNF- α administration on various behavioral parameters (consumption of a palatable chocolate milk solution and sickness symptoms), plasma corticosterone, as well as central biogenic amines, and to determine (b) whether TNF- α has protracted effects on behavior, plasma neuroendocrine and central amine activity, particularly upon re-exposure to a relatively low dose of a previously administered cytokine treatment (i.e. a sensitization effect). Inasmuch as the development of sensitization effects (e.g., to stressors and catecholamine acting agents) may be time dependent (Antelman, 1988), the possibility was explored that the effects of TNF- α would likewise vary over time following initial cytokine treatment.

Proinflammatory cytokines.

Acute phase response.

The acute phase response is a generalized host reaction to various environmental insults such as infection, inflammation as well as to physical or psychological stress (Mandrup-Poulsen, Nerup, Reimers, Pociot, Anderson, Karlsen, Bjerre & Bergholdt, 1995; Zhou et al., 1996). This response involves immune activation followed by changes in metabolic, neurological and endocrine functioning (Mandrup-Poulsen et al., 1995). It has been shown that IL-1 β , TNF- α and IL-6 are important mediators of the acute phase response following various immune challenges (e.g. viral or bacterial infection) and may act as mediators of the brain's response to such challenges. For example, TNF- α has been found to modulate the firing of thermosensitive and glucose-responsive hypothalamic neurons as well as alter feeding and nutrient metabolism in a manner similar to that observed during systemic infection (Breder, Hazuka, Gahayur, Klug, Huginin, Yasuda, Teng, & Saper, 1994). It is thought that the acute phase response engenders profound central neurochemical changes, which may be of adaptive significance (Maness, Banks, Zadina & Kastin, 1995). However, these neurochemical alterations have also been proposed to act like stressors in eliciting behavioral pathologies such as depressive illness (Maes, Vandoorn, Ranja, Bosmans, Bergmans, Desnyder, 1995).

Localization in brain tissue

Cytokines and cytokine receptors appear to be endogenous to the brain, having been identified within neuronal cell bodies, microglia and astrocytes, (Farrar, Kilian, Ruff, Hill, Pert, 1987; Katsuura, Gottschall & Arimura, 1988; Nistico & De Sarro, 1991). Receptors for IL-1 β and IL-6 have been found in several areas of the mouse and rat brain, including the hippocampus, choroid plexus, olfactory bulb, cerebellum and hypothalamus (Cunningham & De Souza, 1993). Receptors for TNF- α have been found to have the highest concentration in the brainstem, thalamus and basal ganglia, while lower

concentrations were present in the cerebellum and cortex (Kinouchi, Brown, Pasternak, & Donner, 1991). Moreover, TNF- α immunoreactivity has been detected in neurons within the hypothalamus, bed nucleus of the stria terminalis and the caudal raphe nuclei (Breder et al., 1993a). It seems likely that cytokines may gain access to the brain from peripheral sites, albeit in limited concentration, and that *de novo* synthesis of cytokines may occur in microglia (Hopkins & Rothwell, 1995; Rothwell & Hopkins, 1995; Schobitz, De Kloet & Holsboer, 1994), astrocytes (Kimberlin, Willis, McCracken, Nisen 1995; Hofman, Hinton, Johnson & Merrill, 1991) and neurons (Gaballec,Griffais, Fillion, & Haour, 1995; Kinouchi et al., 1991; Mandrup-Poulsen et al., 1995; Schobitz et al., 1994). Furthermore, it is thought that while neuronally derived IL-1 β may participate in the transfer of information, glial-produced IL-1 β may play a role in cellular repair, neural growth, and may govern the development of some of the neuropathological changes seen in neurodegenerative diseases (Berkenbosch, de Rijken , Del Rey , Besedovsky,1990; Rothwell, 1991).

Interestingly, in addition to being constitutively expressed in brain, central cytokine expression has been shown to be influenced by stressors as well as various immunological and neurological insults. For example, induction of 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 (Ban et al, 1992; Buttini & Boddeke, 1995; Gatti & Bartfai, 1993; Breder et al., 1994; Gabellec et al, 1995; Laye et al., 1994, 1995; Liu et al., 1996), central LPS injection (De Simoni et al., 1995; Quan et al., 1994; Rajora et al., 1997), viral infection (Sato, Reiner, Jensen,& Roos, 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). Thus, it seems likely that central cytokine activity may play a role in many of the alterations in brain function seen after psychological and physiological pathology.

The question of how the immune system signals the CNS has generated much research, and considerable attention has been devoted to the potential role of cytokines (particularly IL-1 β) in this respect. Several mechanisms have been proposed by which cytokines inform the brain of peripheral challenge. These include the possibility that cytokines gain entry into the brain through circumventricular areas lacking an efficient blood brain barrier (BBB), carrier mediated transport across the BBB, induction of prostaglandins in the brain or induction of de novo synthesis of central cytokines. It has also been suggested that afferent fibers of the vagus nerve may transmit information from activated immune cells (possibly triggered by cytokines) to the brain in response to a physiological or psychological stressor (Dantzer, Bluthé, Aubert, Goodall, Bret-Dibat, Kent, Goujon, Laye, Parnet, Kelley, 1996; Watkins, Maier & Goehler, 1995). Although the particular route or mechanism involved is a question of debate, it has been shown that cytokines do influence brain activity. For instance, systemically administered IL-1 β can activate numerous discrete brain regions and has been found to increase *c-fos* expression in the PVN, bed nucleus of the stria terminalis, central nucleus of the amygdala, lateral parabrachial nucleus and the dorsomedial and ventrolateral medulla (Ericsson et al., 1994).

Cytokine and stressor induced activation of the HPA axis.

It is well established that exogenous stimuli, such as stressors or cytokines activate the HPA axis, and IL-1 β is particularly potent in this respect (Del Rey & Besedovsky, 1992; Dunn, 1989, 1992; Rivier, Chizzonite & Vale, 1989). Systemic and central administration of IL-1 β stimulates the release of CRH and AVP secretion from PVN neurons of the hypothalamus (Mandrup-Poulsen et al., 1995; Tilders et al., 1993; Watanobe & Takebe, 1993). These secretagogues synergistically stimulate the release of pituitary ACTH which, in turn, elicits adrenal glucocorticoid secretion (Johnson, Kamilaris, Chrousos & Gold, 1992; Lightman, 1994; Plotsky, 1991; Rivier & Vale, 1985). The glucocorticoids stimulate hippocampal cells, terminating CRH release from the

PVN (Angelucci & Scaccianoce, 1990; Jacobson & Sapolsky, 1991; Meany et al., 1996; Sapolsky et al., 1990). 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, Guyre & Holbrook, 1984).

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, Brand, Audhya, Nabriski, Hutchinson, Rosenweig & Hollander, 1992; Sapolsky et al., 1987), supporting the notion that this cytokine does not act directly on the pituitary to stimulate ACTH release. Further to the same issue, intravenous administration of IL-1 β has been shown to provoke a time and dose dependent increase in *c-fos* expression in CRH and oxytocin neurons of the PVN (Ericsson, Kovacs, & Sawchenko 1994), supporting the notion 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 suggested that CRH treatment may lead to the sensitization of pituitary cells, such that greater ACTH is elicited upon subsequent IL-1 β challenge (Payne, Weigant, Blalock, 1994). However, subsequent reports indicated 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).

In addition to IL-1 β , other cytokines, including IL-6 and TNF- α , may influence HPA activity (Bernardini, Kamilaris, Calogero, Johnson, Gomez, Gold & Chrousos, 1990; Blalock, 1994; Neta, Sayers, Oppenheim, 1992; Rothwell & Hopkins, 1995; Schobitz et al., 1994; Zhou et al., 1996). Similar to IL-1 β , both intravenous and i.c.v. injection of TNF- α has been found to activate the HPA axis and stimulate ACTH and corticosterone secretion in a dose dependent fashion and this effect was blocked by CRH antiserum (Bernardini et al., 1990; Turnbull et al., 1997). In fact, TNF- α has been found

to stimulate hypothalamic CRH release using both in vivo and in vitro preparations (Johnson et al., 1992). Whether IL-6 influences corticosterone or ACTH release is questionable. There have been reports of increased ACTH release after repeated IL-6 treatment, but little effect after acute administration (Brebner et al., 1998; Zalcman et al., 1994). Despite such reports, it is commonly assumed that IL-6 induces variations of HPA functioning. In part, this likely stems from the findings that IL-6 promotes IL-1 β activity (the latter it will be recalled is a potent HPA stimulant), and from the reports that IL-1 β and IL-6 may act synergistically in promoting ACTH variations (Zhou et al., 1996). Thus, at this juncture the precise effects of IL-6 remain to be elucidated, but it is clear that this cytokine is considerably less effective than IL-1 β in promoting HPA activation (Del Rey & Besedovsky, 1992).

While the acute effects of IL-1 β , IL-6 and TNF- α on the HPA activity have been relatively well documented (the aforementioned disclaimer concerning IL-6 notwithstanding), much less attention has focused on the chronic effects of these substances. In contrast to the elevations in corticosterone and ACTH seen after acute administration, van der Meer and colleagues (1995) found that continuous infusion of IL-1 β only slightly raised corticosterone and ACTH levels, and only on the first day of treatment, and not on two ensuing days. Moreover, TNF- α had no significant effect on these hormones when chronically administered (van der Meer et al., 1995). However, in a subsequent study continuous icv infusion of IL-1 β for three days resulted in persistent elevations of plasma ACTH and corticosterone and such an effect was evident even at very low doses (0.1 ug/day) (van der Meer, Sweep, Pesman, Tilders & Hermus, 1996). Indeed, continuous i.p. infusion of IL-1 β has been shown to cause a prolonged elevation of plasma ACTH and corticosterone levels (Sweep, Van Der Meer, Hermus, Smals, Van Der Meer, Pesman, Willemsen, Benraad & Kloppenborg, 1992).

The route of administration of cytokines may also determine the mechanism by which they impact on HPA processes. It has been suggested 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, Michaud, Kelley, Dantzer, 1996; Dantzer et al., 1996). In contrast, the suppressive effects of vagotomy on IL-1 β elicited social exploration were absent when the cytokine was administered subcutaneously or intravenously (Bluthe et al., 1996). However, cytokines may also effect HPA function through other routes, such as, direct entry into the brain where the blood-brain barrier (BBB) is somewhat compromised (e.g. the organum vasculosum) (Dunn, 1990; Hopkins & Rothwell, 1995; Mandrup-Poulsen et al., 1995). In fact, systemic administration of IL-1 β or TNF- α can lead to a disruption of the BBB (Quagliarello, Wisplwey, Long, & Sheld, 1991) making it easier for these substances to gain entry to the brain. It has been proposed 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). As well, the suggestion has been made that IL-1 β (Banks et al., 1989) and TNF- α (Gutierrez et al., 1993) have saturable transport mechanisms which pump these cytokines into the brain.

The role of proinflammatory cytokines in pathological conditions.

While the primary function of cytokines has been assumed to comprise their role as immune system growth factors [e.g. IL-6 and TNF- α regulation of B cell differentiation (Rieckmann et al., 1991)], they have been shown to act by lysing invading tumors (TNF- α) (Haranaka, Satomi & Sakurai, 1984) and they may even play a permissive role in the development of the brain (e.g. TNF- α) (Kinouchi et al., 1991; Sipe et al., 1996). Additionally cytokines, such as IL-1 β and TNF- α have been implicated in various autoimmune disorders such as rheumatoid arthritis, insulin-dependent diabetes mellitus and inflammatory bowel disease (Deer, 1997). In fact, recent clinical reports show that antibodies directed against TNF- α may be effective in the treatment of rheumatoid arthritis

and Crohns disease (Eigler, Sinha, Hartmann & Endres, 1997). In addition, there is increasing evidence suggesting that the expression of IL-1 β , IL-6 and TNF- α in brain is associated with several CNS pathologies, including Alzheimer's disease (Ershler, 1993; Holden & Mooney, 1995; Vandenbeeke & Fiers, 1991; Wong, Rajora, Boccoli, Catania and Lipton, 1997), head injury (McClain et al., 1991; Wong, et al., 1997), bacterial meningitis and viral infection (Bitsch et al., 1997; Frei et al., 1989; Swiergiel et al., 1997; Wasselingh et al., 1991; Wong, et al., 1997), multiple sclerosis (Frei et al., 1991; Merrill & Benveniste, 1996; Wong, et al., 1997) and CNS HIV (Corley, 1997; Merrill & Chen, 1991; Wong, et al., 1997). While the cytokines (particularly IL-1 β) may be acting in a reparatory capacity, at least at low doses (Rothwell & Strijbos, 1995), it seems that at higher doses these cytokines are actually responsible for at least some of the cellular death associated with these conditions (Rothwell & Hopkins, 1995). A highly redundant biochemical profile for IL-1 β and TNF- α may account for the above mentioned overlapping pathological effects ascribed to these cytokines. Indeed, IL-1 β and TNF- α may share many biological effects owing to the convergence of signal conduction pathways, leading to activation of common transcription factors, namely, nuclear factor κ B and activating protein 1 (AP 1) (Deer, 1997).

