Neurochemical and Anhedonic-like Effects of Continuous Interleukin-2 Infusion

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the degree of

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Abstract

Interleukin-2 (IL-2), a cytokine produced by T cells, has been used as a therapeutic agent in the treatment of several types of cancer. Through stimulation of natural killer and helper T cells, the cytokine is believed to play a major role in militating against tumor formation and progression. Although effective in this capacity, IL-2 therapy is associated with a depressive-like profile, including symptoms of fatigue, reduced appetite, depressed mood, impaired confidence and reduced energy. However, relatively few animal studies have evaluated the behavioral and central neurochemical effects of this cytokine, and still less information is available concerning the effects of chronic IL-2 treatment mimicking the procedures used in humans. The present studies assessed the behavioral, neuroendocrine and neurotransmitter variations associated with chronic systemic infusion of IL-2 (via osmotic minipumps). In addition, as previous studies have reported that the depressive profile associated with other cytokines can be attenuated by pretreatment with an SSRI, paroxetine was administered to determine if this was also the case for IL-2. Finally, it was hypothesized that the superimposition of stressors may exacerbate the effects of protracted cytokine infusion. Indeed, as cancer patients undergoing immune therapy are presumably in a state of considerable distress and uncertainty, the interactive effects of cytokine and stressor treatments would provide valuable insight into such clinical situations.

The present investigation demonstrated that chronic, continuous administration of IL-2 through implanted osmotic minipumps produced an anhedonic-like profile, as indicated by reduced consumption of a palatable substance as well as mild indices of mild

illness. However, IL-2 had limited effects on neuroendocrine and neurotransmitter processes, with its effects being restricted primarily to alterations of dopaminergic functioning in the PFC and substantia nigra. Contrary to the view that IL-2 induces a state like that of clinical depression, pretreatment with paroxetine did not attenuate these behavioral effects. Moreover, IL-2 did not have additive or synergistic effects with stressors regarding either behavioral or central measures. However, due to the numerous differences between the effects of the treatments in animals and human studies, coupled with the limited IL-2 manipulations assessed, it is premature to dismiss a role for IL-2 in depressive-like states.

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In addition to protecting the host organism from various pathogens (e.g. bacterial and viral infections), it has been proposed that the immune system acts as a sensory organ, informing the brain of antigenic challenge (Blalock, 1984; Weigent & Blalock, 1995). The process by which the immune system comes to affect CNS processes has yet to be fully elucidated, although there is reason to believe that multiple routes of interaction between the two systems are possible. In this respect, cytokines, soluble products of an activated immune system, have been shown to act in a signaling capacity to influence neuroendocrine and central neurotransmitter processing, and may thus come to affect behavioral and mood states (Anisman & Merali, 1999).

Human studies supporting a role for the behavioral effects of cytokines have come mainly from reports describing the effects of immunotherapy (e.g. Capuron et al, 2000; Lerner et al., 1999), and from studies assessing cytokines and their receptors in the affective illnesses (e.g. Maes et al., 1993; Maes et al., 1995). In addition, numerous studies have been conducted to assess the effects of cytokines on behavioral outputs and on central neurotransmitter and neuroendocrine functioning. To a significant extent, experimentation in animals has focused mainly on the proinflammatory cytokines interleukin (IL) -1 β and tumor necrosis factor alpha (TNF- α), both of which were found to stimulate the release of various stress-related hormones (e.g. corticosterone) as well as augment neurotransmitter activity within various stressor-reactive brain regions. Thus, it was proposed that IL-1 β and other proinflammatory cytokines may act as immunotransmitters, conveying information between the immune and central nervous systems (Dunn & Wang, 1995; Anisman et al., 2001).

In contrast to the abundance of information concerning the effects of proinflammatory cytokines, there is relatively little information available regarding the behavioral and neurochemical effects of IL-2, a noninflammatory cytokine produced by CD4 Th1 helper cells (Hanisch, 2001). This paucity of information is somewhat surprising, as IL-2 is used as a therapeutic agent in the treatment of cancers such as metastatic renal cell carcinoma (Gore et al., 1994; West et al., 1989) and metastatic melanoma (Legha et al., 1996), and has been reported to promote pronounced adverse effects with respect to neuropsychological functioning (Capuron et al., 2000, 2001; Caraceni et al., 1993; Denicoff et al., 1987; Musselman et al., 2001). Provisionally, the available evidence suggests that the behavioral effects of IL-2 are less pronounced than those of TNF- α and IL-1 β (Anisman & Merali, in press). For example, unlike TNF- α and IL-1 β , IL-2 does not have anxiogenic effects on typical tests of anxiety such as the elevated plus maze (Connor et al., 1998; Pettito et al., 1997). However, this applies to acute administration of the cytokines, as very little information is available regarding the effects of chronic treatments.

Presence of cytokines and cytokine receptors in the brain

Although their levels are low within the brain, cytokines and their receptors are endogenous to neuronal cell bodies, microglia and astrocytes (Farrar et al., 1987; Katsuura et al., 1988; Yabuuchi et al., 1994). Receptors for IL-1 β and TNF- α have been identified in several areas of the mouse and rat brain, including the hippocampus, choroid plexus, cortex, olfactory bulb, cerebellum, hypothalamus, brainstem and thalamus (Schobitz et al., 1994; Kinouchi et al., 1991). Stressors, as well as various immunological

and neurological insults, cause an upregulation in the expression of these proinflammatory cytokines. For example, elevated levels of central IL-1 β and TNF- α mRNA have been reported after restraint stress, lipopolysaccharide (LPS) administration, head injury and cerebrovascular trauma (Yabuuchi et al., 1996; Buttini & Boddeke, 1995; Buttini et al., 1994, 1996; Liu et al., 1996; Sato et al., 1997).

As is the case for IL-1 β and TNF- α , the presence of IL-2 within the brain suggests that the cytokine normally regulates central processes (Lapchak et al., 1991). Indeed, using immunoautoradiographic techniques, IL-2 and its receptors have been localized in several areas of the rat brain (Araujo et al., 1989; Lapchak et al., 1991). A high density of IL-2-like immunoreactivity was detected in the hippocampus, cerebral cortex (lamina IV), lateral septum, neostriatum and cerebellum, while lower levels were demonstrated in the thalamus, medial septum and granule layer of the cerebellar cortex (Lapchak et al., 1991). In keeping with the autocrine and paracrine effects ascribed to IL-2, it was suggested that the cytokine is synthesized and stored in the vicinity of its receptors, allowing for the cytokine to have local modulatory actions. Indeed, immunohistochemical studies revealed positive correlations between IL-2-like and IL-2 receptor-like immunoreactivity within the brain (Lapchak et al., 1991).

Mechanisms of cytokine-induced central effects

Owing to their relatively large size and hydrophilic nature, cytokines do not cross the blood brain barrier (BBB) in appreciable quantities. However, cytokines may gain entry into the brain through circumventricular areas, which lack an efficient BBB (Banks et al., 1989; Quagliarello et al., 1991). Thus, cytokines may passively diffuse through circumventricular organs, such as the organum vasculosum laminae terminalis (OVLT),

area postrema, median eminence and subfornical organ to reach the brain parenchyma (Lee et al., 1998; Laflamme & Rivest, 1999). As well, cytokine receptors are numerous at circumventricular and other vascular regions, and it has been suggested that circulating cytokines may interact with these receptors to generate such secondary messengers inside the brain (Laflamme & Rivest, 1999).

In addition to passive mechanisms, cytokines may also disrupt the BBB (Quagliarello et al., 1991) making it easier for these substances to gain entry to the brain. Furthermore, IL-1 β (Banks et al., 1989) and TNF- α (Guitierrez et al., 1993) have been found to cross the BBB via saturable carrier mediated transport mechanisms, which pump these cytokines into the brain. It was reported that IL-2 is able to cross the BBB (Waguespack et al., 1994); however, unlike IL-1 β and TNF- α , it appears that IL-2 does not act through a saturable, specific transport system, as neither excess unlabelled IL-2 nor IL-2 antibodies affect the rate of transport of radio-labeled IL-2 into the brain from the periphery (Waguespack et al., 1994). Thus, the specific mechanism by which IL-2 is able to cross the BBB remains to be elucidated.

In addition to the potential direct effects, cytokines may also affect CNS functioning without actually penetrating the brain. For instance, vagal afferent fibers may transmit information to the brain after stimulation by circulating cytokines (Dantzer et al., 1996). In this respect, receptors for IL-1 β have been found on peripheral ganglion that project to the brainstem NTS, and *c-fos* expression is elevated in these regions in response to systemic IL-1 β or LPS challenge (Ek et al., 1998; Gaykema et al., 1998). Activation of vagal branches may stimulate the *de novo* synthesis of cytokines within various brainstem and hypothalamic nuclei (Hopkins & Rothwell, 1995; Gaballec et al.,

1995), thus increasing local brain concentrations of these messengers. It is also possible that secondary molecules, such as prostaglandins, may contribute to the central functions of cytokines, as antagonists of these hormones block some of the central actions of IL-1 β (e.g., the NE variations observed within the hypothalamus) (Masana et al., 1990; Tsumori et al., 1998).

