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Inflammatory processes and depressive-like behavior in a syngeneic model of ovarian cancer

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INFLAMMATORY PROCESSES AND DEPRESSIVE-LIKE BEHAVIOR IN A SYNGENEIC MODEL OF OVARIAN CANCER

by

Donald Michael Lamkin

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Psychology
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Thesis Supervisors: Professor Susan K. Lutgendorf
Professor Alan Kim Johnson

ABSTRACT

Considerable data demonstrate a high prevalence of depression symptoms in patients with cancer, with some studies showing the prevalence for major depressive disorder (MDD) to be as high as 50%. Because depression researchers have found that a significant relationship exists between depression symptoms and indices of systemic inflammation and because several cancer types exploit the mechanisms of the body's inflammatory response to aid in their own progression, it was hypothesized that tumor in the body could be a cause of depression symptoms in cancer patients. Examination of this question was conducted using an immunocompetent mouse model of ovarian cancer and several measures of depressive-like and sickness behavior. Initial investigation of the model (Chapter 2) involved a series of pilot experiments that addressed methodology and demonstrated that ID8 murine ovarian carcinoma was capable of inducing elevated levels of systemic IL-6 and depressive-like behavior, specifically anhedonia as measured by a decrease in sucrose solution. In Chapter 3, a larger experiment (Experiment 1) was conducted that examined the effect of ovarian tumor on sucrose intake, food intake, body weight, locomotion, and rotarod performance. Results in the study indicated that sucrose-measured anhedonia in the model was not confounded by anorexia because tumor-bearing mice and control mice exhibited no significant difference in appetite. In Chapter 4, a second experimental factor, social housing, was added alongside tumor condition, and a second measure of depressive-like behavior, tail suspension test (TST) immobility, was added to measures from the previous experiment. The results of this second large experiment (Experiment 2) demonstrated that ovarian tumor had no significant effect on TST immobility, even though it did cause mice to exhibit less motor activity in the home cage. Housing condition did affect TST immobility. Mice that were individually-housed exhibited significantly more TST immobility than group-housed mice. Also, individually-housed mice exhibited less sucrose intake than group-housed

mice. This gave rise to a significant interaction in sucrose preference among the four experimental groups where individually-housed tumor-bearing mice showed less sucrose preference than the other groups. In Chapter 5, systemic proinflammatory and antiinflammatory cytokines from both Experiment 1 and Experiment 2 were examined. Results indicated that both proinflammatory and antiinflammatory cytokines were significantly higher in tumor-bearing mice than in control mice, and these effects were largest for interleukin-6 (IL-6) and IL-10. Among tumor-bearing mice, significant correlations between IL-1β, IL-6, tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF- β) and locomotion were noted, but there was no significant correlation between cytokines and anhedonia. No significant effect of housing condition on cytokines was found. In Chapter 6, principal findings of the project are summarized and discussed with a focus on anhedonia and psychomotor slowing in MDD. Current evidence suggests that dopaminergic and glutamatergic systems in the brain may underlie anhedonic and psychomotor features in inflammation-induced depression. Thus, future investigation of the mediators between ovarian tumor and these depressive-like behaviors in the model may benefit from targeting these specific neural mechanisms.

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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LIST OF ABBREVIATIONS