Central neurochemical effects of immune activation or cytokine challenge.

The proposition that immune activation may influence CNS process was given credence by reports showing that administration of sheep red blood cells (SRBC) culminated in altered hypothalamic unit activity, as well as variations of NE turnover, and that these changes corresponded with an immune response being mounted (Besedovsky et al., 1981,1983). Subsequent studies confirmed that SRBC, in fact, provoked time-dependent variations of hypothalamic NE concentrations, as well as NE variations that were restricted to the PVN and supraoptic nuclei of the hypothalamus (Zalcman et al., 1989b). Moreover, in vivo SRBC was found to influence the utilization of accumbal DA,

indicating that the monoamine variations were neither restricted to the hypothalamus nor to alterations of NE (Lacosta et al., 1994). It has likewise been shown that LPS induces in vivo monoamine variations much like those associated with SRBC challenge (Dunn, 1988; Shintani, Kanba, Nakaki, Nibuya, Kinoshita, Suzuki, Yagi, Kato, & Asai, 1993). Lavicky & Dunn (1995) used in vivo microdialysis to show that i.p. LPS administration in rats results in an increase in interstitial NE and DA concentrations in the medial prefrontal cortex and in the medial hypothalamus. Moreover, other immune challenges, such as viral infection, have been found to alter brain NE and 5-HT activity (Dunn 1993; Dunn, Powell, Meitin & Small, 1989). In fact, viral challenge has been reported to increase NE turnover in hypothalamus, and tryptophan concentrations in parietal cortex and in hypothalamus (Dunn & Vickers, 1994; Dunn et al., 1989). However, it is likely that the mechanisms responsible for neurotransmitter changes may be dependent on the characteristics of the immune challenge.

As in the case of immune activation, cytokines provoke changes in brain monoamines and their metabolites in hypothalamic and extrahypothalamic regions. Systemic IL-1 β administration results in a decrease of NE levels and a parallel increase of MHPG concentration in post mortem hypothalamic tissue (Dunn, 1992). The effects of the cytokine on NE turnover is increased in the PVN and medial basal and lateral hypothalamic nuclei (Dunn, 1988). Likewise, IL-1 β influences serotonergic activity, as indicated by elevations of the metabolite, 5-HIAA, in the hypothalamus (Dunn, 1992) as well as prefrontal cortex and hippocampus (Zalcman et al., 1994). 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, variations in the dopaminergic system have also been found after IL-1 β administration, as reflected by increases of the DA metabolite homovanillic acid (HVA) in

the hypothalamus, as well as augmented cortical DA utilization (Kabiersch et al., 1988; Masana et al., 1990).

As in the case of postmortem studies, cytokine treatments have been shown to influence *in vivo* monoamine functioning. 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., 1993). Interestingly, the augmentation 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., 1993). Likewise, direct injection of IL-1 β into the medial basal hypothalamus augmented serotonergic and dopaminergic activity as reflected by increased interstitial DA, DOPAC and 5-HIAA levels in this region (Mohankumar et al., 1991, 1993). In addition, intravenous IL-1 β administration has also been found to influence anterior hypothalamic unit activity (Bartholomew & Hoffman, 1993), and increases 5-HIAA levels in this brain region (Carmelia et al., 1991). Intracerebroventricular administration of IL-1 β has been shown to provoke an increase in extracellular concentrations of 5-HT and 5-HIAA measured in the hippocampus *in vivo* (Linthorst et al., 1995), and recent 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, Lacosta & Anisman, 1997; Song, Merali & Anisman, 1997).

In addition to IL-1 β , it appears that TNF- α and IL-6 may also provoke alterations of CNS amines, albeit to a lesser extent. Catecholamine release from cultured sympathetic neurons may be influenced by TNF- α (Soliven & Albert, 1992a), and TNF- α may inhibit long term potentiation in slice preparations (Tancredi et al., 1992). Furthermore, in rat brain slice preparation TNF- α was found to markedly reduce firing rate of ventromedial hypothalamic neurons (Katfuchi et al., 1997). Mefford et al. (1991) found that systemic administration of TNF- α caused an increase of hypothalamic NE utilization, but to a lesser

extent than did IL-1 β . Similarly, the IL-6 induced hypothalamic and hippocampal NE turnover was appreciably lower than that elicited by IL-1 β (Zalcman, 1994). In contrast, 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 vivo, however, IL-6 has been shown to profoundly inhibit the release of DA from the nucleus accumbens (Song et al., 1997).

Inasmuch as IL-1 β , IL-6 and TNF- α have potent pyrogenic effects, the possibility that the neuroendocrine and neurotransmitter changes induced by these cytokines were secondary to such effects was considered. 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). In fact, induction of PGE₂ in the brain has been suggested as one possible factor by which cytokines influence CNS amines. However, the effects of such eicosanoid substances may be brain region specific, in fact, it has been suggested that PG may mediate NE turnover in the hypothalamus but not in the cortex or medulla oblongata (Terao, Oikawa & Saito, 1993). Indeed, pretreatment with the prostaglandin inhibitor, indomethacin, was found to block LPS induced alterations of NE and DA concentrations (Lavicky & Dunn, 1995) as well as IL-1 β induced cortical DA utilization (Masana et al., 1990). Likewise, indomethacin has been shown to prevent the increase hypothalamic NE turnover associated with systemic and icv administration of IL-1 β (Terao et al., 1993). 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, 1988b; Kabiersch et al., 1988). Indeed, we have found doses as low as 0.2 μ g of IL-1 β to influence NE turnover in the PVN, locus coeruleus, prefrontal cortex (Lacosta, Merali & Anisman, 1997) 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.

The role of cytokines in the stress response.

Blalock (1984, 1995) suggested that the immune system acted as a sensory organ to inform the brain of viral or bacterial insults. In view of the similarity between the central actions of stressors and those of immune activation, it was suggested that immune challenge (and particularly cytokine administration) was interpreted by the brain as if it were a stressor (Anisman et al., 1993; Dunn, 1990). In support of this contention is the finding that IL-1 β , and to a lesser extent TNF- α and IL-6, can provoke plasma endocrine and central neurochemical alterations comparable to those seen after stressor exposure (Bernardini et al., 1990; Dunn, 1992; Lyson & McCann, 1992; Pearlstein et al., 1993). In fact, IL-1 β has been shown to provoke phenotypic changes in hypothalamic neuropeptide composition (Schmidt et al., 1995), similar to that seen after a chronic psychosocial stressor (De Goeij, Dijkstra, Tilders, 1992). Moreover, Shintani et al., (1995) demonstrated that pretreatment with IL-1Ra blocks the hypothalamic NE alterations and the plasma ACTH increases induced by immobilization stress. Interestingly, IL-1Ra was only effective in this respect when administered shortly before the stressor onset; once the stressor was applied subsequent administration of IL-1Ra was without effect (Shintani et al., 1995). The time dependent nature of IL-1 Ra effects on immobilization stress suggest 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.

It is likely that the PVN plays an important role in the modulation of the brain's response to immune activation and stress. Indeed, both IL-1 β and various stressors have been found to increase *c-fos* expression in CRH-immunoreactive neurons within the PVN (Rivest & Rivier, 1994). In addition to the marked CRH changes in the PVN, it has been shown that stressors give rise to increased CRH mRNA expression at extrahypothalamic sites, including the amygdala and bed nucleus of the stria terminalis (e.g., Sawchenko et al., 1996). Indeed, Pich et al (1993, 1995) and our group (Merali et al., 1997) have shown

that stressors promote increased CRH release at the central amygdala as measured by *in vivo* microdialysis. Consistent with the suggestion that CRH plays a pivotal role in subserving anxiety, there have been numerous reports showing that icv CRH, or infusion into the amygdala, induced behavioral changes (in a variety of behavioral paradigms) reminiscent of anxiety, while CRH antagonists (e.g., α -helical CRH) had the effect of reducing anxiety (Davis, 1992; Gray, 1990). In addition, icv CRH administration has been shown to induce *c-fos* mRNA expression in stressor reactive areas of the brain (Imaki, Shibasaki, Hotta, Demura, 1993). Recently, it has been proposed that fear eliciting stimuli may result in activation of CRH in the central amygdala, whereas diffuse stimuli (leading to anxiety, but not associated with stimulus-evoked fear, i.e., unconditioned stimuli) may engender CRH release from the bed nucleus of the stria terminalis, but without central amygdala activation (Lee & Davis, 1997). Activation of the basolateral amygdala, which innervates both regions, may be fundamental to both conditioned and unconditioned fear responses (Walker & Davis, 1997). It is possible, as well, that the contribution of monoamines in the left and right basolateral amygdala of the two hemispheres may not play identical functions in stressor-related emotional responses (Adamec & Morgan, 1994).

Herman and Cullinan (1997) proposed that the neural circuits involved in the stress response vary in accord with the stimulus attributes of the stressor in question. They 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., bacterial and viral insults as well as cytokines) do so by a limbic-independent mechanism. Instead, input from the autonomic nervous system may play a more predominant role in the relay of chemical signals to the PVN after systemic cytokine administration. In this respect, it was demonstrated that IL-1 β elevates tryptophan (Dunn & Welch, 1991) as well as MHPG concentrations in the PVN (Dunn, 1992), and these effects may be related to projections from brainstem nuclei. In fact, ablation of aminergic projections from the medulla resulted in a significant reduction in

IL-1 β induced c-fos in the PVN (Ericsson et al., 1994). This is not to say that cytokines are without effect on limbic system activity, as it was reported that IL-1 β increased Fos-immunoreactivity in the central amygdala and bed nucleus of the stria terminalis (Ericsson et al., 1994; Sawchenko et al., 1996). Furthermore, in preliminary studies in our laboratory it was observed that systemic IL-1 β increased the in vivo release of CRH from the central amygdala (Merali, Michaud, Kent & Anisman, unpublished results). As indicated by Sawchenko et al (1996), cytokine challenge and neurogenic stressors (restraint, footshock) share numerous similarities, although distinct differences are noted in the general profile of neuropeptide variations. It would seem important at this juncture to determine whether the alterations of CRH induced by these two treatments have comparable behavioral sequelae, as well as comparable effects on other hormones and neurotransmitters. Moreover, it remains to be established whether cytokine challenge influences subsequent neuroendocrine and neurochemical responses to subsequent stressor application, and vice versa.

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

There is reason to believe that cytokines have synergistic effects with respect to neuroendocrine release. For instance, coadministration of IL-1 β and TNF- α can synergistically increase adrenal gland weight in rats (van der Meer et al., 1995) and coadministration of TNF and IL-1 β (Perlstein et al., 1993) or IL-6 and IL-1 β (Zhou et al., 1996) synergistically increase ACTH and plasma corticosteroid release. The coadministration of TNF- α and IL-6 increased ACTH release, but in an additive rather than synergistic fashion (Perlstein et al., 1993). 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).