Behavioural effects of interleukin-2

Cytokines and endotoxins may engender anhedonic-like behaviors in animals, as indicated by reduced consumption of palatable substances or disrupted responding for rewarding intracranial brain stimulation (Borowski et al., 1998; Brebner et al., 2000). Animals will normally emit operant responses for electrical stimulation to brain areas containing high levels of dopamine, such as the medial forebrain bundle, the substantia nigra, the nucleus accumbens, and the ventral tegmentum (Zacharko & Anisman, 1991). Systemic IL-2 was found to disrupt responding for rewarding brain stimulation from the lateral hypothalamus, an effect that persisted for one week following cytokine administration (Anisman et al, 1996, 1998). In these experiments rats were tested in a discriminated task where responding to one stimulus was reinforced, while responding to a second stimulus was not. Following acute IL-2 treatment that disrupted reinforced responses it was observed that non-reinforced responding was unaffected, indicating that IL-2 did not affect performance deficits due to motor or attentional disturbances, or malaise (sickness) that potentially could be induced by cytokines. Paralleling the alterations of lateral hypothalamic self-stimulation observed after systemic treatment with IL-2, icv IL-2 administration was also found to disrupt responding for rewarding stimulation from the ventral tegmental area (Hebb et al., 1998). The latter findings

support the contention that the effects of IL-2 on responding for rewarding stimulation stems from central, rather than peripheral actions of the cytokine.

In addition to disrupted responding for rewarding brain stimulation, anhedonic-like behaviors can be assessed by measurement of consumption of a highly palatable substance, such as chocolate milk or sucrose solution. Reduced consumption following cytokine administration may be indicative of a diminution of the reward value of the substance. Indeed, reduced consumption of a palatable substance has been found following administration of IL-1 β , LPS and TNF- α (Brebner et al., 2000; Shen et al., 1999). However, due to the profound sickness engendered by these cytokines (Dantzer, 2001), it is difficult to dissociate anhedonia from the reduced feeding associated with cytokine-induced illness.

As indicated earlier, despite the fact that human trials indicated that IL-2 promotes adverse psychological effects, remarkably few studies have evaluated the behavioral effects of IL-2 in animals, and evaluation of the impact of chronic treatment with this cytokine has been exceedingly rare. Nevertheless, it has been shown that acute central treatment with IL-2 elicited behavioral inhibition and signs of sickness (Pauli et al., 1998). Moreover, when administered systemically the cytokine altered patterns of exploration of a novel environment (Zalcman, 2001; Zalcman et al., 1998), and provoked hyperactivity in threatening environments such as an elevated plus-maze (Pettito et al., 1997). When IL-2 was administered repeatedly by systemic injection, rats displayed cognitive disturbances, reflected by disruptions of Morris water-maze performance (Lacosta et al., 1999). Few reports exist, however, concerning the impact of IL-2 on depressive-like symptomatology, although acute treatment was shown to promote

anhedonia, as indicated by disrupted responding for rewarding brain stimulation (Anisman et al., 1996, 1998; Hebb et al., 1998). However, it should be underscored that these behavioral reports involved the use of acute bolus injections of IL-2, rather than the schedule of repeated bolus or continuous infusions employed in immunotherapy (e.g., Legha et al., 1996; West et al., 1987). Moreover, as indicated by Anisman, Kokkinidis & Merali (in press), these anhedonic effects were appreciably less pronounced than those of other depressionogenic agents, such as stressors.

Cytokine-induced hypothalamic-pituitary-adrenal axis activation

Acute treatment with IL-1 β and TNF- α reliably provoke rapid and sustained increases of hypothalamic-pituitary-adrenal (HPA) activity (Lacosta et al., 1998; Besedovsky et al., 1991). Like the proinflammatory cytokines, IL-2 induces alterations of neuroendocrine and central neurotransmitter functioning. However, in general, these effects tend not to be as marked as those elicited by IL-1 β and TNF- α (see Anisman & Merali, 1999), and in several ways their effects are distinctively different from those elicited by proinflammatory cytokines. Reports of HPA activation by IL-2 are inconsistent and depend upon such variables as the route of administration, injection schedule (acute vs. repeated), and diurnal patterns.

It was reported that cortisol and ACTH levels were elevated in humans undergoing immunotherapy (Lissoni et al., 1990; Lissoni et al., 1991; Chambrier, 1990; Denicoff et al., 1989). Likewise, application of IL-2 to anterior pituitary cultures induced the release of several hormones, including ACTH (Karanth & McCann, 1991). Although acute central application of IL-2 in rats did not affect HPA activity (Connor et al., 1998),

chronic, continuous icv administration of the cytokine provoked an increase of circulating corticosterone and ACTH concentrations (Hanisch et al., 1994, 1996). However, hormone levels fluctuated during continuous icv infusion over a 14-day period (Hanisch et al. 1996), indicating a complex pattern of hormone secretion when administered chronically rather than a persistent HPA axis activation. Protracted effects of IL-2 on HPA functioning have also been observed. In fact, blood samples taken over a 24-h period following several days of chronic IL-2 infusion revealed elevations of corticosterone over almost the entire diurnal cycle, while ACTH was elevated during the dark phase (Hanisch et al., 1994). In contrast to the marked effects observed when IL-2 was administered centrally, systemic injection of IL-2 did not generally provoke increased plasma corticosterone or ACTH levels, regardless of whether treatment was given acutely or repeatedly over days (Lacosta et al., 2000; Zalcman et al., 1994). To date, however, studies involving systemic IL-2 administration have not assessed the neuroendocrine impact of continuous infusion of the cytokine.

Neurochemical effects of interleukin-2

As well as its effects on stress hormone release, IL-2 administration influences the turnover of monoamines and their metabolites in various hypothalamic and extrahypothalamic brain regions. When administered acutely, IL-2 has limited effects on monoamine activity, which are dependent on whether systemic or central routes are used (Lacosta et al., 2000; Connor et al., 1998; Pauli et al., 1998). In particular, acute systemic IL-2 affected the accumulation of the norepinephrine metabolite MHPG, reflecting increased norepinephrine utilization, in the paraventricular nucleus (PVN) of the

hypothalamus (Lacosta et al., 2000). However, acutely administered icv IL-2 did not affect MHPG in the hippocampus (Connor et al., 1998), but increased levels of hippocampal serotonin (5-HT) and its metabolite 5-HIAA (Pauli et al., 1998).

In contrast to the limited effects of acute treatment, repeated systemic injections provoked a broader range of monoamine alterations. Following one week of repeated systemic injections, IL-2 elevated MHPG within the locus coeruleus, median eminence plus arcuate nucleus, central amygdala and hippocampus (Lacosta et al., 2000). As well, repeated IL-2 administration reduced dopamine levels and turnover within the caudate nucleus and substantia nigra, while the concentration of serotonin was reduced in the medial prefrontal cortex and increased within the hippocampus (Lacosta et al., 2000).

It will be recalled that anhedonia is a primary symptom of depressive illness (DSM-IV). As dopamine is thought to be one of the neurotransmitters subserving reward processes (Wise & Rompre, 1989), it is of particular significance that IL-2 may impact on DA activity. In vivo microdialysis experiments revealed an inhibition of DA release by IL-2 (Anisman et al., 1996). Moreover, as IL-2 has been implicated in several disorders involving the nigrostriatal DA system (e.g. Parkinson's, schizophrenia) (Stypula et al., 1996; Kim et al., 2000) it is again noteworthy that this cytokine influences DA activity at these sites. In particular, IL-2 dose dependently (biphasically) modulates the release of dopamine in striatal slices, augmenting release at lower concentrations while inhibiting it at higher concentrations (Pettito et al., 1997; Alonso et al., 1993).

Immunotherapeutic effects of IL-2

Interleukin-2 is used either alone or in combination with interferon-alpha (IFN- α), another T cell-derived cytokine, in immunotherapeutic treatments for several types of cancer, particularly metastatic renal cell carcinoma (Gore et al., 1994; West et al., 1989) and metastatic melanoma (Legha et al., 1996). Although the precise mechanisms by which IL-2 acts on tumors remain to be fully elucidated, it is known that the cytokine stimulates the production of natural killer (NK) cells and other cytotoxic cells, thereby affecting tumor progression and development (Lerner et al., 1999). As well, IL-2 stimulates the release of other cytokines, including TNF- α , which may themselves possess antitumor properties (List et al., 1992; Mier et al., 1988; Plata Salaman, 1991). Indeed, TNF- α has been shown to induce tumor cell death in vitro (Vigani et al., 1990; Wakabayashi et al., 1997) and has been used therapeutically in the treatment of certain types of cancers, although with considerable toxicity (Spriggs et al., 1988; Steinmetz et al., 1988; Mittelman et al., 1992).