APA: American Psychiatric Association

BBB: blood-brain barrier

cAMP: cyclic adenosine monophosphate

CES-D: Center for Epidemiological Studies-Depression Scale

CMS: chronic mild stress

COMT: catechol-o-methyl transferase

CRH: corticotropin-releasing hormone

CRP: C-reactive protein

CTLs: cytotoxic T-lymphocytes

DMBA: 7,12-dimethylbenz[a]anthracene

DMEM: Dulbecco's Modified Eagle Medium

DSM-I: Diagnostic and Statistical Manual of Mental Disorders, 1st Edition

DSM-III: Diagnostic and Statistical Manual of Mental Disorders, 3rd Edition

DSM-IV: Diagnostic and Statistical Manual of Mental Disorders, 4th Edition

ECM: extracellular matrix

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

FDA: Food and Drug Administration

FGFs: fibroblast growth factors

FST: forced swim test

HADS: Hospital Anxiety and Depression Scale

HAM-D: Hamilton Rating Scale for Depression

HPA: hypothalamic pituitary adrenal

HVA: homovanillic acid

ICSS: intracranial self stimulation

IFN-α: interferon alpha

IFN-β: interferon beta

IFN-γ: interferon gamma

IL-1: interleukin-1

IL-4: interleukin-4

IL-6: interleukin-6

IL-10: interleukin-10

IL-17: interleukin-17

IDO: indoleamine 2,3 dioxygenase

i.p.: intraperitoneal

LPS: lipopolysaccharide

MADRS: Montgomery-Asberg Depressive Rating Scale

MAO: monoamine oxidase

MAOI: monoamine oxidase inhibitor

MDD: major depressive disorder

MMP: matrix metalloproteinase

MMP-9: matrix metalloproteinase 9

NF-κB: nuclear factor-kappa B

NK: natural killer

NMDA: N-methyl-D-aspartic acid

PDGF: platelet derived growth factor

POMS: Profile of Mood States

PMA: phorbol-12-myristate-13-acetate

SEM: standard error of the mean

SQRT: square root transformed

SSRI: serotonin reuptake inhibitor

Std diff: standardized difference in mean

TGF-β: transforming growth factor beta

TNF-α: tumor necrosis factor alpha

TST: tail suspension test

VEGF: vascular endothelial growth factor

VTA: ventral tegmental area

CHAPTER 1

GENERAL INTRODUCTION

Depression

Considerable data demonstrate a high prevalence of depression symptoms in patients with cancer.[1] Some studies have shown that the prevalence for depression symptoms in cancer patients can range as high as 50%.[2] A recent review of the literature estimates the median prevalence for major depressive disorder (MDD) in cancer patients at 15% and the median prevalence for all depressive disorders at 30%.[3] These rates are substantially higher than that found in the general population for MDD, which ranges from 2-4%.[4]

Classifying the Disorder

While transient feelings of sadness are intrinsic to the human condition, occasionally these feelings may become more intense and/or persist for a sustained period of time. In such cases, if the phenomenon begins to interfere with normal functioning, then it may be termed a *mood disorder.* According to the current fourth edition *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) of the American Psychiatric Association (APA), the subcategory of mood disorders known as *depressive disorders* are defined, in part, by the signs and symptoms of a *major depressive episode*, presented in Table 1-1.[5] Of the nine signs and symptoms that may constitute a major depressive episode, at least five have to be present in a given individual for at least two weeks to qualify the episode as such, and at least one of the five has to be criterion 1 or 2. Persons who suffer one or more such episodes are deemed to have MDD. Persons who do not experience a full major depressive episode but experience a chronic depressed mood and/or some of the other features that define a major depressive episode may be diagnosed with *dysthymic disorder, depressive disorder not otherwise specified,* or *adjustment disorder with depressed mood.*

Table 1-1. Criteria for a major depressive episode

1. depressed mood most of the day, nearly every day
2. markedly diminished interest or pleasure in activities most of day, nearly every day
3. significant weight change and/or significant change in appetite, nearly every day
4. insomnia or hypersomnia, nearly every day
5. psychomotor retardation or agitation, nearly every day
6. fatigue or loss of energy, nearly every day
7. feelings of worthlessness or excessive or inappropriate guilt, nearly every day
8. diminished ability to think or concentrate, or indecisiveness, nearly every day
9. recurrent thoughts of death or suicidal ideation or suicidal attempt

Neurological Correlates of Depression

In the 1950s, it was discovered that the hypertension drug, reserpine, could cause severe depression in some users.[6,7] Around the same time, it was learned that the drug, iproniazid (originally tested for treating tuberculosis), provided relief to patients with depression and seemed to make mentally normal patients happier.[8,9,10] These accidental discoveries, along with the discovery that the drug, imipramine, was effective at alleviating depressive symptoms in schizophrenic patients, gave rise to what is known today as the *monoamine hypothesis of affective disorders.*[11] Synaptic levels of monoamine neurotransmitters, i.e., dopamine, norepinephrine, and serotonin, are affected by the drugs reserpine, iproniazid, and imipramine. Reserpine prevents the intracellular packaging of these neurotransmitters into vesicles for eventual release into the synaptic cleft, leaving the neurotransmitters in the cytoplasm of the neuron where the enzyme, monoamine oxidase (MAO), degrades them. The result is lower levels of monoamines in neuronal synapses. Conversely, iproniazid is a monoamine oxidase inhibitor (MAOI). Thus, it allows for more vesicles to be packaged for eventual release into the synaptic