Commensurate with the suggestion that the neuroendocrine and/or neurochemical effects of cytokines and stressors involve common underlying mechanisms, it was shown that IL-6 may act in a synergistic manner with a psychological stressor. 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. This finding is all the more intriguing given that CRH has been suggested to act through CNS pathways that subserve behavioral responsiveness to novelty (Menzaghi et al., 1994). However, it would appear that restraint may utilize a separate pathway, which is not readily amenable to the neuroendocrine actions of IL-6. In addition to the neuroendocrine effects, cytokines may also have synergistic effects on central amine activity. For example, IL-1 β has been found to act synergistically with a mild stressor (air puff) in determining central neurochemical alterations, including elevated 5-HIAA, HVA and MHPG concentrations at several limbic sites (Merali et al., 1997; Song et al., 1997). In a similar fashion, TNF- α may synergistically impact on central neurochemical processes. In particular, TNF- α may augment the action of Beta-amyloid protein on the induction of nitric oxide in astrocytes (which has been proposed to play a role in the pathology seen in neurodegenerative disorders such as Alzheimer's disease) (Rossi & Bianchini, 1996).

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

In concert with immunological changes, pathogens promote behavioral styles that may be beneficial in order for the organism to deal with the insult. Yet, the biological or behavioral strategies deployed are not without cost, and may have both immediate and protracted adverse consequences, including increased vulnerability to psychological disturbances. Experimental activation of the immune system (e.g., by bacterial endotoxins) or direct administration of products of an activated immune system (e.g., cytokines such as IL-1 β , IL-6 and TNF- α) induce an array of behavioral symptoms commonly referred to as

sickness behavior (Kent et al. 1992). In addition to fever and curled body posture, systemic IL-1 β and TNF- α provoke anorexia (Bluthe et al. 1989; Dantzer et al., 1996; Hellerstein et al. 1989; Holden & Pakula, 1996; Kent et al., 1994; McCarthy et al. 1985; Plata-Salaman 1989, 1994; Sonti et al., 1996; Van der Meer et al., 1995), sleep (Krueger et al. 1995; Moldofsky 1995; Payne et al. 1993), and reduced locomotor activity (Linthorst et al. 1995), each of which minimizes energy expenditure and sustains body temperature. Interestingly, some of the responses to IL-1 β treatment (e.g., food and water intake; social exploration, sleep and soporific effects) directly or indirectly involve central mechanisms (e.g., Bluthe et al., 1997; De Sarro et al., 1997; Kent et al., 1996; Masotto et al., 1992), although peripheral IL-1 β and effects on vagal afferents appear to contribute in this respect as well (e.g., Dantzer et al., 1996; Kent et al., 1996; Watkins, Maier & Goehler, 1995).

In addition to these behaviors, cytokines disrupt operant responding for food reinforcement (Bluthe et al. 1989; Bret-Dibat et al. 1995; Crestani et al. 1991; Kent et al., 1994), exploratory behaviors (Dunn 1992; Spadaro and Dunn 1990), as well as social interaction (Bluthe et al. 1996). While these effects may be secondary to the sickness-inducing effects elicited by cytokines (e.g., IL-1 β), they may also reflect motivational actions (e.g., anhedonia) elicited by the treatments (Dantzer et al. 1996; Kent et al. 1992). In addition, it seems likely that both IL-1 β and TNF- α (but not IL-2 or IL-6) applied systemically or by intraventricular route induced anxiogenic-like responses (e.g., in a plus maze) (Connor, Song, Leonard, Merali & Anisman, 1998; Lacosta, Merali & Anisman, 1997, 1998). Interestingly, however, neither IL-1 β , IL-6 (Anisman et al., 1998) nor TNF- α (unpublished findings), disrupted responding for rewarding brain stimulation, while IL-2 markedly interfered with responding. Yet, LPS, which stimulates the secretion of all three proinflammatory cytokines, disrupted responding for rewarding brain stimulation, possibly reflecting the synergistic actions of these cytokines (Borowski et al., 1997). Commensurate with the view that cytokines may act synergistically in the provocation of behavioral changes, coinjection of IL-1 β and TNF- α have been found to synergistically reduce body

weight and food consumption and to increase rectal temperature in a dose dependent fashion (Van Der Meer et al., 1995). Likewise, studies reported by Sonti, Ilyin and Plata-Salaman (1996), as well as experiments in this laboratory, indicated that systemic injection of TNF- α or IL-1 β reduced food consumption, as well as intake of a highly preferred substance (chocolate milk). Moreover, these effects were enhanced in a synergistic manner when the cytokines were co-administered (Brebner, 1997; Sonti et al., 1996). Whether such a suppression in feeding reflects a response of an anhedonic nature or simply a product of a general malaise remains to be determined. Provisionally, the data suggest that beyond their illness inducing actions, cytokines may elicit anxiogenic and anhedonic effects, and these may be pertinent to the analysis mood disorders that have been associated with cytokine alterations (Anisman et al., 1998; Maes, 1995), as well as to neuropsychiatric sequelae of immunotherapy (Hanisch et al., 1997).

Cytokine and stressor sensitization effects.

Sensitization refers to an increased response of a biological system to a stimulus, given prior exposure to that particular stimulus. For instance, in classical immunology, 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. In the fields of behavioral neuroscience or psychopharmacology, the term implies that a 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. Moreover, 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). It appears that sensitization may impact upon a wide spectrum of biological systems including the CNS, neuroendocrine and immune systems.

Sensitization in response to stressors

There have been numerous reports showing that exposure to stressors will enhance the subsequent neurochemical response to mild stressors (Anisman et al., 1993; Finlay et al., 1997; Gresch, Sved, Zigmond & Finlay, 1994; Jordan, Kramer, Zukas & Petty, 1994; Robinson, Becker, Young, Akil, & Castaneda, 1987). Such effects have been documented with respect to hypothalamic, hippocampal and amygdaloid NE, as well as DA activity within the prefrontal cortex (Anisman & Sklar, 1979; Finlay et al., 1997; Gresch et al., 1994; Irwin et al., 1986; Jordan et al., 1997). Indeed, among animals that had been exposed to chronic cold stress, later stressor exposure in the form of tail-shock, promoted an increase of NE release in the hippocampus and medial prefrontal cortex, as well as an increase of DA release from cortically projecting neurons (Gresch et al., 1994; Nisenbaum, Zigmond, Sved & Abercrombie, 1991). Likewise, tyrosine hydroxylase activity in the hippocampus also increased after rats that had been chronically treated with cold stress were later exposed to a tail shock stressor (Nisenbaum & Abercrombie, 1992). Furthermore, acute stressor exposure may also lead to a sensitization of central amines. It has been shown that while first exposure to swim stress does not cause dramatic changes in biogenic amine release, a second exposure to the stressor elicits profound *in vivo* increases in NE, 5-HT and DA in the medial prefrontal cortex (Jordan et al., 1994). Of particular importance from the present perspective is that the two sessions need not involve the same stressors (i.e., cross-stressor sensitization effects are readily observed) and the stressor may be either of a chronic or acute nature.

It might be noted at this juncture that the sensitization that occurs following a chronic stressor regimen is not entirely congruent with that provoked by an acute stressor. Ordinarily, following chronic stressor exposure the levels of NE and DA are not reduced as they are following an acute uncontrollable stressor. In the case of NE, it seems that the chronic stressor may result in provocation of a compensatory increase of synthesis, possibly owing to autoreceptor subsensitivity. Interestingly, if animals were subjected to a chronic stressor regimen, and then subsequently exposed to an acute stressor, the amine

levels remained constant, while the utilization of NE was greatly increased (Irwin, Ahluwalia & Anisman, 1986). Thus it was suggested that while acute stressor exposure leads to sensitization of mechanisms associated with NE release, chronic stressor regimens also provoked sensitization of the mechanisms promoting NE synthesis. Thus, upon stressor re-exposure after a chronic regimen the greatly increased NE utilization is met by a comparable increase of synthesis (Anisman et al., 1993).

Analysis of the behavioral effects of stressors revealed that the impact of aversive challenges become progressively more pronounced with the passage of time (e.g., Anisman, deCatanzaro & Remington, 1978; Glazer & Weiss, 1976). While these findings were frequently attributed to factors such as incubation of fear, time-dependent variations of competing motor responses, and biphasic variations of more than a single transmitter (e.g., Anisman, 1975), the view was offered that the passage of time may be an essential factor for the generation of neurochemical changes which govern or facilitate sensitization effects (Antelman, 1988). Indeed, acute footshock has been shown to result in a time dependent impairment of behavioral functioning which is not present 1 or 4 hours after the stressor but is apparent after 24 hours and is still seen 21 days later (van Dijken et al., 1992).

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). Moreover, as in the case of stressors, administration of proinflammatory cytokines (e.g. IL-1 β , IL-6 and TNF- α) may result in a sensitizing effect such that subsequent responses to these cytokines is increased (Schmidt et al., 1995; Tilders et al., 1993). 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, Kvetnansky, Whitnall, Jezova, Berkenbosch & Tilders, 1991; Schmidt, Janszen, Wouterlood & Tilders,

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 (Schmidt et al., 1995). Tilders and his associates (de Goeij, Jezova & Tilders, 1992a; Schmidt et al., 1995) reported that following a chronic stressor regimen, or several days after exposure to either a single stressor experience or after systemic IL-1 β administration, characteristic changes were observed in hypothalamic CRH terminals in the external zone of the median eminence (ZEME). Ordinarily approximately 50% of the CRH terminals in the ZEME contain AVP (Whitnall, Smyth & Gainer, 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). The increased colocalization of these neuropeptides only became apparent 4 days after IL-1 β administration, and was still greater 11 days following the treatment. The AVP stores 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). It was suggested that IL-1 β ordinarily activates non-AVP producing CRH neurons in naive rats; however, in animals previously treated with the cytokine the re-exposure results in a shift toward the activation of CRH neurons coproducing AVP (Schmidt et al., 1995). In effect, it was proposed that pretreatment with IL-1 β caused a sensitizing effect manifested as an enhanced secretion of AVP and a subsequent augmentation of the signal driving ACTH release (Schmidt et al., 1995).

Not surprisingly, enhanced co-release of AVP and CRH into the hypophyseal portal system results in an increase in plasma corticosterone concentrations owing to the synergistic effects of these secretagogues on pituitary ACTH release (Rivier & Vale, 1983; Schmidt et al., 1995). Inasmuch as glucocorticoid disturbances have been implicated in numerous behavioral pathologies, including several affective disorders, post traumatic

stress disorder, as well as chronic fatigue syndrome (Pezeshki, Pohl & Schobitz, 1996 ; Propes & Johnson, 1997; Servatius, Ottenweller , Natelson, 1995) it would be of interest to establish whether cytokine alterations, like stressors, render the organism more vulnerable to such pathologies.

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 chronic intermittent immobilization stress (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). It is not clear what mechanism may be responsible for the increased AVP stores observed in CRH terminals in the ZEME, but it has been suggested that an absence of glucocorticoid inhibitory effects on AVP production (Sawchenko et al., 1987) or hyperactivity of CRH neurons in the PVN may cause such a phenotypic shift (Tilders et al., 1993). Moreover, it is presumed that the apparent sensitizing effects of IL-1 β and stressors stem from these phenotypic variations of CRH neurons. Yet, the possibility needs to be considered that the neuroendocrine consequences of various challenges, including pathogenic or chemical 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, Klostergaard, Khodadadian, Wu, Wu, Fung, Carper & Tomasovic, 1996). Thus, it is possible that treatment with a cytokine may affect subsequent thermogenic responses to this cytokine, and hence may come to influence later HPA responses induced by re-exposure to the cytokine or to stressors.

In addition to the sensitizing effects described thus far, it was reported that a similar enhancement of circulating ACTH and corticosterone levels was evoked by electric

footshock in rats pretreated with IL-1 β (Schmidt et al., 1995). Such a cross sensitization was presumably mediated by the phenotypic variations of AVP within CRH neurons, resulting in a subsequent augmentation of HPA activity induced by stressor exposure. Like IL-1, TNF- α may also have cross-sensitizing actions. In particular, TNF- α application to isolated sensory neuron cultures enhanced the current amplitude of these neurons to a subsequently applied dose of the chemical agent capsaicin (Nicol et al., 1997). Parenthetically, certain aspects of drug abuse may also be subject to sensitization (i.e. enhanced drug effect after pre-exposure to that same substance) or cross-sensitization (i.e. enhanced drug effect after pre-exposure to a different substance or challenge, e.g. stress) (Phillips, Roberts & Lessov, 1997). Moreover, stressor exposure has been found to sensitize mice to the rewarding effects of ethanol and it is likely that the HPA axis may play a role in this cross-sensitization effect (Phillips et al., 1997).