Although effective as an antitumor agent, therapeutic use of IL-2 is complicated by side effects, which may be of sufficient severity to necessitate the discontinuation of therapy. For instance, IL-2 therapy is often accompanied by adverse psychiatric and psychological symptoms, including neurocognitive disturbances, such as deficits in spatial tasks (Capuron et al., 2001), schizophrenic-like symptoms (Denicoff et al., 1987), and particularly marked symptoms reminiscent of depression (Caraceni et al., 1993; Capuron et al., 2000; Denicoff et al., 1987; Lerner et al., 1999). The latter comprises symptoms of fatigue, reduced appetite, depressed mood, impaired confidence, reduced energy and suicidal ideation (Baron et al., 1993; Dutcher et al., 2000; Eton et al., 1996;

Vlasveld et al., 1992; Walker et al., 1997). This depressive symptomatology is not a direct result of chemotherapy itself, as patients receiving chemotherapy in combination with IL-2 reported increased depressive-like symptoms relative to those receiving chemotherapy alone (Walker et al., 1997). Moreover, these depressive symptoms occur early in the treatment, are more pronounced when the cytokines are given in combination (Capuron et al., 2000), and cease following termination of the treatment (Denicoff et al., 1987). Commensurate with a role of cytokines in affective states, the depressive characteristics observed in cancer patients undergoing immunotherapy with IFN- α were abated by pretreatment with a selective serotonin reuptake inhibitor, paroxetine (Musselman et al., 2001).

Routes of administration

When used in cancer patients, IL-2 is delivered either by bolus injections, subcutaneous injections, or continuous infusion (Lerner et al., 1999). Clinical reports indicate that continuous IL-2 infusion has antitumor activity that is comparable to that of bolus injections, but is associated with less toxicity (e.g. hypotension, capillary leak) (West, 1989; Clark et al., 1990; Legha et al., 1996; Caligiuri et al., 1991, 1993). As well, IL-2 is biologically more active when given on a continuous infusion schedule as opposed to bolus administration (Thompson et al., 1988; Clark et al., 1990), thus potentially reducing the dose necessary for antitumor activity. Given the apparent benefits of utilizing a continuous infusion schedule in IL-2 immunotherapy, there is an obvious need to assess the behavioral and neurochemical consequences of this treatment.

Experimental Prospectus

The present series of experiments will be undertaken to assess the influence of chronically infused IL-2 on behavioral, neurochemical and neuroendocrine functioning. In this respect, the effects of IL-2 will be assessed on the consumption of a highly favored solution (chocolate milk), as this behavioral change may be a reflection of sickness and/or anhedonia. Indeed, it has been shown previously that this behavior is sensitive to the effects of proinflammatory cytokines (Brebner et al., 2000) and endotoxin treatment (Yirmiya, 1996), and the latter effects are attenuated by chronic antidepressant administration (Yirmiya, 1996). In the present investigation we will establish whether chronic infusion of IL-2 also impacts on this behavior.

It is suggested that antidepressant treatments may ameliorate the behavioral and neurochemical effects of the aforementioned cytokine treatments. Thus, chronic antidepressant treatment was given concurrently with IL-2 to address this question. There is reason to believe that cytokines and stressors may have synergistic effects (Song, Merali & Anisman, 1999). Likewise, as indicated earlier, there is evidence suggesting that the behavioral effects of IL-2 are dependent upon whether animals are tested in a threatening or non-threatening environment (Pettito et al., 1997; Zalcman, 2001; Zalcman et al., 1998). Thus, there is reason to believe that IL-2 and stressors may also have additive or synergistic effects. Indeed, as cancer patients undergoing immune therapy are presumably in a state of considerable distress and uncertainty, the interactive effects of cytokine and stressor treatments needs to be considered. In view of these factors, it is expected that the superimposition of various, unpredictable, mild stressors may exacerbate the central and behavioral effects of protracted IL-2 infusion.

Materials and Methods

Subjects

Male CD-1 mice, approximately 90 days of age, were obtained from Charles River Laboratories (St Constant, Quebec, Canada). Mice were housed individually in standard polypropylene cages and allowed two weeks to acclimate to the laboratory before being used as experimental subjects. Animals were given *ad libitum* access to pellet mouse chow (5075 Ralston Purina) and tap water. Lighting was maintained on a 12-hour light, 12-h dark cycle (lights on at 0800 h), and room temperature was maintained at 21°C.

Measurement of consumption of a palatable substance and determination of sickness behaviors

In addition to the normal supply of mouse chow and water, mice were offered chocolate milk (Neilson, 1% partly skimmed) for one hour daily during the light cycle (at approximately 1000 h). Bottles were weighed before and after the one-hour period in order to determine total consumption. This procedure was maintained for 10 to 14 days prior to surgery, permitting a stable baseline of chocolate milk consumption to be reached (three consecutive days over which consumption varied by less than 10%). Thereafter osmotic mini-pumps containing IL-2 or vehicle were surgically implanted and consumption measured for 7 further days.

Commencing the day following surgery and continuing through to day 6, the day before decapitation, sickness behaviors were assessed once per day using a four-point rating scale: 1 = normal (locomotion, appearance); 2 = slight signs of illness (lethargy,

ptosis or piloerection); 3 = obvious illness (very little movement, ptosis and piloerection, curled body posture); 4 = very sick animal (virtually no movement, impaired breathing, unresponsiveness, plus the above symptoms). This procedure has been found, in our laboratory, to yield better than 90% agreement between raters.

Cytokine administration

Once stable consumption was established, mice were anesthetized using halothane via inhalation, and osmotic mini-pumps (model 1007D with a delivery rate of 0.5 μ l/h, Alza Corp., Palo Alto, CA) were implanted subcutaneously. The osmotic pumps allowed for a continuous flow of solutions over a 7-day period, thereby ensuring a constant level of cytokine in the blood over this time period. Pumps were implanted, as recommended by the manufacturer, through a small incision made in the skin between the scapulae. Pumps were filled with sterile solutions of either human recombinant IL-2 (50,000 IU/day), or saline (100 μ l). IL-2 was obtained in lyophilized form (provided by C. Reynolds, National Cancer Institute, Frederick, Maryland) and dissolved in sterile phosphate buffered saline.

Experiment 1: Behavioral effects of chronic, continuous infusion IL-2. Mice (n=10/group) received continuous infusion of either human recombinant IL-2 (50,000 IU/day) or saline (100 μ l) over a 7-day period. As previously indicated, chocolate milk consumption and sickness behaviors were monitored during this period.

Experiment 2: Effect of paroxetine on the neurochemical, neuroendocrine, and anhedonic effects of continuous infusion IL-2. In order to determine whether the depressive-like effects of IL-2 treatment could be attenuated by antidepressant administration, mice (n = 10/group) received paroxetine (15 mg/kg) or vehicle daily by gavage (1" 22-gauge, CDMV) for a period of 14 days prior to implantation of osmotic minipumps containing either IL-2 or saline, and thereafter during the period of minipump infusion. Chocolate milk consumption and sickness behaviors were monitored during this time. Following the experiment (day 7 of minipump infusion) animals were decapitated and trunk blood and brains collected for neuroendocrine and monoamine determinations.

Experiment 3: Synergistic effects of chronic, continuous IL-2 infusion and chronic, unpredictable mild stress. To determine the interactive effects of IL-2 and stress, mice (n = 10/group) were subdivided into 6 groups such that animals received either no stress, acute stress or chronic stress. All were implanted with an osmotic minipump containing either vehicle or IL-2 (3 x 2 ANOVA). Chronic stress comprised a daily mild stressor (restraint, forced swim, novel cagemates, open field, tail suspension, elevated plus maze with closed arms covered, Y maze, exposure to predator odor (rat feces), novel bedding, restraint, food deprivation, change of light cycle) for 14 days prior to the implantation of osmotic minipumps, and thereafter for each of the seven days of minipumps infusion. Restraint (10 min) followed by 5 min in the home cage acted as the acute stressor immediately prior to decapitation as well as the final stressor for the chronically stressed mice.

Plasma corticosterone

Following decapitation 7 days after mini-pump implantation, trunk blood was collected in tubes containing EDTA and centrifuged at 3600 RPM. The resultant plasma was aliquoted into tubes for corticosterone determinations, and stored at -80°C until the assay was performed. All decapitations were performed between 0800 h and 1200 h to minimize effects attributable to diurnal variations in hormone release. Plasma corticosterone levels were assayed, in duplicate, using commercially available radioimmunoassay kits (ICN Biomedicals Inc., Calif., USA). The assay procedure yielded less than 10% intra-assay variability. To eliminate interassay variability all samples were assayed within a single run.