cleft, thereby raising levels of all the monoamines. Imipramine, categorized as a tricyclic antidepressant because of its molecular shape, binds to presynaptic transporter proteins, blocking the reuptake of neurotransmitters (particularly, norepinephrine and serotonin), which also then results in raising the levels of the neurotransmitters in the synaptic cleft.

Although selective serotonin reuptake inhibitors (SSRIs) have become the most predominantly prescribed antidepressant in recent years, many depression researchers today conclude that no single monoamine neurotransmitter appears to be more important than the other.[12] There is increasing evidence of anatomical and functional interaction between the noradrenergic system emanating from the locus coerulus and the serotonergic system of the raphe nuclei that suggests the two systems are capable of regulating each other.[11] Even more recently, depression research has come to focus on the glutamatergic system.[13,14] Abnormal glutamate levels have been found in multiple brain regions of persons with MDD.[15,16] Further investigation into this area of research has found that some of the genes responsible for glutamate clearance from the synaptic cleft for recycling are downregulated in persons with depression.[17]

Inflammation and Depression

An early debate over the classification of depression centered on an etiological distinction between *reactive depression* and *endogenous depression*, a distinction that continues to generate discussion today.[18,19] A depression may be considered reactive when it follows a stressful event in one's life (e.g., death of a spouse). Conversely, endogenous depression may be thought of as due to one or more internal factors that ultimately impact the neurological brain systems underlying mood.

It is in this context of an endogenous etiological perspective that some depression researchers have looked outside the brain but still inside the body for answers to what may be the cause, *or at least a cause,* of depression. One such cause may be chronic systemic inflammation.

The Nature of Inflammation

The inflammatory response of the immune system is a reaction to pathogen and/or tissue injury.[20] It involves a rather complex set of mediators that together orchestrate the full response. Under normal conditions, the response successfully functions to eliminate any pathogen that has gained entry to the body and/or to repair damage to the body's tissues.

Within minutes after an incident of tissue injury, at least five plasma protein systems are activated to address the injury.[20] First, perturbed phospholipids on damaged cellular membrane give rise to prostaglandins and thromboxanes, the latter causing platelet aggregation at the site of injury. The kinin system gives rise to the vasodilator bradykinin. The clotting system gives rise to fibrin that works with platelets to form clots for containing any pathogen that enters the body and stanching continued bleeding from ruptured capillaries. The fibrinolytic system acts upon clotting elements to produce chemotactic proteins that facilitate the arrival of neutrophils and other circulating immune cells to the site of injury. The complement system also provides chemotactic proteins for circulating immune cells and causes local tissue mast cells to degranulate histamine, which in turn potently causes vascular permeability. The coordinated action of these immediate plasma systems quickly gives rise to the five classical hallmarks of a local inflammatory response: *tumor* (swelling), *rubor* (redness), *calor* (heat), *dolor* (pain), and *functio laesa* (loss of function).

In addition to the chemotactic proteins mentioned above, other chemokines and the proinflammatory cytokines interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α) may be secreted by local tissue macrophages that become activated upon recognition of pathogens entering the site of injury.[20] Together, these molecules facilitate the activation and homing of several other immune cell types to the local area to stop the invasion of pathogen. First among these to arrive are neutrophils, followed by additional monocytes that differentiate into additional local tissue macrophages. An

influx of other granulocytes (i.e., eosinophils, basophils, mast cells) and lymphocytes also respond to the presence of proinflammatory cytokines and chemokines emanating from the local area.