Purpose of the present investigation

The data presented by Tilders et al. (de Goeij et al., 1991; Schmidt et al., 1995; Tilders et al., 1993) makes it clear that IL-1 β , like stressors, may have a sensitizing effect with respect to HPA functioning. There is likewise reason to believe that TNF- α may have effects of a sensitizing nature on some biological systems. For example, various pathogens, such as bacillus Calmette-Guerin, have been found to sensitize mice to the lethal effects of LPS, whose actions are presumed to be mediated primarily by TNF- α and IL-1 (Wallach et al., 1988). In addition, infection with the lymphocytic choriomeningitis virus sensitized mice to the lethal effects of LPS, and this hypersensitivity correlated with hyperproduction of TNF- α (Nansen, Christensen, Marker, Thomsen, 1997). Furthermore, cells normally resistant to the apoptotic effects of TNF- α were made susceptible to such actions after exposure to the Hepatitis B virus (Su & Schneider, 1997), indicating a sensitizing role for the virus in TNF- α mediated apoptosis. Finally, low doses of endotoxin (Wallach, 1988) or the transcription inhibitor actinomycin D (Sipe et al.,

1996) were found to sensitize cells to the apoptotic effects of $\text{TNF-}\alpha$. To our knowledge, however, there are no published reports that have examined whether a sensitization effect occurs with respect to direct administration of $\text{TNF-}\alpha$ (as opposed to viral insults), whether such an effect is evident with respect to either behavior, neuroendocrine processes, or central neurotransmitters, and whether such a sensitization is dependent on temporal factors.

In view of these considerations, coupled with the possibility that $\text{TNF-}\alpha$, like IL-1, can act as an immunotransmitter between the immune system and the CNS, it was of particular interest in the present investigation to assess the effects of $\text{TNF-}\alpha$ on central biogenic amine functioning. Given the lack of attention previously devoted to $\text{TNF-}\alpha$ induced central amine and neuroendocrine changes, a preliminary study assessed the dose-dependent immediate effects on neurochemical functioning. In parallel with the neurochemical analyses, an experiment was conducted to examine the possibility that an initial administration of $\text{TNF-}\alpha$ would lead to sensitization of the behavioral effects (sickness profile) of this cytokine.

Experiment 1: Acute neurochemical effects of TNF- α

Materials and methods

Subjects

Male CD-1 mice, 8-10 weeks of age were obtained from Charles River Inc, Laprairie Quebec. Mice were housed in groups of four in standard propylene cages, and given at least two weeks to acclimate to the laboratory. Animals were maintained on a 12 hour light/dark cycle with lights on at 0800, room temperature was kept at 21°C. An ad libitum diet of 5075 Ralston Purina mouse chow and tap water was available.

Procedure

In order to minimize the contribution of diurnal variations associated with neurotransmitter or neuroendocrine processes, or potential effects of cytokines, all experimental procedures were conducted between 0800 and 1200 hr. Mice (n=10/group) were injected intraperitoneally (i.p.) with either sterile saline (0.9 %) or human rTNF- α (1.0, 2.0 or 4.0 μ g) (R & D Systems, specific activity = 1.1×10^5 units/mg, dissolved in sterile saline), and then decapitated 1 hr later. Recombinant human TNF- α was chosen because of its specific stimulation of the p55 receptor and its less toxic systemic effects compared to the murine form of the cytokine. Brains were immediately frozen in isopropyl alcohol, and then stored at -80°C until later sectioning and removal of nuclei for analysis of CNS amines. Trunk blood was collected and centrifuged (3500 rpm for 15 minutes) in tubes containing 10 μ l of EDTA, and the supernatant was stored at -80°C until assayed.

Plasma corticosterone assay

Plasma corticosterone levels were determined (in duplicate) using a commercially available radioimmunoassay (RIA) kit (ICN Biomedicals Inc., CA). Using this method and cross checking with internal standards (W.H.O 74/555), we typically obtain intra-assay and inter-assay variations of 6% and 10%, respectively. However, to eliminate interassay

variability, the hormone analyses were conducted in a single run. The corticosterone antiserum shows cross reactivity of 6.1% with desoxycorticosterone, 0.29% with progesterone, 0.19% with cortisol, and negligible (<0.1%) with other steroids including aldosterone, dihydroprogesterone, testosterone, estradiol.

Brain dissection technique

Frozen brains were placed on a stainless steel dissecting block (cortex side down) and aligned with olfactory bulbs just protruding past the anterior aspect of the block. Sharp razor blades were then inserted into the first and last slots of the block, thereby effectively dissociating the olfactory bulbs and the lower brainstem while anchoring the rest of the brain to prevent any movement in the block. When properly anchored, blades were sequentially slid into adjacent slots (spaced approximately 0.5 mm apart), sectioning the brain into a progressively descending series of coronal slices. The dissection of the brain was conducted in a cold chamber, with the brain resting on stage containing dry ice. Brain sections were mounted on glass slides and placed on a petri dish filled with powered dry ice which served as a cold stage. Utilizing the mouse brain atlas of Franklin and Paxinos (1997), brain nuclei were removed by micropunch using a hollow 16 or 20 gauge needle with a beveled tip. Tissue was taken bilaterally from the PVN, locus coeruleus, hippocampus, central amygdala, prefrontal cortex, and nucleus accumbens, and then stored at -80° C until processed using HPLC.

HPLC procedure for analysis of brain amine 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 which was comprised of 14.17 g monochloroacetic acid, 0.0186 g 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 (5 μ , C18 reverse phase, 8 mm x 10 cm), and a three cell coulometric electrochemical detector (ESA Model 5100,A) 20 μ l 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 1.3 g of heptane sulfonic acid, 0.1g 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. 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).

Experiment 2: Sensitization to the effects of TNF- α

Behavioral analysis

It will be recalled that TNF- α , like IL-1 β , induces a series of behavioral effects, including decreased locomotor activity and exploration, diminished food and water intake, piloerection, ptosis, curled body posture, and soporific effects, collectively referred to as sickness behavior (Kent et al., 1992, 1996). Like Bluthé et al (1996), we observed that systemic administration of TNF- α induces a similar profile. In this respect, particularly marked reductions in the consumption of a highly palatable food (chocolate milk) is evident in TNF- α treated animals (Brebner et al., 1998). Accordingly, in addition to the neuroendocrine and neurotransmitter alterations, in the present investigation we also assessed whether variations in behavioral outputs would be associated with reexposure to cytokine treatment. In this respect, we assessed (a) whether consumption of the palatable food was subject to a sensitization effect, and (b) in a separate set of animals (n=10 group), whether the initial treatment or reexposure to TNF- α would influence social interaction, which may be reflective of the sickness profile.

To assess the effects of TNF- α on consumption of the palatable solution, mice that received ad lib food were given free access to chocolate milk (Sealtest, 1% partly skimmed) for one hour each day during the light phase (commencing at 1000 hr). Bottles were weighed at the beginning and end of the hour in order to determine total consumption (weights were converted to volume). Following the establishment of a steady rate of drinking (3 consecutive days over which consumption varied by less than 10%), mice received an acute i.p. injection of either saline or TNF- α (1.0, 2.0 or 4.0 μ g). Mice were then returned to their home cages. Two weeks later mice from each group were subdivided and treated with either saline or the lowest dosage (1.0 μ g) of TNF- α (n = 10/group). One hour later chocolate milk was presented to the mice individually and the consumption over a 1 hr period was recorded.

The design of the second behavioral study is shown in Table 1. In this sickness symptom/social interaction test, mice ($n = 10/\text{group}$) received TNF- α (4.0 μg) and then at 1, 7, 14 or 28 days later they were reexposed to the 1.0 μg dosage of the cytokine. Three additional groups, which acted as controls, received either saline on 2 occasions, saline followed 2 weeks later by TNF- α (1.0 μg), or TNF- α (4.0 μg) followed two weeks later by saline. Mice were then individually returned to their home cages, and their behavioral profiles and social interactions with noninjected cage-mates was recorded. Commencing 15 min after the second i.p. injection, and at the 3 successive 15 min intervals, the following behaviors were rated on 4 point scales (low values denoting low levels of given behavior, and high values denoting high levels of behavior): locomotor activity, social interaction (huddling with other mice vs isolation from cage mates), and overall appearance (normal, lethargic, mild sickness as defined by slow movement, ruffled fur, ptosis; severe sickness as defined by non-movement, curled body posture, ruffled fur, marked ptosis, difficulty breathing, and general nonresponsiveness to cage mates).

Table 1.

Research design for time-dependent sensitization effects

<u>Initial injection</u>	<u>Final injection</u>	<u>Time Interval</u>
TNF- α (4.0 μ g)	TNF- α (1.0 μ g)	1 day
TNF- α (4.0 μ g)	TNF- α (1.0 μ g)	7 days
TNF- α (4.0 μ g)	TNF- α (1.0 μ g)	14 days
TNF- α (4.0 μ g)	TNF- α (1.0 μ g)	28 days
TNF- α (4.0 μ g)	Saline	14 days
Saline	TNF- α (1.0 μ g)	14 days
Saline	Saline	14 days

Neurochemical analyses

The experimental design was the same as that of the social interaction test. Male CD-1 mice ($n = 10/\text{group}$) received an initial i.p. injection of $4\text{ }\mu\text{g}$ TNF- α followed by a second injection of $1\text{ }\mu\text{g}$ of the cytokine either 1, 7, 14 or 28 days later. As shown in Table 1, additional groups received either saline on two occasions 14 days apart, saline followed 14 days later by a low dose of TNF- α ($1.0\text{ }\mu\text{g}$), or TNF- α ($4.0\text{ }\mu\text{g}$) followed 14 days later by saline. At 1.0 hr after the second injection mice were decapitated, brains collected, and discrete nuclei removed and analyzed for CNS monoamines as described earlier. Trunk blood was also collected for the determination of plasma corticosterone levels.

Statistical Analyses

The dependent measures for the dose response study were analyzed by a single factor analysis of variance, followed by Newman-Keuls multiple comparisons ($\alpha=0.05$) of the significant main effects. The analysis of the immediate and proactive effects of TNF- α on chocolate milk consumption were analyzed in two phases. First, variations of chocolate milk consumption at baseline, 1 hr after cytokine treatment and 24 hr after treatment were determined by a repeated measures analysis of variance. Then, the effects of reexposure to the cytokine were determined as a 4 (initial TNF- α treatment) $\times$ 2 (reexposure treatment) analysis of variance with repeated measures (baseline, test day, and 24 hr retest). In both cases, comparisons between means comprising the simple effects of significant interactions were determined by Newman-Keuls multiple comparisons.

The TNF- α induced sensitization of the neuroendocrine and central biogenic amines, as well as the sickness/social behaviors, were analyzed in two phases. First, the data of the groups that received either saline on two occasions, or only a single TNF- α injection (either on the initial injection day or on the reexposure day) were analyzed by a one-way analysis of variance. Newman-Keuls multiple comparisons were conducted to assess the differences between individual means in order to determine whether the low dose of the cytokine had immediate neurochemical or behavioral effects and whether the higher dose had proactive consequences. A separate one-way

analysis of variance was then conducted to evaluate whether the four groups that received TNF- α on two occasions (at different reexposure intervals) exhibited different behavioral and neurochemical profiles. Finally, Dunnett's tests were used to determine whether reexposure to TNF- α induced behavioral and neurochemical effects beyond those observed in animals that received only saline treatment or a single injection of TNF- α (the latter would reflect a sensitization effect).

Results

Experiment 1: Acute effects of TNF- α

Administration of TNF- α induced a dose-dependent increase of plasma corticosterone collected 1 hr after treatment, $F(3, 36) = 10.94$, $p < 0.01$. The multiple comparisons indicated that relative to saline treated mice [$(x \pm \text{SEM}) = 5.83 \pm 1.39$], both the 1.0 and 2.0 μg doses of TNF- α increased corticosterone levels (22.43 ± 4.67 and 21.15 ± 3.29), and the extent of the enhancement was still greater following the 4.0 μg dosage (37.96 ± 5.33).