Measurement of plasma IL-2

To ensure that viable levels of IL-2 remained in the system over the 7-day period, plasma human IL-2 was measured in a subgroup of animals using a commercially available enzyme-linked immunosorbent assay (ELISA, R & D Systems). Mice ($n = 3$) were implanted with minipumps containing IL-2 or saline, as described above, and trunk blood was collected following 3 or 6 days of cytokine administration. Blood was centrifuged and plasma collected as described earlier, and subsequently stored at -80°C until the assay was performed.

Brain dissection technique

Following decapitation brains were removed and sliced. Brains were sectioned in coronal slices on a stainless steel dissecting block with adjacent blocks approximately

100 μm apart. The PVN, central amygdala, substantia nigra, nucleus accumbens and locus coeruleus were obtained by micropunch using a hollow, 16-gauge microdissection needle with a beveled tip, using the mouse brain atlas of Franklin & Paxinos (1997). The prefrontal cortex and hippocampus were removed in their entirety using razor blades. Samples were stored at -80°C until monoamine and metabolite determinations by high performance liquid chromatography (HPLC).

HPLC procedure for analysis of brain monoamine and metabolite levels

Levels of dopamine (DA), norepinephrine (NE) and serotonin (5-HT) and their respective metabolites 3-methoxy-4-hydroxyphenylglycol (MHPG), 3,4-dihydroxyphenylacetic acid (DOPAC), and 5-hydroxyindole acetic acid (5-HIAA) were determined by HPLC. Tissue punches were sonicated with homogenizing solution containing 14.17 gm of monochloroacetic acid, 0.0186 gm of EDTA, 5.0 ml of methanol and 500 ml of H_2O . Samples were centrifuged and resultant supernatants were used for HPLC analysis. Using a Waters Associates (Milford, MA) M-6000 pump, guard column, radial compression column (5 m, C18 reverse phase, 8 mm $\times$ 10 cm), and a three-cell coulometric electrochemical detector (ESA model 5100A), 20 μl of the supernatant was passed through the system at a flow rate of 1.5 ml/min (1400-1600 psi). The mobile phase was then filtered (0.22 mm filter paper) and degassed, after which the pH was adjusted to 2.5 with phosphoric acid. The area and height of peaks was determined using a Hewlett-Packard (Palo Alto, CA) integrator. The protein content of each sample was determined using bicinchoninic acid with a protein analysis kit (Pierce Scientific, Brockville, Ontario, Canada) and a spectrophotometer (PC800 colorimeter, Brinkman

Intruments, Westbury, NY). The lower limit of detection of amines and metabolites was approximately 5.0 pg.

Statistical analyses

Consumption of chocolate milk was analyzed by mixed measures analysis of variance (ANOVA) with Treatment (e.g., cytokine, stressor, antidepressant) as the between-groups variable and Days as the within group measure. The mean consumption on the three days immediately prior to implantation of the minipumps served as the baseline. Sickness behaviors were also analyzed by mixed measures ANOVA with Days as the within group variable. Plasma corticosterone and ACTH concentrations, as well as brain monoamines and metabolites, were analyzed by single-factor ANOVA. Post hoc comparisons of predicted differences were analyzed by Tukey and Dunnett tests.

Results

Measurement of Human IL-2 Levels in Plasma

As expected, levels of human IL-2 were substantially increased following 3 days of IL-2 treatment (1125.0, 1094.0 and 407.0 pg/ml) and began to drop after 7 days (157.8, 119.7 and 269.2 pg/ml). It is uncertain, however, whether this difference reflects a reduction in the viability of the IL-2 remaining in the pumps, or whether the pump reservoirs were empty as their 7-day infusion period had elapsed. Saline-treated mice showed no detectable levels of human IL-2 at either time point, indicating a lack of cross-reactivity with murine IL-2. Thus, the IL-2 detected by the ELISA was only that which came from the minipumps.

Experiment 1: Behavioral Effects of Chronic IL-2 Treatment

Chocolate milk consumption and sickness behaviors. Chocolate milk consumption was found to vary as a function of the IL-2 treatment x Days interaction, $F(6, 108) = 2.615$; $p < 0.05$. The post hoc multiple comparisons revealed that in saline-treated animals consumption was reduced below baseline on Day 1 following minipump implantation, but increased to baseline levels thereafter (see Figure 1, upper panel). Among IL-2-treated animals the reduction of chocolate milk consumption appeared to be more sustained than that of saline-treated mice, but this reduction was only statistically significant relative to baseline at Days 2 and 4 following surgery.

Ratings of sickness varied as a function of the IL-2 treatment x Days interaction, $F(5, 90) = 2.99$; $p < 0.05$. The multiple comparisons revealed increased ratings of sickness in IL-2 treated mice on days 1 to 5, relative to saline-treated animals. It ought to be underscored however, as shown in Figure 1 (lower panel), that although significant, the level of sickness observed in this experiment was much less pronounced than that seen in previous experiments in this laboratory using the proinflammatory cytokines, TNF- α and IL-1 β , or by endotoxin administration (Lacosta et al., 1998; Hayley et al., 1999, 2001). In the main, the sickness was characterized by slight lethargy and ruffled appearance. Body weight was found to be stable throughout the experiment.

Figure 1. IL-2 reduced the consumption of a palatable substance (chocolate milk; top panel). * $p < .05$ relative to baseline (IL-2) and ° $p < .05$ relative to baseline (saline). IL-2 also increased ratings of illness, as reflected by changes in overall appearance. * $p < .05$ relative to saline-treated controls.

Experiment 2: Effect of Paroxetine Treatment on IL-2-induced Anhedonic-like Effects

Chocolate milk consumption and sickness behaviors. Chocolate milk consumption was reduced following treatment with IL-2, as indicated by a significant main effect of cytokine treatment, $F(1, 55) = 5.45$; $p < 0.05$. As well, consumption was reduced by paroxetine treatment, $F(1, 55) = 13.45$; $p < 0.01$. However, the interaction between these variables did not approach significance and it was clear that the reduced chocolate milk consumption elicited by the cytokine was not attenuated by the antidepressant (see Figure 2, upper panel).

In addition to the altered consumption, mice that received IL-2 also showed an increase in the severity of illness, as indicated by ratings of overall appearance $F(1, 55) = 34.43$, $p < 0.01$. Curiously, the paroxetine treatment also caused an increase in sickness behaviors, $F(1, 55) = 4.90$; $p < 0.05$, although there was no change in body weight throughout the experiment. As was the case for the chocolate milk consumption, paroxetine did not attenuate the sickness elicited by IL-2. In fact, the cytokine and paroxetine appeared to have additive effects, although the signs of illness were, in fact, modest (see Figure 2, lower panel).

Plasma corticosterone. Both the cytokine and paroxetine treatments elevated plasma corticosterone levels, F 's $(1, 54) = 8.42$ and 5.37 ; $p < 0.01$ and 0.05 , respectively. Moreover, these treatments had additive effects in this respect (see Table 1), although in both instances the corticosterone elevation elicited by the cytokine was small. Due to the lack of effect of paroxetine on IL-2-induced anhedonic-like behavior, monoamine concentrations were not evaluated in this experiment.

Figure 2. Mean ($\pm$ SEM) chocolate milk consumption (top panel) and ratings of overall appearance (bottom panel) in mice administered systemic IL-2 or saline, in addition to either the SSRI paroxetine, or saline, by gavage.

Table 1
Effects of IL-2 and Paroxetine Treatment on Plasma Corticosterone

Minipump Treatment	Gavage Treatment	Hormone Level ($\mu\text{g/dl}$)
IL-2	Paroxetine	15.88 ± 2.64
IL-2	Saline	11.44 ± 1.15
Saline	Paroxetine	10.45 ± 1.39
Saline	Saline	7.10 ± 0.81

Note: Values are expressed as the mean $\pm$ SEM.

Experiment 3: Synergistic Effects of Chronic IL-2 and Chronic Mild Stress

Chocolate milk consumption and sickness behaviors. Chocolate milk consumption was found to vary as a function of the IL-2 x Stressor treatment interaction, $F(1, 23) = 5.71$; $p < 0.05$. The multiple comparisons indicated that among mice that received saline, those that were chronically stressed consumed less chocolate milk than non-stressed mice, while the consumption of chocolate milk by mice treated with IL-2 was not further affected by stressor condition (see Figure 3, upper panel). Chocolate milk consumption also varied as a function of the IL-2 x Days interaction, $F(6, 138) = 2.70$; $p < 0.05$. The multiple comparisons revealed that although consumption was reduced below baseline only on Day 1 following minipump implantation in saline-treated animals, IL-2 infusion from the minipump reduced consumption levels below baseline on Days 2, 3, 4 and 5 following surgery (see Figure 3, upper panel). Chocolate milk consumption also varied as a function of the Stressor x Days interaction, $F(6, 138) = 2.59$; $p < 0.05$. In this case, chocolate milk consumption was reduced relative to baseline on all six days following surgery in non-stressed mice. Surprisingly, among chronically stressed mice, chocolate milk consumption was reduced below baseline only on day 3 following surgery (see Figure 3, upper panel). However, it must be noted that baseline measures of chocolate milk consumption were lower in chronically stressed mice than that of non-stressed mice, possibly due to a cumulative effect of the 14 days of stressor exposure prior to surgery. Thus, a floor effect may have been realized, such that any further stressor-induced reductions in chocolate milk consumption following minipump implantation were masked.