A local acute inflammatory response is often accompanied by a systemic response, which may include fever, increased production of white blood cells, and production of a large number of acute phase proteins by the liver.[20] These events are caused by the action of IL-1, IL-6, and TNF-α emanating from the site of injury. These cytokines act on the hypothalamus to induce fever, which makes the body less amenable to many types of pathogen. The action of these cytokines on the hypothalamus also triggers the hypothalamic pituitary adrenal (HPA) axis which in turn generates immunosuppressive cortisol for regulation of the inflammatory response. IL-6 and TNF-α act upon vascular endothelial cells and macrophages to induce production of colony stimulating factors, which in turn upregulate the production of white blood cells in the bone marrow for fighting the pathogen. All three of the cytokines also cause the liver to upregulate production of positive acute phase proteins such as C-reactive protein (CRP) and serum amyloid A, which among other things, participate in the removal of damaged tissue cells and breakdown of the elaborate structure of proteins that exists between cells in tissue, a structure known as the extracellular matrix (ECM). These activities help to rebuild new tissue at the site of injury.

Not Always a Good Thing?

While the activities of IL-1, IL-6, and TNF-α presented above are essential to the immune system and, thus, a person's survival, depression researchers have steadily been taking note of a relationship between such indices of systemic inflammation and the presence of depression symptoms. In 1991, Roger S. Smith was the first investigator to articulate a full synthesis of the initial findings surrounding systemic inflammation and depression that suggested chronic systemic inflammation may indeed be a cause of

MDD, a hypothesis he termed the *macrophage theory of depression*.[21] Smith pointed to the research that showed the prototypical macrophage cytokine, TNF-α, when administered to cancer patients, caused many of the features found in depression, including malaise, fatigue, anorexia, and myalgia.[22] He also noted epidemiological research that showed a correlation between MDD and medical conditions marked by elevated levels of systemic inflammation, such as cardiovascular disease and rheumatoid arthritis.[23,24] The finding that populations that consume higher amounts of fish oil, with its macrophage suppressing effect, exhibit both a lower prevalence of depression and these inflammatory medical conditions were also evidence that chronic systemic inflammation may cause depression.[25,26,27]

Around the same time, psychiatrist Michael Maes began reporting in a series of papers his findings that showed a positive relationship between depression and positive acute phase proteins, all of which culminated with his conclusion that the inflammatory response was a contributor to MDD.[28] However, not all investigators found a connection, and other studies came out that contradicted the observations made by Maes.[29]

The hypothesis that higher levels of systemic inflammation, as indicated by elevated levels of IL-6, IL-1, and CRP, are positively correlated with the presence of MDD and/or depressive symptoms in general was recently tested in a study that collected all available data on this relationship in the English language and meta-analyzed it. A total of 127 data reports on depression and systemic IL-1, IL-6, and CRP were analyzed and are presented in Figures 1-1, 1-2, and 1-3, respectively.[30] All three inflammatory mediators were found to significantly correlate with depression. The size of the effect, while reliable in both clinical samples with a diagnosis of MDD and community-based samples completing self-report measures of depression symptoms, was larger in clinical samples with MDD.

Study name	Std diff in means	Lower limit	Upper limit	p-Value
Brambilla & Maggioni, 1998 (94)	-0.109	-0.986	0.768	0.807
Eskandari et al., 2007 (101)	1.795	0.958	2.631	0.000
Ferketich et al., 2005 (102)	-0.081	-0.809	0.648	0.828
Hekler et al., 2007 (108)	0.226	-0.349	0.802	0.441
Huang & Lee 2007 (111)	0.241	-0.194	0.675	0.277
Kagaya et al., 2001 (118)	-0.678	-1.501	0.145	0.106
Leo et al., 2006 (128)	0.985	0.552	1.418	0.000
Levine et al., 1999 (39)	1.870	0.173	3.567	0.031
Lyness et al., 2001 (134)	0.442	-0.247	1.130	0.209
Miller et al., 2002 (59)	0.576	0.162	0.990	0.006
Moorman et al., 2007 (143)	-0.197	-0.556	0.163	0.284
Owen et al., 2001 (146)	0.768	0.126	1.410	0.019
Thomas et al., 2005 (166)	0.882	0.232	1.532	0.008
Yang et al., 2007 (172)	-0.010	-0.542	0.522	0.971