In contrast to the marked variations of corticosterone, the immediate effects of TNF- α on central monoamines were limited. Within the PVN, neither NE nor MHPG levels were significantly affected by TNF- α . However, the levels of 5-HT were reduced by the cytokine, $F(3, 27) = 3.98$, $p < 0.05$ (see Figure 1). Within the locus coeruleus, the accumulation of MHPG was likewise unaffected by the cytokine, although NE levels were altered, $F(3, 33) = 4.10$, $p < 0.05$. The multiple comparisons indicated that each of the doses provoked a reduction of the NE concentrations relative to that seen in saline treated mice (Figure 2). Within the hippocampus and in the prefrontal cortex, the utilization of NE and 5-HT, as reflected by MHPG and 5-HIAA accumulation, respectively, was not altered by the cytokine treatment. However, at the PFC levels of 5-HT were altered by TNF- α treatment $F(3,36) = 3.25$, $p < 0.50$. Indeed, the multiple comparisons indicated that the 1 and 2 μg doses elevated 5-HT concentrations ($p < 0.05$) (Figure 3). Within the hippocampus, however, there was a modest effect on NE concentrations, $F(3, 34) = 3.55$, $p < 0.05$. The multiple comparisons confirmed that NE levels were reduced by each of the TNF- α doses relative to saline treated animals (see Figure 2). As in the other limbic structures, the turnover of NE and DA were not altered by the TNF- α within the central amygdala. Likewise, the concentrations of 5-HT were not affected by the treatment. However, 5-HIAA accumulation within the central amygdala was influenced by the

cytokine, $F(3, 33) = 3.56$, $p < 0.02$. This stemmed from marked elevations of 5-HIAA in mice that received the 1.0, 2.0 and 4.0 μg doses of $\text{TNF-}\alpha$, relative to saline treated mice (see Figure 4). Finally, while the level and utilization of DA (reflected by DOPAC accumulation) in the PFC was unaltered by the cytokine, within the nucleus accumbens the accumulation of DOPAC was affected, $F(3,32) = 3.54$, $p < 0.05$. The multiple comparisons indicated that relative to saline treated mice the 1.0 μg dosage was without significant effect, while both the 2.0 μg and 4.0 μg doses increased DOPAC accumulation (see Figure 5). The level of DA within the accumbens, however, was unaffected by the $\text{TNF-}\alpha$ treatment.

 Insert Figures 1-5 about here

Experiment 2: Behavioral changes associated with $\text{TNF-}\alpha$ exposure

The analysis of variance revealed that consumption of chocolate milk in $\text{TNF-}\alpha$ treated animals varied over the course of the test sessions, $F(6, 152) = 7.04$, $p < 0.01$. As seen in Table 2 and confirmed by Newman-Keuls multiple comparisons, at baseline the groups displayed comparable levels of chocolate milk consumption. However, a dose dependent reduction of consumption was evident 1 hr after $\text{TNF-}\alpha$ administration, such that the 2.0 and 4.0 μg groups displayed lower levels of consumption than saline treated animals. As well, the $\text{TNF-}\alpha$ groups exhibited consumption levels below that observed at baseline. Upon subsequent testing 24 hr later, consumption in the 4.0 μg group returned to baseline levels, while that of the 2.0 μg group, surprisingly, remained depressed. However, in a partial replication of this study (Brebner et al., 1998), we observed that within 24 hr of treatment, chocolate milk consumption was comparable to that seen prior to such treatment.

Figure 1. Mean ($\pm$ S.E.M.) concentrations of NE and 5-HT (Left panels) and their respective metabolites MHPG and 5-HIAA (right panels) within the paraventricular nucleus of the hypothalamus (PVN) of post mortem tissue collected from mice treated with varying doses of TNF- α . * $p < .05$ relative to saline treated animals.

Figure 2. Mean ($\pm$ S.E.M.) concentrations of NE and MHPG within the locus coeruleus (LC) (upper panel) and dorsal hippocampus (HIPPO) (lower panel) following intraperitoneal injection of TNF- α (1.0-4.0 μ g). * $p < .05$ relative to saline treated animals.

Figure 3. Mean ($\pm$ S.E.M.) concentrations of 5-HT and 5-HIAA within the medial prefrontal cortex (PFC) in response to multiple doses of TNF- α . * $p < .05$ relative to saline treated animals.

Figure 4. Mean ($\pm$ S.E.M.) concentrations of 5-HT and 5-HIAA within the central amygdala (AMY) after acute administration of varying doses of TNF- α . * $p < .05$ relative to saline treated animal.

Figure 5. Mean ($\pm$ S.E.M.) concentrations of DA and the metabolite, DOPAC, within the nucleus accumbens (NAC) in response to TNF- α (1.0-4.0 ug). * $p < .05$ relative to saline treated animals.

Table 2.

Dose-dependent reduction of consumption of a palatable solution (chocolate milk) in response to systemic administration of TNF- α [Mean consumption $\pm$ SEM (ml)].

	Baseline	1 hr Post TNF	24 hr Post TNF
Saline	3.48 $\pm$ (0.22)	3.95 $\pm$ (0.27)	3.21 $\pm$ (0.24)
TNF- α (1 ug)	3.83 $\pm$ (0.24)	3.38 $\pm$ (0.22)	3.31 $\pm$ (0.27)
TNF- α (2 ug)	3.55 $\pm$ (0.18)	2.60 $\pm$ (0.23)*	2.65 $\pm$ (0.21)*
TNF- α (4 ug)	3.42 $\pm$ (0.17)	2.18 $\pm$ (0.22)*	3.01 $\pm$ (0.22)

 * P < .05 relative to baseline

Figure 6 shows the chocolate milk consumption in mice initially treated with TNF- α (1.0, 2.0 or 4.0 μ g) or saline, and then reexposed to either TNF- α (1.0 μ g) or saline. Although a low dose of TNF- α (1.0 μ g) did not itself influence consumption of chocolate milk (i.e., in animals with a history of saline injection), among animals previously treated with TNF- α , reexposure to 1.0 μ g of the cytokine profoundly reduced consumption of chocolate milk. The analysis of variance indeed revealed a significant Initial TNF x Reexposure treatment interaction, $F(3, 72) = 2.97$, $p < 0.05$. The multiple comparisons indicated that in the absence of prior TNF- α exposure, the 1.0 μ g treatment provoked a nonsignificant decline of chocolate milk consumption ($.10 < p < 0.05$). Among mice that received TNF- α treatment 2 weeks earlier, irrespective of the dosage, reexposure to the 1.0 μ g dosage of TNF- α profoundly reduced chocolate milk consumption. Clearly, TNF- α exerted a sensitization effect such that the behavioral consequences of the cytokine were greatly increased upon reexposure 2 weeks after initial treatment.

 Insert Figure 6 about here

The general sickness profile of mice, like the consumption of the palatable substance, was altered upon reexposure to the cytokine. However, it was clear that such an outcome was dependent upon the time between the initial and reexposure treatments (see Table 3). Analysis of variance revealed that the overall sickness profile, locomotor activity and social interaction varied as a function of the Time between TNF- α treatments F 's (3, 23; 3, 22 and 3, 23) = 15.25, 4.20 and 9.56, P 's < 0.05 , respectively. Those animals that received the second injection of the cytokine 14 or 28 days after the initial treatment displayed greater illness, reduced motor activity and diminished exploration relative to mice that received the reexposure treatment 1 or 7 days after initial administration of TNF- α ($p < 0.05$). Moreover, Dunnett's tests confirmed that reexposure to the TNF- α at 14 and 28

Figure 6. Mean (+SEM) concentrations of chocolate milk consumption as a function of the initial and reexposure treatment mice received. The two treatments were administered 2 weeks apart.

days after the initial treatment generated greater indications of sickness and reduced motor activity. As well, reexposure to TNF- α at these times reduced social interactions relative to that seen among mice that received saline on both occasions or only a single TNF- α treatment.

Neuroendocrine Changes upon TNF- α reexposure

Levels of plasma corticosterone were significantly altered in response to acute TNF- α treatment, $F(2,27)=15.89$, $p < 0.01$. As confirmed by multiple comparisons, the acute dose of TNF- α (1.0 μg) provoked a significant rise in plasma corticosterone ($p < 0.05$), while mice injected with saline after prior exposure to the cytokine 14 days earlier did not significantly differ from saline only treated animals. Likewise, in mice pretreated with TNF- α , the concentration of corticosterone varied as a function of the time elapsed before subsequent reexposure ($F(3,37)=5.1$, $p < 0.05$). The multiple comparisons revealed that reexposure to TNF- α after 7, 14 or 28 days elevated corticosterone levels above those seen in saline-treated animals ($p < 0.05$) (see Figure 7). However, only upon reexposure to TNF- α 28 days after initial challenge did corticosterone levels significantly exceed those observed in mice receiving a single acute injection of the cytokine (i.e. a genuine sensitization effect). Interestingly, a desensitization effect was evident upon reexposure to TNF- α after only 24 hours. Indeed, corticoid levels in these mice were lower than those seen in animals that received the single acute cytokine dose ($p < 0.05$).

Insert Figure 7 about here

Figure 7. Mean ($\pm$ S.E.M) concentration of of plasma corticosterone as a function of TNF- α treatments. All mice received 2 intraperitoneal injections. The three groups to the left received either saline only, saline followed two weeks later by a relatively low dose of TNF- α (1.0 ug) or a high dose of TNF- α (4.0 ug) followed 2 weeks later by saline treatment. The four groups on the right were administered two injections of TNF- α ; an initial 4.0 ug dose followed by a second 1.0 ug dose either 1, 7, 14 or 28 days later. * p < .01 relative to saline-saline treated animals. ** p < .05 relative to saline-TNF treated animals.

Table 3.

Behavioral Scores as a function of the initial TNF- α (4.0 μ g) or reexposure (1.0 μ g) treatment.

	sickness profile	motor activity	social interaction
sal/sal	1.00 + 0	3.00 + 0	1.75 + 0.25
Sal/TNF	1.14 + 0.38	2.57 + 0.20	1.83 + 0.31
TNF/sal	1.29 + 0.76	2.86 + 0.14	1.67 + 0.33
TNF/TNF (1 day)	1.80 + 0.20	2.00 + 0*	1.40 + 0.40
TNF/TNF (7 days)	1.67 + 0.33	2.00 + 0.31*	1.67 + 0.33
TNF/TNF (14 days)	2.83 + 0.17**	1.67 + 0.21	2.83 + 0.40*
TNF/TNF (28 days)	3.57 + 0.20**	1.00 + 0**	3.71 + 0.29**

 * P < .05 relative to saline-saline treated mice

** p < .05 relative to saline-TNF treated mice

Neurochemical Changes upon TNF- α reexposure

In contrast to the modest effects seen after the initial TNF- α administration, marked alterations of the amines were evident following reexposure to the cytokine. However, it appeared that these effects were dependent upon the time between the two TNF- α treatments.

Within the PVN the levels of NE and MHPG were unaffected in mice that received only a single TNF- α injection either 1 hr earlier (1.0 μ g) or 2 weeks previously (4.0 μ g) (see Figure 8). In mice previously treated with the high dose of TNF- α and then reexposed to the low dose, levels of NE did not significantly differ from one another or from that of mice that received saline or a single injection of TNF- α . However, it appeared that MHPG accumulation increased progressively between 1 and 28 days following the initial treatment. It is particularly significant that the Dunnett's tests indicated that mice that received the TNF- α reexposure treatment either 14 or 28 days after the initial administration exhibited MHPG levels that exceeded those of saline treated mice. As well, MHPG levels in mice reexposed to the TNF- α 28 days after initial treatment exceeded that seen in mice that received only a single injection of TNF- α ($p < 0.05$).