In addition to the altered chocolate milk consumption, IL-2 treatment influenced sickness behaviors, $F(1, 42) = 23.93$, $p < 0.0001$. Stressor treatment itself did not influence ratings of sickness, nor did it interact with the cytokine to affect this measure, as indicated in Figure 3 (lower panel). As in the previous experiments, the degree of sickness observed was relatively modest, and was less pronounced than that seen in experiments involving the proinflammatory cytokines and bacterial endotoxin. Once again, there was no change in body weight throughout the experiment.

Plasma corticosterone. The analysis of corticosterone levels included animals that received no stressor exposure, acute stressor and chronic stressor treatment. The ANOVA revealed that the stressor treatment significantly elevated plasma corticosterone levels, $F(2, 38) = 33.66$, $p < 0.05$ (see Table 2). The multiple comparisons indicated that corticosterone levels of both chronic and acute stressor groups were elevated relative to non-stressed mice, while not differing significantly from one another. In contrast to the stressor effects, IL-2 treatment did not influence plasma corticosterone levels.

Figure 3. Mice receiving IL-2 or saline by minipump also underwent either 21 days of stress (Chronic) or no stress. IL-2 decreased chocolate milk consumption over the period of minipump infusion, and chronic stressor treatment appeared to decrease baseline levels of consumption, prior to minipump treatment (top panel). IL-2, but not stressor treatment, increased ratings of illness (bottom panel).

Table 2
Effects of IL-2 and Stressor Treatment on Plasma Corticosterone

Minipump Treatment	Stressor Treatment	Hormone level ($\mu\text{g/dl}$)
IL-2	Acute	30.24 ± 3.54
IL-2	Chronic	29.50 ± 3.03
IL-2	No Stress	11.05 ± 2.93
Saline	Acute	27.01 ± 2.60
Saline	Chronic	31.10 ± 3.44
Saline	No Stress	5.91 ± 0.67

Note: Values are expressed as the mean $\pm$ SEM.

Brain monoamines and metabolites. Within the PVN, neither the cytokine nor stressor influenced NE levels, however, concentrations of MHPG varied as a function of the stressor condition, $F(2, 33) = 12.14$, $p < 0.001$, while IL-2 was without affect (see Figure 4). The multiple comparisons indicated that both the acute and chronic stressors increased levels of the metabolite relative to control levels. Unlike the utilization of NE, utilization of 5-HT and DA in the PVN, as reflected by metabolite accumulation, were not influenced by either the stressor or cytokine treatments (see Tables 3 and 4, respectively).

IL-2 treatment reduced the utilization of DA in the PFC, as indicated by a reduction in the level of DOPAC, $F(1, 38) = 8.87$, $p < 0.01$, although there was no effect of IL-2 on DA (see Figure 5). As well, the chronic stressor regimen reduced DA levels, although this reduction did not reach significance, $F(1, 40) = 3.18$; $p = 0.05$. Although neither IL-2 nor the stressor treatment affected levels of NE within the PFC, accumulation of MHPG varied as a function of stressor condition, $F(2, 40) = 3.60$; $p < 0.05$, but was unaffected by IL-2 (see Figure 6). Multiple comparisons indicated that chronic, but not acute stressor exposure, increased PFC MHPG levels relative to control animals. The levels of the 5-HT metabolite 5-HIAA were not affected by cytokine or stressor treatments (see Table 5).

Within the locus coeruleus, NE levels varied as a function of stressor condition, $F(2, 37) = 3.81$; $p < 0.05$, while IL-2 did not affect this measure. Multiple comparisons indicated that both the acute and chronic stressors increased NE concentrations relative to control levels. In contrast, levels of MHPG were unaffected by the stressor or cytokine treatments (see Figure 7).

Analysis of monoamine levels within the substantia nigra indicated an increase in DA utilization due to IL-2 treatment, reflected as an increase in the concentration of DOPAC, although this increase just missed significance $F(1, 36) = 3.88$; $p = 0.06$ (see Figure 8, lower panel). In the central amygdala, DA levels were found to vary as a function of the IL-2 x Stressor Treatment interaction, $F(2, 34) = 3.88$; $p < 0.05$. As indicated in Table 6, chronic stress reduced dopaminergic activity with respect to both DA and DOPAC, while DA appeared to be modified by IL-2 treatment. In effect, the stressor treatment augmented the effects of IL-2 on amygdaloid DA activity. Within the central amygdala, NE, 5-HT and their respective metabolites remained unaffected by the IL-2 or stressor treatments (see Tables 7 and 8).

Finally, neither stressor nor IL-2 treatment altered monoamine utilization within the dorsal hippocampus or nucleus accumbens (see Tables 9, 10 and 11).

Paraventricular Nucleus

Figure 4. Mean ($\pm$ SEM) concentrations of NE (top) and MHPG (bottom) within the PVN as a function of IL-2 and stressor treatments. Mice were systemically treated with either IL-2 or saline and also received either 21 days of stress (Chronic), an acute stressor, or no stress.

Table 3

Effects of IL-2 and Stressor Treatment on PVN 5-HT and 5-HIAA

Minipump Treatment	Stressor Treatment	5-HT (ng/mg protein)	5-HIAA (ng/mg protein)
IL-2	Chronic	34.33 $\pm$ 11.22	16.85 $\pm$ 4.83
IL-2	Acute	37.81 $\pm$ 9.40	21.00 $\pm$ 4.53
IL-2	No Stress	47.18 $\pm$ 17.32	12.39 $\pm$ 5.74
Saline	Chronic	38.55 $\pm$ 14.92	6.02 $\pm$ 2.17
Saline	Acute	25.16 $\pm$ 11.21	18.25 $\pm$ 4.68
Saline	No Stress	26.04 $\pm$ 4.09	12.74 $\pm$ 3.14

Note: Values are expressed as the mean $\pm$ SEM.

Table 4
Effects of IL-2 and Stressor Treatment on PVN DA and DOPAC

Minipump Treatment	Stressor Treatment	DA (ng/mg protein)	DOPAC (ng/mg protein)
IL-2	Chronic	31.07 $\pm$ 4.32	19.41 $\pm$ 7.42
IL-2	Acute	26.19 $\pm$ 5.57	8.82 $\pm$ 3.00
IL-2	No Stress	16.85 $\pm$ 6.22	22.30 $\pm$ 10.69
Saline	Chronic	26.55 $\pm$ 7.17	21.47 $\pm$ 11.04
Saline	Acute	31.23 $\pm$ 5.59	11.46 $\pm$ 3.07
Saline	No Stress	30.85 $\pm$ 4.62	16.78 $\pm$ 5.20

Note: Values are expressed as the mean $\pm$ SEM.

Prefrontal Cortex

Figure 5. Mean ($\pm$ SEM) concentrations of DA (top) and DOPAC (bottom) within the PFC as a function of the stressor and IL-2 treatments.

Prefrontal Cortex

Figure 6. Mean ($\pm$ SEM) concentrations of NE (top) and MHPG (bottom) within the PFC as a function of the stressor and IL-2 treatments.

Table 5
Effects of IL-2 and Stressor Treatment on PFC 5-HT and 5-HIAA

Minipump Treatment	Stressor Treatment	5-HT (ng/mg protein)	5-HIAA (ng/mg protein)
IL-2	Chronic	$5.78 \pm .69$	9.17 ± 1.35
IL-2	Acute	$4.58 \pm .75$	7.56 ± 1.57
IL-2	No Stress	$4.81 \pm .85$	7.22 ± 2.17
Saline	Chronic	6.90 ± 1.26	10.66 ± 1.54
Saline	Acute	5.57 ± 1.40	9.16 ± 1.03
Saline	No Stress	$5.85 \pm .93$	9.78 ± 2.10

Note: Values are expressed as the mean $\pm$ SEM.

Locus Coeruleus

Figure 7. Mean ($\pm$ SEM) NE (top) and MHPG (bottom) within the locus coeruleus among mice receiving either IL-2 or saline along with either chronic, acute, or no stress.

Substantia Nigra

Figure 8. Mean (+ SEM) DA (top) and DOPAC (bottom) within the substantia nigra among mice receiving either IL-2 or saline along with either chronic, acute or no stress.