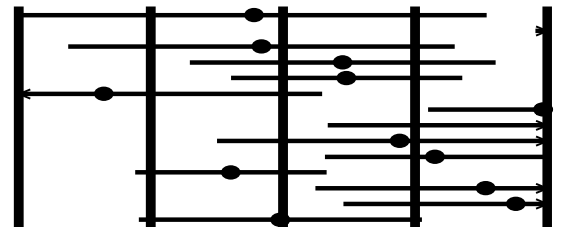

Figure 1-1. Forest plot of individual reports on the relationship between IL-1 and depression. Values to the right of the zero line for standardized difference in mean (Std diff) indicate a positive correlation between systemic levels of IL-1 and depression. Aggregate effect size was *Std diff* = .35, $P < .05$.

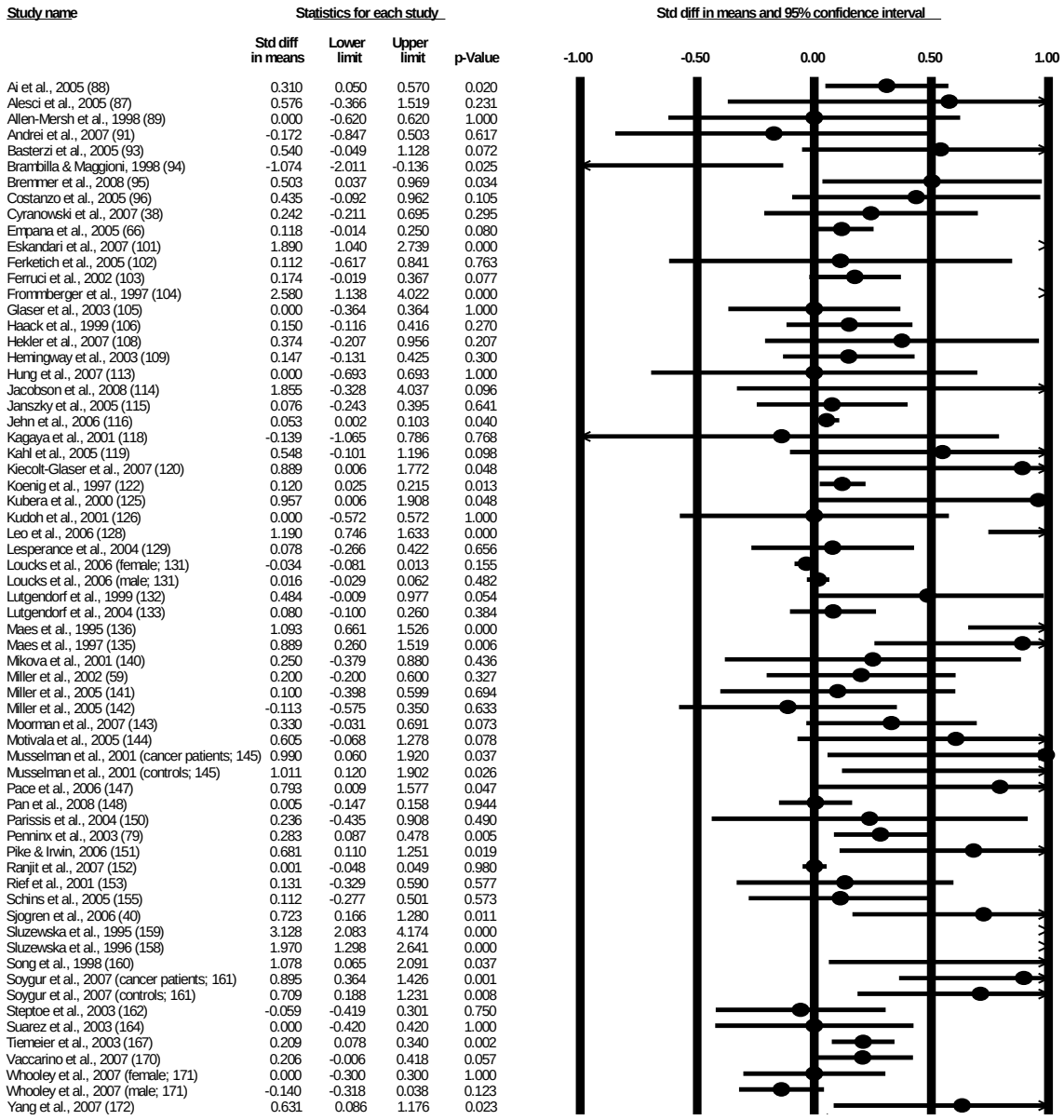

Figure 1-2. Forest plot of individual reports on the relationship between IL-6 and depression. Values to the right of the zero line for standardized difference in mean (Std diff) indicate a positive correlation between systemic levels of IL-6 and depression. Aggregate effect size was *Std diff* = .25, $P < .001$.