 Insert Figure 8 about here

The amine variations within limbic sites could readily be dissociated from the effects seen in PVN. Within the locus coeruleus a single administration of TNF- α did not affect the levels of NE, whereas MHPG was affected by the treatment, $F(2, 21) = 6.02$, $p < 0.01$. The Newman-Keuls multiple comparisons indicated that 1.0 μ g of TNF- α administered 1 hr earlier provoked a marked elevation in the accumulation of the NE metabolite (see Figure 9). In contrast to the lack of an immediate effect on NE levels, upon reexposure to the cytokine at the different intervals the concentrations of NE were altered

Figure 8. Mean ($\pm$ S.E.M) concentration of NE (top) and MHPG (bottom) within the PVN as a function of TNF- α treatments. All mice received 2 intraperitoneal injections. The three groups to the left received either saline only, saline followed two weeks later by a relatively low dose of TNF- α (1.0 ug) or a high dose of TNF- α (4.0 ug) followed 2 weeks later by saline treatment. The four groups on the right were administered two injections of TNF- α : an initial 4.0 ug dose followed by a second 1.0 ug dose either 1, 7, 14 or 28 days later. * $p < .01$ relative to saline-saline treated animals. ** $p < .05$ relative to saline-TNF treated animals.

significantly, $F(3,29) = 4.05$, $p = 0.01$. This effect was found to be attributable to an appreciable decline of NE in mice that received the second TNF- α administration 1 day after initial treatment. Dunnett's tests also indicated that the NE levels were reduced relative to those evident of mice that received a single injection of TNF- α (1.0 μ g). However, the reductions of NE associated with reexposure to TNF- α were not accompanied by significant variations of MHPG. Thus, while acute TNF- α elicits an increase of MHPG accumulation, this effect was not evident in mice previously treated with TNF- α .

 Insert Figure 9 about here

Acute treatment with TNF- α did not significantly affect the concentrations of either NE or MHPG within the PFC, although there appeared to be a modest rise of NE concentrations in mice that had received the 1.0 μ g dose 1 hr earlier (see Figure 10). Interestingly, the levels of both NE and MHPG varied as a function of the time of reexposure to TNF- α , F 's (3, 33) = 19.49 and 10.06, p 's < 0.01, respectively. The Newman-Keuls multiple comparisons confirmed that among mice reexposed to TNF- α at the 1 day interval the amine and metabolite concentrations were elevated relative to each of the remaining groups. Likewise, the NE and MHPG concentrations were significantly greater than those of mice that received either saline or only a single TNF- α treatment.

 Insert Figure 10 about here

The profile of DA and DOPAC variations within the PFC, which are shown in Figure 11. Levels of DA were found to vary as a function of TNF- α treatment

Figure 9. Mean ($\pm$ S.E.M) concentration of NE (top) and MHPG (bottom) within the locus coeruleus as a function of TNF- α treatments. All mice received 2 intraperitoneal injections. The three groups to the left received either saline only, saline followed two weeks later by a relatively low dose of TNF- α (1.0 ug) or a high dose of TNF- α (4.0 ug) followed 2 weeks later by saline treatment. The four groups on the right were administered two injections of TNF- α ; an initial 4.0 ug dose followed by a second 1.0 ug dose either 1, 7, 14 or 28 days later. * $p < .01$ relative to saline-saline treated animals. ** $p < .05$ relative to saline-TNF treated animals.

Figure 10. Mean ($\pm$ S.E.M) concentration of NE (top) and MHPG (bottom) within the prefrontal cortex as a function of TNF- α treatments. All mice received 2 intraperitoneal injections. The three groups to the left received either saline only, saline followed two weeks later by a relatively low dose of TNF- α (1.0 ug) or a high dose of TNF- α (4.0 ug) followed 2 weeks later by saline treatment. The four groups on the right were administered two injections of TNF- α ; an initial 4.0 ug dose followed by a second 1.0 ug dose either 1, 7, 14 or 28 days later. * $p < .01$ relative to saline-saline treated animals. ** $p < .05$ relative to saline-TNF treated animals.

$F(6,62)=2.64$, $p < 0.05$). While acute exposure to the cytokine did not alter levels of the amine, at the 1 and 14 day intervals there was a significant rise of DA levels compared to saline only treated animals, but these increases did not differ from those of mice that had received a single injection of TNF- α . The accumulation of DOPAC following acute TNF- α treatment did not differ among groups that received saline treatment or a single administration of TNF- α . There was again, however, a modest rise of DOPAC in mice that had received the 4.0 μg dose of TNF- α , followed 2 weeks later by saline ($p < 0.06$). Among mice that had received the initial TNF- α treatment and then reexposed to the low dosage at various times afterward, the accumulation of DOPAC varied significantly across groups, $F(3, 33) = 3.76$, $p < 0.05$. The Newman-Keuls multiple comparisons indicated that at the 1 day reexposure interval DOPAC accumulation exceeded that seen in the remaining groups. As well, the DOPAC level was greater than that evident in saline treated mice and those that had received a single injection of TNF- α (1.0 μg) 1 hr earlier. The accumulation of the metabolite upon reexposure to TNF- α relative to that seen in animals treated with TNF- α and then reexposed to saline did not reach statistical significance.

 Insert Figure 11 about here

Variations of 5-HT and 5-HIAA, in the PFC, associated with the cytokine treatment were particularly dramatic. The analysis of variance indicated that a single administration of TNF- α influenced the concentrations of both 5-HT and its metabolite 5-HIAA, $F_s(2, 24) = 6.92, 10.74$, $P's < 0.01$, respectively. The multiple comparisons indicated that 1.0 μg of TNF- α 1 hr before decapitation resulted in a significant rise of 5-HT. While a rise of 5-HT was also evident in mice that received TNF- α (4.0 μg) 2 weeks earlier, this effect just missed significance ($p < 0.06$). Interestingly, however, the latter treatment markedly increased 5-HIAA accumulation, while the effect of TNF- α 1 hr earlier

Figure 11. Mean ($\pm$ S.E.M) concentration of DA (top) and DOPAC (bottom) within the prefrontal cortex as a function of TNF- α treatments. All mice received 2 intraperitoneal injections. The three groups to the left received either saline only, saline followed two weeks later by a relatively low dose of TNF- α (1.0 ug) or a high dose of TNF- α (4.0 ug) followed 2 weeks later by saline treatment. The four groups on the right were administered two injections of TNF- α ; an initial 4.0 ug dose followed by a second 1.0 ug dose either 1, 7, 14 or 28 days later. * $p < .01$ relative to saline-saline treated animals. ** $p < .05$ relative to saline-TNF treated animals.

was less pronounced, and did not reach significance. Comparisons between the groups that received TNF- α on two occasions indicated that the time of the second administration greatly influenced 5-HT and 5-HIAA levels, $F(3, 23 \text{ and } 3, 26) = 11.41, 13.56$, p 's < 0.01. The multiple comparisons indicated that the levels of the amine and its metabolite varied as a U-shaped function of the time between treatments (see Figure 12). When the second injection was administered 1 day after initial treatment an exceptionally marked elevation of 5-HIAA was evident, which was accompanied by an increase of 5-HT. Levels of both 5-HT and 5-HIAA then declined to saline values in mice that received the TNF- α treatments at 7 and 14 day intervals following initial treatment. Thereafter, a further rise was seen (28 day reexposure), such that the amine and metabolite exceeded that of saline treated animals. It is important to note that while the U-shaped function for 5-HT was marked, the level of the amine (at the 1 and 28 day intervals) did not surpass that associated with acute treatment. With respect to 5-HIAA, a genuine sensitization effect (i.e., levels exceeding those of acutely treated animals) was only evident in mice that received the second TNF- α treatment 1 day after the first such administration.

 Insert Figure 12 about here

Unlike the effects seen in the PFC, acute administration of TNF- α did not affect the concentrations of hippocampal NE. However, MHPG accumulation was affected by the treatment, $F(2,22) = 4.40$, $p < 0.05$. The multiple comparisons indicated, however, that while the low dose of the cytokine administered 1 hr before decapitation was without effect, 4.0 μ g administered 2 weeks earlier significantly increased the accumulation of hippocampal MHPG. Among the groups reexposed to the cytokine there were no differences as a function of the time of reexposure. However, as seen in Figure 13, NE

Figure 12. Mean ($\pm$ S.E.M) concentration of 5-HT (top) and 5-HIAA (bottom) within the prefrontal cortex as a function of TNF- α treatments. All mice received 2 intraperitoneal injections. The three groups to the left received either saline only, saline followed two weeks later by a relatively low dose of TNF- α (1.0 ug) or a high dose of TNF- α (4.0 ug) followed 2 weeks later by saline treatment. The four groups on the right were administered two injections of TNF- α ; an initial 4.0 ug dose followed by a second 1.0 ug dose either 1, 7, 14 or 28 days later. * $p < .01$ relative to saline-saline treated animals. ** $p < .05$ relative to saline-TNF treated animals.

levels in each of these groups was lower than observed after saline treatment or acute TNF- α . MHPG levels, however, were not correspondingly affected.

Insert Figure 13 about here

In contrast to the variations of hippocampal NE activity associated with TNF- α administration, neither acute administration of this cytokine, nor reexposure to TNF- α significantly affected 5-HT or 5-HIAA concentrations.

Within the central amygdala the concentrations of NE and DA were not found to vary as a function of either the acute TNF- α or reexposure treatments. Likewise, MHPG and DOPAC accumulation were unaffected by the acute TNF- α treatment. While there was some indication of reexposure to TNF- α augmenting MHPG accumulation, this effect was shy of statistical significance. It must be underlined that the MHPG accumulation was, in fact, quite marked (approximately 80% above control values), but the variability among many of the groups prevented detection of a significant overall effect.

Within the nucleus accumbens the concentrations of DA did not vary as a function of the cytokine treatments. The acute administration of TNF- α 1 hr before decapitation provoked a modest rise of DOPAC accumulation, but the overall analysis of variance was not statistically significant, $F(2, 23) = 2.84$, $p = 0.07$. Reexposure to TNF- α did not influence DOPAC accumulation as a function of the time of the reexposure treatment. While these groups exhibited elevated levels of DOPAC relative to the saline treated mice, they did not display effects that were greater than that elicited by the acute treatment..

Insert Figure 14 about here

Figure 13. Mean ($\pm$ S.E.M) concentration of NE (top) and MHPG (bottom) within the dorsal hippocampus as a function of TNF- α treatments. All mice received 2 intraperitoneal injections. The three groups to the left received either saline only, saline followed two weeks later by a relatively low dose of TNF- α (1.0 ug) or a high dose of TNF- α (4.0 ug) followed 2 weeks later by saline treatment. The four groups on the right were administered two injections of TNF- α ; an initial 4.0 ug dose followed by a second 1.0 ug dose either 1, 7, 14 or 28 days later. * $p < .01$ relative to saline-saline treated animals. ** $p < .05$ relative to saline-TNF treated animals.

Figure 14. Mean ($\pm$ S.E.M) concentration of DA (top) and DOPAC (bottom) within the nucleus accumbens as a function of TNF- α treatments. All mice received 2 intraperitoneal injections. The three groups to the left received either saline only, saline followed two weeks later by a relatively low dose of TNF- α (1.0 ug) or a high dose of TNF- α (4.0 ug) followed 2 weeks later by saline treatment. The four groups on the right were administered two injections of TNF- α ; an initial 4.0 ug dose followed by a second 1.0 ug dose either 1, 7, 14 or 28 days later. * $p < .01$ relative to saline-saline treated animals. ** $p < .05$ relative to saline-TNF treated animals.

General discussion

Behavioral effects of TNF- α

It will be recalled that proinflammatory cytokines, such as IL-1, IL-6 and TNF- α , provoke sickness behaviors (Bluthe et al., 1994; Kent et al., 1996). In the present investigation, using human TNF- α , this sickness profile was confirmed employing two different behavioral paradigms. The cytokine induced a marked reduction of consumption of a palatable chocolate milk solution, and also provoked modest alterations of locomotion and social exploration in the home cage setting. Interestingly, the sickness behaviors elicited by TNF- α were subject to a time-dependent sensitization. In general, animals exposed to a single acute dose of TNF- α (4.0 μ g) exhibited few signs of sickness. Likewise, few sickness symptoms, with the exception of slight lethargy, were apparent among mice that received the 4.0 μ g dose and then reexposed to a lower dose (1.0 μ g) 1 or 7 days later. However, mice reexposed to TNF- α 14 or 28 days after previous exposure to the cytokine showed pronounced sickness behavior, including ptosis, respiratory disturbances, ruffled fur, curled body posture, immobility and reduced social exploration. The severity of the symptoms and the rapidity of onset increased with the passage of time, being exacerbated 28 days after the initial treatment relative to that seen after 14 days. In fact, in preliminary studies it was observed that reexposure to a 2.0 μ g dosage 28 days after initial treatment with TNF- α (4.0 μ g) was lethal in a small proportion of animals.