Table 6
Effects of IL-2 and Stressor Treatment on Central Amygdala DA and DOPAC

Minipump Treatment	Stressor Treatment	DA (ng/mg protein)	DOPAC (ng/mg protein)
IL-2	Chronic	46.81 $\pm$ 10.99	26.36 $\pm$ 9.05
IL-2	Acute	22.69 $\pm$ 5.17	24.11 $\pm$ 3.58
IL-2	No Stress	31.50 $\pm$ 11.05	44.73 $\pm$ 14.85
Saline	Chronic	7.44 $\pm$ 4.62	5.81 $\pm$ 2.06
Saline	Acute	33.84 $\pm$ 5.57	22.16 $\pm$ 6.13
Saline	No Stress	26.31 $\pm$ 7.14	32.94 $\pm$ 14.19

Note: Values are expressed as the mean $\pm$ SEM.

Table 7
Effects of IL-2 and Stressor Treatment on Central Amygdala NE and MHPG

Minipump Treatment	Stressor Treatment	NE (ng/mg protein)	MHPG (ng/mg protein)
IL-2	Chronic	26.25 $\pm$ 4.30	6.92 $\pm$ 1.01
IL-2	Acute	17.95 $\pm$ 4.61	5.66 $\pm$.59
IL-2	No Stress	31.37 $\pm$ 14.49	6.77 $\pm$.83
Saline	Chronic	171.88 $\pm$ 69.09	6.64 $\pm$.73
Saline	Acute	24.31 $\pm$ 4.35	6.92 $\pm$ 1.02
Saline	No Stress	24.88 $\pm$ 3.71	6.56 $\pm$.53

Note: Values are expressed as the mean $\pm$ SEM.

Table 8
Effects of IL-2 and Stressor Treatment on Central Amygdala 5-HT and 5-HIAA

Minipump Treatment	Stressor Treatment	5-HT (ng/mg protein)	5-HIAA (ng/mg protein)
IL-2	Chronic	26.62 ± 11.17	20.07 ± 5.74
IL-2	Acute	35.57 ± 7.85	28.43 ± 6.51
IL-2	No Stress	36.26 ± 3.85	39.90 ± 10.85
Saline	Chronic	18.69 ± 4.47	35.01 ± 11.18
Saline	Acute	23.49 ± 6.73	34.00 ± 8.21
Saline	No Stress	26.15 ± 8.39	28.74 ± 7.32

Note: Values are expressed as the mean $\pm$ SEM.

Table 9
Effects of IL-2 and Stressor Treatment on Hippocampal NE and MHPG

Minipump Treatment	Stressor Treatment	NE (ng/mg protein)	MHPG (ng/mg protein)
IL-2	Chronic	5.05 $\pm$ 1.01	5.12 $\pm$.76
IL-2	Acute	7.81 $\pm$ 1.25	5.00 $\pm$.88
IL-2	No Stress	7.14 $\pm$.86	4.25 $\pm$.44
Saline	Chronic	6.32 $\pm$ 1.00	5.72 $\pm$.35
Saline	Acute	5.51 $\pm$.48	4.01 $\pm$.77
Saline	No Stress	6.49 $\pm$.52	4.48 $\pm$.84

Note: Values are expressed as the mean $\pm$ SEM.

Table 10

Effects of IL-2 and Stressor Treatment on Hippocampal 5-HT and 5-HIAA

Minipump Treatment	Stressor Treatment	5-HT (ng/mg protein)	5-HIAA (ng/mg protein)
IL-2	Chronic	9.28 ± 2.25	$3.75 \pm .87$
IL-2	Acute	11.41 ± 2.34	$4.93 \pm .76$
IL-2	No Stress	$8.29 \pm .88$	4.65 ± 1.00
Saline	Chronic	9.60 ± 3.85	$3.10 \pm .66$
Saline	Acute	10.43 ± 2.68	$3.17 \pm .59$
Saline	No Stress	10.67 ± 2.37	4.34 ± 1.25

Note: Values are expressed as the mean $\pm$ SEM.

Table 11
Effects of IL-2 and Stressor Treatment on Nucleus Accumbens DA and DOPAC

Minipump Treatment	Stressor Treatment	DA (ng/mg protein)	DOPAC (ng/mg protein)
IL-2	Chronic	111.51 $\pm$ 19.38	16.33 $\pm$ 3.28
IL-2	Acute	110.93 $\pm$ 12.46	16.47 $\pm$ 2.31
IL-2	No Stress	115.90 $\pm$ 15.05	15.68 $\pm$ 3.46
Saline	Chronic	115.69 $\pm$ 17.47	19.20 $\pm$ 1.23
Saline	Acute	138.63 $\pm$ 14.71	20.83 $\pm$ 3.30
Saline	No Stress	103.47 $\pm$ 12.31	15.38 $\pm$ 3.10

Note: Values are expressed as the mean $\pm$ SEM.

Discussion

Cytokine-induced sickness behaviors and depressive symptomatology

Among humans undergoing IL-2 immunotherapy, neuropsychiatric and cognitive disturbances may occur, which may be sufficiently severe to necessitate the discontinuation of therapy (Meyers & Valentine, 1995). One of the common side effects of IL-2 therapy is depression, often manifested as fatigue, reduced appetite, depressed mood, impaired confidence, reduced energy and suicidal ideation (Baron et al., 1993; Dutcher et al., 2000; Eton et al., 1996; Vlasveld et al., 1992; Walker et al., 1997). These depressive-like effects of IL-2 tend to occur early in therapy, appearing as soon as five days into the treatment (Capuron et al., 2000, 2001a, 2001b, 2002).

As one of the characteristic symptoms of depression is that of anhedonia, in the present investigation we assessed the impact of chronic IL-2 infusion on one potential index of hedonic effects, namely consumption of a highly palatable and preferred food, chocolate milk. Indeed, it was observed that consumption of chocolate milk was reduced, and this occurred as early as the first day following surgery. Commensurate with the human literature, the anhedonia observed was accompanied by signs of malaise, but these appeared at an exceedingly low level. Thus, it is unlikely that the reduction in chocolate milk consumption was entirely due to the malaise produced by the cytokine treatment. In fact, body weight, which is usually a reliable index of sickness, remained constant, thus it is unlikely that the reduced chocolate milk consumption was accompanied by a corresponding decrease in the intake of normal lab chow.

Interestingly, in patients undergoing immunotherapy with the related cytokine, IFN- α , symptoms appear at different times during the course of therapy and are

differentially responsive to antidepressant treatment. Specifically, neurovegetative or somatic symptoms, including fatigue, anorexia and pain, appeared during the first 2 weeks of IFN- α therapy, whereas the neuropsychiatric symptoms of depressed mood, anxiety and cognitive disturbances occurred later in the treatment. Antidepressant treatment was most effective in eliminating the mood and cognitive symptoms, as well as the pain induced by IFN- α therapy in humans, while it was less efficient in alleviating the other symptoms (Capuron et al., 2002). Thus, it appears that different mechanisms may be involved in the responses to cytokine therapy. Although there have been no systematic studies conducted regarding the various dimensions of symptoms associated with IL-2 therapy, the two cytokines appear to share a similar symptom profile, and may conceivably involve related (or overlapping) mechanisms.

It is essential to underscore that the mild malaise associated with chronic IL-2 treatment in the present investigation was very different from the sickness ordinarily elicited by IL-1 β and TNF- α . As indicated earlier, acute injections of the proinflammatory cytokines give rise to a combination of behavioral disturbances referred to as sickness behavior, comprising symptoms such as reduced locomotor activity, reduced social exploration, anhedonia, decreased food-motivated behaviors, and impaired learning and memory (Pauli et al., 1998; Kent et al., 1992, 1996; Aubert et al., 1995). These are thought to correspond to the symptoms seen in humans in response to infection or inflammation, typically comprising depression, lethargy, lack of interest in surroundings, and reduced eating and drinking (Dantzer, 2001). In contrast to the effects of the proinflammatory cytokines, IL-2 generally does not elicit marked or wide-ranging sickness behaviors. To the contrary, as observed in the present investigation, the signs of

malaise engendered by IL-2 were relatively mild, with the most common symptoms being a slight reduction in locomotor activity and piloerection in some, but not all IL-2 treated animals. The apparently more profound effects of IL-2 in humans undergoing immunotherapy raises the question as to whether the dose of cytokine used in the present investigation was, in any fashion, a parallel to that used in humans. Further to this same point, however, the effects seen in humans are not necessarily a reflection of the cytokine effects alone. As these patients may be suffering from certain types of cancer (and the treatment usually applied when other treatments have failed) or hepatitis C, the possibility exists that the effects of the cytokine may be interacting with the active disease state, thereby leading to the illness observed. For example, certain types of cancer are associated with elevations of IL-6 (Maes et al., 2001; Martin et al., 1999) as well as IL-1 and TNF- α (Mantovani et al., 1998, 2000), which may contribute to any effects of the IL-2 itself. Indeed, synergistic effects have been observed among cytokines (Brebner et al., 2000; McIntosh et al., 1999), raising the possibility that this may contribute to the more pronounced effects of IL-2 found in humans. In addition, cachexia, often a side effect of the cancer itself (Strassmann & Kambayashi, 1995), may make cancer patients more vulnerable to cytokine-induced changes in central functioning.