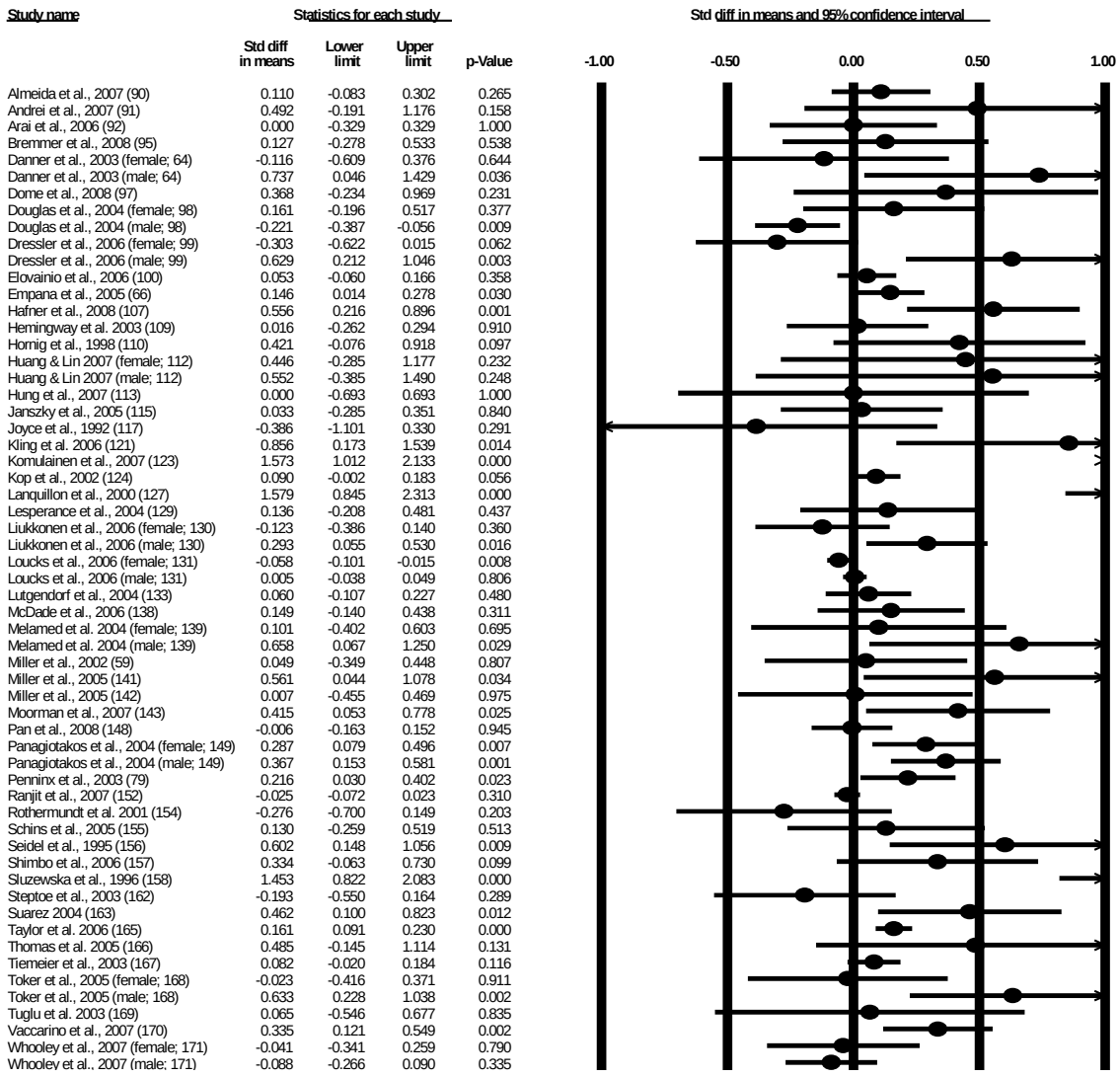

Figure 1-3. Forest plot of individual reports on the relationship between CRP and depression. Values to the right of the zero line for standardized difference in mean (Std diff) indicate a positive correlation between systemic levels of CRP and depression. Aggregate effect size was *Std diff* = .22, *P* < .001.

Inflammation, Sickness Behavior, and Depression

A New Antiviral Drug

In 1991, the Food and Drug Administration (FDA) approved a drug, marketed as Intron A®, for the treatment of hepatitis C. The drug is a recombinant form of the proinflammatory cytokine, interferon alpha (IFN-α), one of the three types of interferons (the others being interferon beta [IFN-β] and interferon gamma [IFN-γ]) named as such because of their ability to *interfere* with viral replication.[31] While the drug provided a welcomed increase in success rates for controlling hepatitis C virus, it also became notorious for inducing fatigue in almost all patients and severe depression in some.[29]

Andrew H. Miller and fellow researchers at Emory University have studied this phenomenon in great detail. They have found that 30-50% of patients on cytokine therapies develop a full complement of the criteria used to make a diagnosis of MDD.[32] In addition to these criteria, Table 1-2 presents other signs and symptoms they have measured in patients undergoing IFN-α treatment.[33] This research group published a landmark double-blind controlled trial in 2001 that showed they could significantly attenuate the development of MDD in patients undergoing IFN-α treatment by administering the SSRI, paroxetine, two weeks before beginning IFN-α and continuing the antidepressant for the duration of the 12-week IFN-α therapy.[34] Interestingly, however, the antidepressant was not able to attenuate significant increases in two of the neurovegetative symptoms listed in Table 1-2. These symptoms were fatigue and loss of appetite.