Acute effects of TNF- α : Plasma Corticosterone

Like stressors, immune challenges (e.g., LPS) have been shown to alter HPA functioning (Dunn et al., 1989; Rivier, 1991). Similarly, augmented circulating corticoid levels have been observed after systemic IL-1 (Besedovsky et al., 1991; Dunn, 1988a, 1990; Zalcman et al., 1994), as well as TNF- α administration (Benigni et al., 1996;

Bernardini et al., 1990; van der Meer et al., 1996). In addition, i.c.v. administration of antibodies directed against TNF- α reduce the onset and overall magnitude of the ACTH response to LPS (Turnbull & Rivier, 1998). Consistent with these earlier findings, in the present investigation a dose-dependent increase of plasma corticosterone was elicited by systemic TNF- α administration. Inasmuch as icv TNF- α has also been shown to elicit an anxiogenic effect in a plus-maze test (Connor et al., 1998), these data are consistent with the presumption that TNF- α engenders a stress-like response. This is not to say that psychogenic, neurogenic and systemic stressors elicit identical effects. As indicated by Herman and Cullinen (1997) the HPA alterations elicited by processive stressors (both psychogenic and neurogenic) may involve the activation of limbic forebrain regions, which in turn, may stimulate GABAergic neurons at an intervening synapse (e.g. bed nucleus of the stria terminalis) before acting upon corticotropic PVN cells. In contrast, the HPA alterations elicited by a systemic stressor (such as IL-1 and TNF- α) may reflect the stimulation of brainstem nuclei which directly activate the PVN (Herman & Cullinan, 1997). Nevertheless, the parallel between the effects of processive and systemic stressors raises the possibility that elements of the stress response are also elicited by TNF- α .

In addition to the independent effects of TNF- α on sickness behaviors and plasma corticosterone concentrations, additional experiments conducted in our laboratory revealed synergistic actions of IL-1 and TNF- α with respect to these variables. In contrast, no such synergistic actions were evident after coadministration of IL-1 and IL-6 or the conjoint treatment with IL-6 and TNF- α . In experiments conducted in parallel with the primary studies of the present investigation, we also assessed the potential synergistic effects of low doses of IL-1, IL-6 and TNF- α on central amine activity. Unlike the effects evident with respect to IL-1 plus TNF- α on plasma corticosterone concentrations, there was no evidence for such synergistic effects with respect to amines within several brain regions, including the locus coeruleus, central amygdala, PVN and prefrontal cortex. However, in these experiments relatively low doses of the cytokines were employed and it remains to

be determined whether synergisms would be evident at higher doses. Nevertheless, these data suggest that the HPA axis and central amine responses to TNF- α (at least within the brain regions assessed) are independent of one another. Furthermore, since the combination of IL-1 and TNF- α induced a sickness profile, these data make it clear that despite the illness and pronounced hormonal variations provoked by the cytokine treatments, one need not expect such effects to be translated into neurochemical changes within the brain.

Acute effects of TNF- α : Brain amines

Systemic TNF- α elicited amine variations at several hypothalamic and extrahypothalamic sites. The PVN was expected to be particularly sensitive to TNF- α induced amine alterations given the high density of TNF- α immunoreactive neurons found in this region (Breder et al., 1993a). While TNF- α modestly reduced the levels of NE, a more pronounced reduction of 5-HT was evident within this region. In neither case, however was the accumulation of MHPG and 5-HIAA significantly affected by the cytokine. The altered amine levels observed within the PVN are particularly intriguing in light of their important regulatory control over HPA activity (Dinan, 1996; Fuller, 1992; Pacak, Armando, Fukuhara, Kvetnansky, Palkovits, Kopin, Goldstein, 1992; Pacak, Palkovits, Kopin, Goldstein, 1995).

In contrast to the PVN, pronounced reductions of NE were evident in the dorsal hippocampus and locus coeruleus and 5-HT levels were increased rather than decreased in the PFC. The reduced NE levels in the locus coeruleus may reflect increased release of the amine onto forebrain terminal fields. Indeed, TNF- α has been shown to modify NE release from the locus coeruleus and hippocampus within electrically stimulated tissue sections (Ignatowski et al., 1997). In addition to altering NE release, TNF- α itself has been shown to be released from the hippocampus and locus coeruleus of tissue sections obtained from rodents treated with the tricyclic antidepressant, desimipramine (Ignatowski

et al., 1997). Thus, in addition to altering amine activity, $\text{TNF-}\alpha$ may act as central-transmitter in response to changes in local amine concentrations. An explanation of why PFC 5-HT levels would increase in the absence of changes in the metabolite, 5-HIAA, is not readily apparent. However, it may be the case that $\text{TNF-}\alpha$ selectively provokes the synthesis of the amine in the PFC. In line with this suggestion, previous studies have reported elevated levels of the 5-HT precursor, tryptophan, within the PFC and parietal cortex after IL-1 treatment (Dunn, 1988; Dunn & Welch, 1991).

In contrast to the effects observed in other brain regions, $\text{TNF-}\alpha$ increased 5-HIAA levels in the central amygdala. Interestingly, increased serotonergic activity in the central amygdala has been noted after immobilization stress (Beaulieu et al., 1987) and this brain region may be especially important with respect to fear and anxiety behaviors (Adamec & Morgan, 1994). Within the nucleus accumbens $\text{TNF-}\alpha$ provoked a marked elevation of the DA metabolite, DOPAC. Like the amygdala 5-HIAA variations, the elevated DOPAC levels observed in the nucleus accumbens are reminiscent of the effects elicited in response to psychogenic and neurogenic stressors (Anisman & Zacharko, 1986). However, it ought to be noted that the effects of the $\text{TNF-}\alpha$ administration can also be distinguished from some effects ordinarily associated with stressors. Most prominently, unlike the effects of $\text{TNF-}\alpha$, processive stressors tend to have more profound effects on DA turnover within the PFC than the nucleus accumbens (Deutch & Roth, 1990).

To our knowledge, the presently observed neurochemical variations represent the first reports of systematically-induced extrahypothalamic amine variations induced by $\text{TNF-}\alpha$. As such, these data provide a framework from which to explore the contribution of $\text{TNF-}\alpha$ in modulating the potential cognitive and affective consequences of this cytokine. However, in assessing the effects of $\text{TNF-}\alpha$ on amine turnover, it is essential to consider the fate of amine metabolites, particularly given the lengthy interval between $\text{TNF-}\alpha$ administration and subsequent collection of tissues (1 hr). Specifically, it is possible that amine utilization was not increased, hence the absence of increased

metabolites. Alternatively, utilization may have been increased, but dispersion of the metabolite, coupled with uptake into surrounding tissues precluded detection of elevated MHPG or 5-HIAA accumulation. Ultimately, the use of *in vivo* methods, such as microdialysis, may help circumvent some of the problems associated with measuring metabolite levels. In fact, recent data collected in our laboratory (Borowski, Hayley, Anisman & Merali, unpublished results) employing *in vivo* microdialysis, revealed that TNF- α was effective in augmenting transmitter utilization in limbic sites among rats.

While the distribution half-life for human TNF- α measured in mice is approximately 30 minutes (Bocci, 1991), the maximal elevation of plasma corticosterone in response to the cytokine occurs approximately 1 hr following bolus injection (Bernardini et al., 1990). In the present investigation, postmortem tissue was collected 1 hr after TNF- α treatment, but it is not necessarily the case that peak illness and hormonal changes temporally coincide with the central amine variations. In fact, in our *in vivo* dialysis studies (Borowski, Hayley, Anisman & Merali, unpublished observations) DA release at the nucleus accumbens was not detected until 90 min after systemic TNF- α administration. In effect, it is possible that the failure to detect variations of NE and 5-HT metabolites in post mortem tissue of the present investigation may reflect the time at which amines and metabolites were sampled, rather than lack of effects of the treatments.

The central circuitry associated with an acute phase response (i.e., after endotoxin or cytokine treatment) is fairly complex, involving several pathways and sites of entry, culminating in activation of neurons within numerous brain regions (Rivest, 1995). Moreover, following IL-1 β or LPS challenge *c-fos* mRNA expression across brain regions may follow a biphasic temporal course (Brady et al., 1994; Rivest et al., 1995). The early set of changes may reflect the initial entry into the brain, promoting neuroendocrine changes and the neural transduction of peripheral signals, while the late phase may reflect cellular activation associated with IL-1 β (or bioactive products) along various diffusion routes or secondary to *de novo* synthesis (Brady et al., 1994; Gaillard et al., 1995). In a

similar fashion, it is possible that a finer temporal resolution of the effects of TNF- α may have revealed amine variations at times other than 1 hr following systemic treatment.

Sensitization effects: Neuroendocrine alterations

It will be recalled that upon reexposure to TNF- α in mice that had previously been treated with the cytokine, a marked sickness profile was evident. This effect, however, was time-dependent, being manifested primarily in mice that were reexposed to the cytokine 14-28 days after the initial treatment. Commensurate with the behavioral data, a progressive increase in plasma corticosterone was seen upon reexposure to TNF- α . The corticoid levels were greatest upon reexposure to the cytokine after 28 days, where a sensitization effects was evident. Given the correspondence between the manifestation of sickness symptoms, the hormonal variations may be related to the sickness profile (i.e., they may be secondary to the illness or may be causally related to the development of the malaise).

Interestingly, upon reexposure to TNF- α 24 hr after initial treatment, the corticosterone levels were reduced when compared to that of mice exposed to only a single dose of the cytokine. Thus, there may be a critical early period where TNF- α has a desensitizing effects on corticoid levels. Such an early time-dependent desensitization effect for TNF- α is not altogether surprising. For example, pre-treatment with TNF- α has been shown to limit the lethal effects of co-administration of a very high dose of the cytokine together with the RNA synthesis inhibitors D-galactosamine or actinomycin-D (Wallach et al., 1988). Furthermore, while such a prophylactic effect became evident 1 h after TNF- α preexposure, it was diminished after 24 h, and entirely absent after 48 h (Wallach et al., 1988). Further, a cross-desensitization effect has also been demonstrated, whereby IL-1 pretreatment can substitute for TNF, and conversely TNF can substitute for IL-1 in protecting mice against the lethal effects of coadministration of either of these cytokines together with D-galactosamine or actinomycin-D (Wallach et al., 1988).

Some of the hormonal and behavioral (in particular sickness) sensitization effects observed in the current study may reflect the interaction between TNF- α with other endogenous cytokines. Indeed, both interferon- γ (IFN- γ) and IL-12 have been shown to mediate the sensitization to the lethal effects of recombinant human TNF- α elicited by bacillus Calmette-Guerin bacterial infection (Cauwels et al., 1995,1996). Also, mice pretreated with IFN- γ have been shown to display an exaggerated TNF- α response to LPS (Bundschuh et al., 1997; Nansen et al., 1997). Thus it was suggested that LPS may sensitize cells of the monocyte/macrophage lineage for the hyperproduction of TNF- α by stimulating T-cells to produce IFN- γ which subsequently prime the mononuclear cells (Nansen et al.,1997). However, application of either IL-1 β or IFN- γ alone did not induce TNF- α production from isolated rat astrocytes, whereas coadministration of the two cytokines provoked a synergistic effect with respect to astrocytic TNF- α production (Chung & Benveniste, 1990). With respect to the present investigation, it is possible that the initial administration of TNF- α may have provoked increased IFN- γ or IL-1 β levels, which then acted synergistically with the subsequently administered TNF- α . Likewise, it is possible that initial TNF- α administration provoked an increase of endogenous TNF- α stores through the production of cytokine intermediates such as IFN- γ or IL-12. The increased endogenous levels of TNF- α may have rendered the animal susceptible to subsequent suboptimal challenge with the cytokine.