Antidepressant effects on cytokine-induced anhedonia

Consistent with the view that consumption of palatable solutions may reflect anhedonia and depression, it has been reported that consumption of favorable solutions is ordinarily reduced by proinflammatory cytokines (Brebner et al., 2000) and by endotoxin treatment (Yirmiya, 1996), and the latter effects were attenuated by chronic

antidepressant administration (Yirmiya, 1996). In addition, chronic IFN- α treatment altered consumption of saccharin solutions as shown in the three-bottle test in rats, an effect that was reversed by a selective 5-HT reuptake inhibitor, paroxetine, which acts as an effective antidepressant agent (Sammur et al., 2001). Paralleling such findings, the depressive symptoms in humans undergoing immunotherapy with IFN- α were reversed by chronic paroxetine administration (Miller et al., 1999; Musselman et al., 2001). Contrary to these findings, however, antidepressant treatment in the present investigation did not attenuate the effects of IL-2. However, the SSRI itself was found to promote diminished chocolate milk consumption, an effect not uncommon in animals treated with SSRIs (e.g., Grignaschi & Samanin, 1993; Konkle & Bielajew, 1999). Thus, the action of paroxetine itself may have precluded detection of the antidepressant actions of the cytokine with respect to chocolate milk intake. Clearly, as SSRIs affect consumption, alternative approaches will be needed to assess the effects of antidepressant treatments on cytokine-induced behaviors. It ought to be noted that gavage was used to administer paroxetine in the present investigation, as earlier experiments in our laboratory yielded still more profound anorexia when the drug was administered systemically. Ultimately, further experiments will be needed involving different drug delivery systems and varied drug doses to determine whether the effects of IL-2 can indeed be reversed by pretreatment with a selective serotonin reuptake inhibitor.

Although antidepressants have been shown to attenuate the effects of cytokine and bacterial endotoxin treatment, inconsistent results have been reported in this respect. In particular, chronic imipramine or fluoxetine treatment abolished the reduced saccharine preference and the diminished social exploration, elicited by LPS (Yirmiya,

1996; Yirmiya et al., 2001), while the atypical antidepressant, tianeptine, attenuated the reduced locomotion and social exploration provoked by systemic LPS and IL-1 β (Castanon et al., 2001). Other investigators, however, reported that in contrast to chronic desipramine, which prevented the LPS-induced anorexia and the reduced saccharine consumption, paroxetine had only modest effects, and venlafaxin was entirely ineffective. Likewise, Dunn & Swiergel (2001) reported that neither chronic imipramine nor venlafaxine attenuated the reduced sweetened milk consumption elicited by either LPS or IL-1 β , although a modest increase of milk intake was provoked by imipramine. Thus, attenuation of the effects of LPS and IL-1 β may be specific only to some types of antidepressants.

Alternatively, the lack of antidepressant effect in the present investigation may reflect the possibility that an alteration in the consumption of a highly palatable substance is not an adequate indicator of anhedonia and/or depression. For example, it was recently shown that IL-1 β provoked both a reduction of food intake and responding for sucrose on a progressive ratio (PR) schedule (Merali et al., 2002). In this particular paradigm the number of responses required to obtain a reward becomes progressively greater, making it possible to determine the amount of work animals are willing or prepared to emit in order to obtain a fixed reward (incentive motivation). Thus, the critical measure is not how much an animal consumes, but rather the motivation to obtain the rewarding substance. In rats that had been chronically treated with the SSRI fluoxetine, the reduced food consumption elicited by the cytokine was unaffected by the antidepressant, whereas the disrupted responding for sucrose on the PR schedule was attenuated. These investigators suggested that responding on the PR schedule was more closely aligned

with incentive motivation than effects related to sickness alone, and as such may have been more sensitive to the effects of antidepressant treatment than simply assessing the actual consumption of a sweetened substance. It may similarly be the case that the effects of IL-2 in the present investigation were more closely tied to the anorexia induced by the cytokine than to potential anhedonic effects, and hence were less amenable to modification by an antidepressant. Although consumption of regular chow was not assessed in the present investigation, it will be useful in future studies to determine if this is also affected by IL-2 administration.

HPA activity

It has been demonstrated that IL-2 remains viable at physiological temperatures for up to 14 days (Boehringer Mannheim, technical service information), thus making it unlikely that the absence of particular effects in the present investigation was related to diminished action of the cytokine within the osmotic pumps. However, the possibility exists that the limited effects of IL-2 may have stemmed from the minipump reservoir becoming depleted by the time brain regions or blood were taken for analysis. Yet, measurement of IL-2 levels in the plasma (by ELISA) revealed that the blood concentration of IL-2 was still relatively high 7 days after osmotic pump implantation, although the level was reduced from that seen after three days. Thus it may be the case that most of the cytokine had been metabolized by the time of decapitation. It may therefore be profitable to measure monoamine and hormone levels at somewhat earlier time points during the 7-day period of minipump infusion, when circulating levels of the cytokine were relatively high.

Typically, IL-2 has far less pronounced effects on HPA activity relative to those provoked by IL-1 β and TNF- α (Borowski et al., 1998; Lacosta et al., 1995; Anisman et al., 2001). Accordingly, it was expected that IL-2 would have relatively modest effects on HPA activity in the present investigation. Although IL-2 increased corticosterone levels in one experiment, mainly in mice that also received paroxetine, this effect was weak, and indeed was not evident in a subsequent experiment. In contrast to these findings, previous studies revealed elevations of ACTH and cortisol levels in patients undergoing IL-2 therapy (Lotze et al., 1985; Denicoff et al., 1989; Lissoni et al., 1990, 1991). As well, it has been shown that IL-2 can induce the release of ACTH when applied to pituitary cells in vitro (Karanth & McCann, 1991), and when administered centrally it may increase circulating ACTH and corticosterone levels (Hanisch et al., 1994, 1996). However, when administered systemically to mice by intraperitoneal injection, IL-2 did not increase HPA hormone levels, regardless of whether the cytokine was given acutely or repeatedly (Lacosta et al., 2000). Thus, the possibility ought to be considered that the route of administration and species under investigation might influence the ability of IL-2 to stimulate HPA activity.

It is possible that the dose of IL-2 used in this experiment was not adequate to cause a significant elevation of corticosterone. It must also be considered that animals in the present experiment received IL-2 for only one week, whereas in humans undergoing immunotherapy, the cytokine is given for much longer periods. Thus, it may take longer for noticeable increases in hormone levels to occur. Finally, in the present investigation corticosterone determinations were made in blood collected during the light phase of the diurnal cycle. It has previously been reported that elevations of corticosterone and ACTH

elicited by centrally administered IL-2 are particularly marked during the evening (Hanisch et al., 1994). Thus, it is conceivable that hormone elevations elicited by IL-2 may be restricted to only certain portions of the diurnal cycle.

IL-2 effects on neurochemistry

In contrast to the continuous infusion schedule used in the present study, most previous experiments that examined the effects of IL-2 on monoamine turnover used acute systemic injections. When administered acutely, IL-2 increased hypothalamic and hippocampal MHPG (reflecting an increase of NE utilization) and enhanced DA turnover in the prefrontal cortex (Zalcman et al., 1998). Acute IL-2 has also been found to increase NE utilization within the PVN (Lacosta et al., 2000). However, with repeated injections, IL-2 failed to influence NE or MHPG levels within the PVN (Lacosta et al., 2000). In contrast, repeated injections of IL-2 increased NE utilization in the hippocampus, as well as inducing small increases of NE turnover in the mPFC and central amygdala and reducing NE levels in the locus coeruleus. As well, following repeated IL-2 treatment, 5-HT levels were increased in the hippocampus and reduced in the mPFC (Lacosta et al., 2000).

Consistent with the previous findings using repeated injections, continuously infused IL-2 did not affect PVN monoamine activity. However, contrary to the previous findings, the present investigation found no effect of IL-2 on NE within the locus coeruleus, central amygdala or hippocampus. In addition, unlike previous findings with repeated injections (Lacosta et al., 2000), in the present investigation IL-2 failed to influence 5-HT levels in the hippocampus and PFC. It is not certain what factors may

have accounted for such an outcome given the differences in the regimen of IL-2 administration (repeated injection in the earlier studies vs continuous infusion in the present report). Likewise, it is not known what levels of circulating IL-2 were achieved in the earlier studies.