It may be recalled, that although the effects of endogenous TNF- α are mediated by two receptor sub-types (p55 and p75), the human form of TNF- α activates only the p55 receptor and not the p75 type. Use of the selective antagonist for the p55 receptor in the present investigation, recombinant human TNF- α , suggests that any sensitization effects observed may be due to activation of this receptor subtype. Indeed, the p55 TNF- α receptor may play a greater role than the p75 subtype in neuroendocrine and neurotoxic effects of the cytokine (Benigni et al., 1996; Sipe et al., 1996). However, there have been reports that some of the synergistic effects of rhTNF- α and rhIL-1 β coadministration

results from the ability of IL-1 to mimic activation of the p75 receptor (Bluthe et al., 1994). The initial TNF- α administration applied in the present investigation may have induced the release of IL-1 (due to its well known ability to stimulate the production of other cytokines) which may subsequently have stimulated the p75 receptor, culminating in a potentiated effect upon reexposure to the cytokine. Indeed, there have been reports that activation of the p75 receptor may potentiate some of the effects of the p55 receptor (Benigini et al., 1996). Yet another alternative for the sensitization effect comes from studies showing that viruses may sensitize neurons to some of the cytotoxic effects of TNF- α by inhibiting the production of some endogenous protective factor (Sipe et al., 1996). It is possible that the initial TNF- α administration in the present investigation, may have suppressed the expression or activity of such protective mechanisms, thereby rendering the animal susceptible to subsequent challenge with the cytokine. Of course, each of the aforementioned suggestions is highly speculative and are offered only as provisional hypotheses.

Sensitization effects: Central neurochemical variations

Like stressors, the administration of IL-1 may provoke protracted neurochemical effects, which increase with the passage of time (Schmidt et al., 1995; Tilders et al., 1993). Specifically, it was observed that over time following IL-1 treatment (as well as following stressor exposure) the HPA response to subsequent IL-1 or stressor challenge was greatly increased (e.g., enhanced corticosterone levels). Further, with the passage of time following an acute stressor or IL-1 β administration, phenotypic variations occurred in CRH terminals within the external zone of the median eminence, such that they came to coexpress AVP. As CRH and AVP synergistically provoke ACTH release, such a mechanism may account for the sensitization of HPA activity associated with stressor or IL-1 β treatment (Bartanusz et al., 1993; Schmidt et al., 1995; Tilders et al., 1993). Likewise, in preliminary studies we observed an upregulation of AVP stores in CRH

terminals in the ZEME in response to reexposure to TNF- α 14 days after initial treatment. Moreover, it appeared that AVP was colocalized with CRH in these terminals (Hayley, Staines, Merali & Anisman, 1998). It is possible that paralleling the effects of IL-1, the sensitizing actions of TNF- α on neuroendocrine activity may involve phenotypic changes of hypothalamic neuropeptide composition. However, such a conclusion must await our current more detailed analysis of CRH/AVP coexpression following TNF administration.

Of particular interest, in the present investigation, was the finding of two distinct sensitization patterns with respect to central amine variations. Unlike the progressive increase of plasma corticosterone levels seen upon TNF- α reexposure, the sensitization patterns for the central amines varied as a function of the brain region and the specific neurotransmitter assessed. For instance, within the PVN the accumulation of MHPG paralleled the plasma corticosterone variations, being maximal when the TNF- α was re-administered 28 days after initial treatment. At this time, the elevated utilization was accompanied by a reduction of NE levels.

In contrast to the progressive rise of plasma corticosterone and PVN NE utilization, a second pattern of amine variations was observed. This profile comprised an early increase of amine utilization (i.e., upon reexposure to TNF- α 24 hr after the initial treatment), without a comparable sensitization effect at other time points. In particular, within the PFC the concentrations of both NE and MHPG were markedly elevated upon reexposure to TNF- α at the 24 hr time period (> 300% and 500%, respectively). The sensitization, however, was entirely absent at longer intervals.

The source for the different sensitization patterns is not immediately evident. However, it is possible that the progressive rise of amine functioning seen with respect to PVN NE activity (which paralleled the corticosterone sensitization) may be related to illness symptoms. In contrast, the early sensitization profile (i.e., the enhanced PFC and locus coeruleus amine increases) was clearly independent of illness factors, as mice did not display any obvious malaise at the early time point. Such amine variations could ostensibly

promote other types of behavioral outcomes, including cognitive or anxiogenic effects which have been associated with cytokine treatments (Anisman & Merali, 1998).

The early sensitization of aminergic activity in the PFC coincided with the desensitization of plasma corticosterone levels upon reexposure to TNF- α at this time point. Interestingly, the PFC has been proposed to exert inhibitory control over neuroendocrine actions of the PVN (Herman & Cullinan, 1997). Indeed, ablation of the PFC has been reported to enhance HPA responsiveness to acute stressors as well as increased basal ACTH and corticosterone secretion (see Herman & Cullinan, 1997). The PFC may exert inhibitory action over the PVN through a bisynaptic circuit, whereby excitatory PFC projections stimulate GABAergic neurons in the bed nucleus of the stria terminalis, preoptic area or local hypothalamic neurons, which in turn, may inhibit corticotrophic PVN neurons (Herman & Cullinan, 1997). With respect to the present investigation, it is tempting to speculate that the sensitization of aminergic activity in the PFC may activate such an inhibitory neural circuit, which in turn may desensitize HPA activity upon reexposure to TNF- α after the 24 h period. It remains unclear as to why a sensitization of NE turnover occurs upon TNF- α reexposure at the 24 hr time point, but not at later intervals. In fact, it will be recalled that sensitization effects (e.g., those associated with IL-1, stressors, and even antidepressant treatments) typically become progressively more pronounced with the passage of time (Antelman, 1988; Schmidt et al., 1995; Tilders et al., 1993; Van Dijken et al., 1992), as observed with respect to the PVN variations of NE activity in the present investigation.

Biphasic Behavioral and Neurochemical effects of TNF- α

The levels and/or turnover of several amines fluctuated in a biphasic manner over the reexposure intervals. For instance, 5-HT and 5-HIAA concentrations at the PFC and PVN showed a large early peak (24 h), but at the 7 and/or 14 day reexposure periods the amine response was comparable to that of acutely treated or saline treated mice. Upon

reexposure to the cytokine 28 days after initial treatment a second peak (albeit less pronounced than the initial one) was evident. The biphasic amine sensitization responses (early and late sensitization peaks) seen in the current investigation may reflect the operation of two distinct processes. The latter peak may reflect (or may be secondary to) the cytokine provoked sickness, whereas the early peak involves other mechanisms.

Biphasic temporal patterns are not unique to the present investigation, although the temporal dynamics that have been reported occurred over less protracted time frames. For instance, Romanovsky et al., 1996 reported that two different sickness patterns occur in rats exposed to acute systemic LPS administration: (1) an early phase (after approximately 1 hr) syndrome occurs wherein fever is accompanied by high levels of activity and low tail flick latency to a noxious stimulus and (2), a later phase (peaking 2.5-3 hr after LPS) during which the rise in body temperature is accompanied by low levels of activity and high tail flick latency to a noxious stimulus (Romanovsky et al., 1996). Interestingly, while the mechanisms subserving the early sickness response to LPS remains to be determined, it has been proposed that the latter response is mediated by the release of prostaglandins (Romanovsky et al., 1996). In a similar fashion, it has been reported that TNF- α may act directly in the brain to produce biphasic alterations in behavior. For example, it has been reported that microinjection of TNF- α into the locus coeruleus of rats produces an initial transient (20-30 min) phase of behavioral arousal followed by a later longer lasting (45-80 min) soporific period (De Sarro et al., 1997).

Biphasic time-dependent changes in the expression of TNF- α and other cytokines, including IL-1, have been described in several brain regions. For example, shortly after LPS administration (1.5-6 hr), TNF- α mRNA expression was greatest in discrete brain regions, including the perivascular cells and neurons in the circumventricular organs including the OVLT and area postrema. In contrast, later febrile responses to the endotoxin (15-18 hr) were characterized by increased expression of the cytokine in the anteroventral, periventricular and arcuate nucleus of the hypothalamus, as well as the nucleus of the

solitary tract (Breder et al., 1994). More recently, Quan et al. (1998) demonstrated that LPS leads to two time-dependent phases of IL-1 β expression in the CNS. They describe an early IL-1 mRNA signal in the circumventricular organs appearing at 0.5 h and peaking 2 h after LPS administration, followed by a later signal, scattered throughout the brain parenchyma, between 8-12 h following treatment, and declining to control values by 24 h after LPS administration (Quan et al., 1998). Such time-dependent activation effects within different brain regions may reflect the recruitment of temporally-sensitive specific neural circuits. The results of the present investigation need to be distinguished from the biphasic effects seen over a 1-3 hr period (Romanovsky et al., 1996; De Sarro et al., 1997), as that evident over a 24 hr period (Breder et al., 1994; Quan et al., 1998). Specifically, the biphasic variations in the present study occurred over a 28 day period, and were evident upon reexposure to TNF- α . Nevertheless, it is conceivable that these time-dependent sensitization effects may have been mediated by distinct CNS brain circuits. Whether anatomical- and neurotransmitter-sensitive sensitization patterns translate into functional differences remains to be determined.

As discussed earlier, there are any number of mechanisms that can be posited as potential factor contributing to the development of the sensitization effect (e.g., effects on P55 plus P75 receptors, recruitment of intermediate cytokines, involvement of GABAergic neurons). At this juncture, however, there is simply insufficient information available to favor one hypothesis over another. At the very least, studies are necessary to establish whether the sensitization effects stem from neurochemical and endocrine alterations engendered by TNF- α itself, or are the result of a profound antibody response which develops over time to counteract the antigenic properties of the exogenously applied cytokine. That is, the initial administration of human TNFa may set into motion an immunologic response (antibody production to TNF- α), so that a secondary response is provoked upon later reexposure to the same "antigen". We are currently conducting an experiment assessing whether pretreatment with LPS influences the central and

neuroendocrine actions of later TNF- α treatment. If a cross-sensitization of neurochemical and neuroendocrine activity is seen, then it is safe to assume that the sensitization effect in the current investigation was not due to a humoral immune reaction, since the initial LPS treatment will have provoked production of murine, rather than human, TNF- α .

Conclusions

It is clear that systemic administration of TNF- α has behavioral, neuroendocrine and central neurochemical effects and that such effects are subject to a time-dependent sensitization. In particular, HPA activity, as reflected by levels of circulating corticosterone, may be stimulated upon reexposure to TNF- α after relatively long time intervals, whereas suppression of neuroendocrine activity is evident when reexposure occurs after shorter time intervals. These findings have potentially important implications for immunotherapy, just as the effects of IL-1 are pertinent in this respect. For instance, some of the psychiatric disturbances seen in patients receiving immunotherapy (e.g., after IL-2) may be related to alterations in CNS neurotransmitter activity and such effects may be exacerbated if the individual already had elevated cytokine levels prior to such treatment. Additionally, while locoregional administration of TNF- α has been used in cancer therapy, the well documented toxic effects of TNF- α preclude its systemic use in such treatment. The present investigation further addresses the issue of toxicity from a sensitizing perspective. More specifically, any bacterial or infectious agents that provokes the release of endogenous TNF- α may render such individuals susceptible to some of the behavioral, endocrine and neurochemical disturbances seen in the present investigation. Subsequent reexposure to the cytokine would lead to potentially profound deleterious consequences, depending on the time interval elapsed between the initial and final exposures.

The chronicity of the insult also deserves to be addressed. In particular, chronic persistent infection may have vastly different sensitizing consequences relative to that elicited by the two acute injections employed in the current investigation. Indeed, patients

treated with immunotherapy (e.g. cancer) often have persistent chronic secondary bacterial infections which may render them particularly vulnerable to some of the harmful effects of treatment with cytokines such as, TNF- α . Likewise, cytokine immunotherapy would likely involve the repeated administration of the cytokine over a protracted time period potentially leading to a sensitization or desensitization syndrome, depending on the duration of treatment.

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IMAGE EVALUATION TEST TARGET (QA-3)

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
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Fax: 716/288-5989

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