In contrast to other cytokines, such as IL-1 and TNF- α , that typically have few effects on central DA activity, IL-2 has been reported to potently influence dopaminergic function. Indeed, acute IL-2 was shown to reduce dopamine release within the nucleus accumbens (Song et al., 1999) and following repeated IL-2 injection DA and DOPAC levels were reduced within the substantia nigra and caudate nucleus (Lacosta et al., 2000). Likewise, an acute injection of IL-2 was shown to increase DA turnover within the PFC (Zalcman et al., 1994, 1998). In the present investigation, continuous administration of IL-2 for 7 days had no effect on DA utilization within the nucleus accumbens. As well, although acute IL-2 was previously shown to reduce DA utilization in the substantia nigra, in the present investigation DA utilization was increased in this area. However, as reported for acute administration, IL-2 infusion reduced the utilization of DA at the PFC. In addition, IL-2 interacted synergistically with the chronic stressor treatment to raise the concentration of DA in the central amygdala. Given the presumed behavioral role of DA at these brain areas, these findings raise the possibility that chronic administration of the cytokine may influence central processes involved in cognitive appraisal (Kulisevsky, 2000) or rewarding behavior (You et al., 2001; Chevette et al., 2002).

It is tempting to speculate that the cellular origin (T cell derived IL-2 vs macrophage derived IL-1, TNF- α) or the pyrogenic actions of these cytokines may influence the differences in their central actions. However, sufficient data do not exist to

draw definitive conclusions in this respect. Interestingly, it has been reported that the central effects of IL-2 varied in a biphasic manner as a function of the cytokine dosage (Alonso et al., 1993; Lapchak, 1992). Although the cytokine may increase DA activity at low concentrations, at higher doses an inhibitory effect was provoked (Pettito et al., 1997). Thus, endogenous IL-2 may have dual regulatory actions on central neurotransmission, such that it may stimulate DA-mediated behaviors under physiological conditions, but reduce behavioral activity in response to challenges that appreciably increase IL-2 synthesis (e.g. gram positive challenges such as SEB) (Arad et al., 2001). However, given such divergent effects of IL-2, coupled with the limited data regarding continuous infusion of this cytokine, it is unclear where the dose of IL-2 used in the present investigation lies on a dose response curve.

In addition to its effects on monoamines, it appears that IL-2 has dose-dependent effects on other biological processes. For example, a low dose of IL-2 in humans caused a coordinated increase in expression of IL-2 and IFN- γ mRNA, while higher dosages also induced the expression of IL-6 and TNF- α mRNAs (Hladik et al., 1994). As well, when IL-2 was given acutely it not only had biphasic dose response effects with respect to the release of DA (Pettito et al., 1997), but also had similar actions with respect to acetylcholine (Hanisch et al., 1993). In each of these instances there was virtually no information available concerning the impact of chronic infusion of the cytokine. Given the increasing use of IL-2 as an immunotherapeutic agent, it is essential to determine its effects on central neurochemical activity at a range of therapeutic doses administered over sustained periods.

Stressor effects

As indicated earlier, cancer and hepatitis C patients undergoing immunotherapy frequently exhibit profound neuropsychiatric disturbances. Inasmuch as these patients are in a state of considerable distress and uncertainty, the possibility ought to be considered that stressors may exacerbate the effects of IL-2 on central neurochemical functioning and resultant behavioral pathology. Indeed, it has been reported that cytokines act synergistically to influence behavior and monoamine turnover (Brebner et al., 2000), and similarly, synergistic effects have been reported concerning the effects of stressors and cytokines (Anisman & Merali, 1999). Thus, the possibility was considered that chronic cytokine treatment would also interact with the actions of stressors in provoking exaggerated behavioral and neurochemical alterations.

To our knowledge, only one other study has examined the interactive effects of chronic IL-2 and stressors. This study, however, showed that rats given a restraint stressor following 11 days of intraventricular IL-2 treatment, displayed increases of corticosterone and ACTH levels comparable to that of animals that received only the restraint (Hanisch et al., 1994). As chronic central IL-2 infusion induced massive leukocyte infiltration coupled with activation of local glial cells (Hanish et al., 1994), which in itself may affect neuroendocrine functioning, the contribution of the stressor may have been obfuscated. Regardless, these data do not speak to the effects of chronic stressor exposure following chronic systemic IL-2 treatment. Indeed, a stressor paradigm, in which animals are given daily unpredictable stressors for several weeks, may be more

appropriate to model the experiences of cancer patients than that of a single stressor episode.

Commensurate with previous reports demonstrating the suppressive effects of chronic mild stress on consumption of palatable substances (Monleon et al., 1995), the present investigation revealed that the chronic differential stressor regimen reduced chocolate milk consumption irrespective of whether mice had received IL-2 or saline treatment. In effect, the anhedonic-like effects of IL-2 were not exacerbated in mice that had been exposed to the chronic stressor regimen.

In addition to its effects on behavior, the stressor itself, independent of any effect of the cytokine treatment, increased plasma corticosterone and influenced monoamine utilization at several brain regions. Specifically, as previously observed (Shanks et al., 1991), the stressor increased MHPG in the PFC and PVN. In the PFC, it was mainly the chronic stressor that caused the increase of MHPG, as acute stressor exposure did not elevate levels above saline-treated animals. However, the effects of the stressor were not influenced by the cytokine treatment. Likewise, there was no evidence of the stressor and cytokine treatments have synergistic effects with respect to DA changes within the PVN, PFC, or substantia nigra. This outcome was unexpected, as earlier reports from this laboratory indicated that stressors and cytokine treatments synergistically stimulated monoamine release at extrahypothalamic sites (Merali et al., 1997; Song et al., 1999). However, in these studies amines were determined *in vivo* by microdialysis, whereas amines were determined in post mortem tissue in the present investigation. It is well established that analyses of neurotransmitters in post-mortem tissue collected at a single time point may not provide an adequate reflection of the dynamic changes that may be

occurring, particularly in response to stressors. To be sure, post-mortem analyses may be useful in that they permit the determination of proteins in a wide sampling of brain regions; however, the absence of an effect does not necessarily imply that variations of the neurotransmitter are not occurring as a result of the treatment. In contrast, *in vivo* microdialysis is an effective method of determining ongoing amine variations over time following a given treatment. Thus, at this juncture it is tempting to suggest that analyses of the synergistic effects of IL-2 and chronic stressors need to employ such a technique (or voltametry), particularly as the time course of the treatment effects may vary with the cytokine dosage and the chronicity of the stressor or cytokine treatments.

Conclusions and clinical considerations

Chronic IL-2 clearly reduced consumption of chocolate milk, without appreciably provoking signs of illness. Thus, these findings support the proposition that the cytokine was provoking anhedonic-like actions, possibly reflecting depressive-like effects. However, chronic paroxetine treatment did not influence the effects of IL-2, leaving doubt as to the parallel to depression. Moreover, the profile of neurochemical changes elicited by the cytokine was not consistent with the profile one would have expected if the treatment were, in fact, engendering a depressive-like state (e.g., increased HPA activity, coupled with diminished 5-HT activity in the PFC and DA activity within the PFC and nucleus accumbens). Unfortunately, the paucity of available information regarding the dose-dependent effects of acute treatment with IL-2, to say nothing of the absence of information concerning the impact of chronic infusion, makes it difficult to

discern what factors (if any) may have limited the detection of parallels to depressive-like states.

While it has been our contention that stressors exacerbate the actions of cytokines, in the present studies the stressor treatments did not interact with IL-2 to influence the behaviors measured, although the treatments did appear to interact with respect to DA within the central amygdala. However, the chronic stressor paradigm used in the present experiment may not have adequately modeled the sustained psychosocial aspects of the stressor experienced by cancer patients, including the uncertainty and situational ambiguity associated with the illness. Although care was taken to ensure that the stressor schedule was unpredictable, it is still possible that the animals habituated to the stressors. Cancer patients are presumably stressed continuously, and the stressors experiences are likely to be ambiguous and continually changing, with individuals having little control over them. To the extent that mice in the present investigation received only brief stressor experiences on each day, this model may not adequately mirror that of cancer patients. Future studies may require the use of stressors that are more persistent and psychosocial in nature, to more closely mirror the ambiguity and uncertainty of the stressors experienced by cancer patients. Of course, other significant environmental factors faced by cancer patients (e.g. social and economic disruptions) cannot be adequately modeled in animal studies play a role in provoking behavioral, hormonal and monoamine variations. As well as environmental factors, the disease state itself may exacerbate the effects of IL-2 on the central nervous system, a factor that was not considered in the present investigation. Indeed, tumor bearing mice that experienced cachexia displayed pronounced central monoaminergic effects (Uomoto et al., 1998). Thus, future studies

Thus, future studies may benefit from investigation of the impact of stressor and cytokine treatments in tumor bearing mice, allowing for a closer parallel to the human clinical condition. In addition, given to the biphasic nature of some of its effects, it will be beneficial in future studies to use a variety of doses of IL-2. Furthermore, as IL-2 is normally given for periods longer than one week in cancer patients, it may be profitable in future investigations to administer the cytokine over a more extended time period. Such variations may reveal more pronounced changes in monoamine and neuroendocrine activity than those seen in the present investigation.

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