[33] Furthermore, when examining the temporal occurrence of the symptoms in just the placebo group, the researchers found that the neurovegetative and somatic symptoms occurred early during treatment while the depressive, anxious, and cognitive symptoms occurred later. Both of these results led the investigators to conclude that the effects of proinflammatory cytokine treatment on different symptom dimensions are mediated by different mechanisms.

Table 1-2. Percentage of patients experiencing moderate to severe intensity of the listed symptoms during IFN-α therapy

	%
Depressive Symptoms	
Depressed mood	60
Anhedonia	30
Suicidal Thoughts	10
Feelings of Guilt	5
Anxious Symptoms	
Tension/irritability	50
Anxious mood	45
Fear	15
Cognitive Symptoms	
Loss of concentration	30
Memory disturbances	15
Word-finding problems	15
Episodes of confusion	10
Indecisiveness	10
Neurovegetative Symptoms	
Fatigue/loss of energy	80
Abnormal sleep	45
Psychomotor retardation	40
Abnormal appetite	35
Somatic Symptoms	
Pain	55
Gastrointestinal symptoms	50

Sickness Behavior and Depressive-like Behavior

Investigation into the mechanisms that may underlie inflammation-induced changes in depressive mood or other behaviors has been extensively carried out in the sickness behavior area of research. Following the first definitive review on the behavior of sick animals as an evolved adaptive response,[35] the term *sickness behavior* was introduced by Kent, Bluthe, Kelley, and Dantzer to define the specific behaviors that

accompany infection and subsequent inflammation.[36] These behaviors include decreases in food intake, water intake, locomotion, grooming, social interaction, and increased sleep. The agents used to induce a peripheral inflammatory response have typically been lipopolysaccharide (LPS), which potently stimulates production of IL-1, IL-6, TNF-α, and IFN-γ, or injection of the individual recombinant proinflammatory cytokines produced by LPS. Of the individual proinflammatory cytokines studied in this literature, Adrian Dunn and colleagues conclude that IL-1 is the most potent inducer of sickness behavior.[37] However, they also note that while IL-1 alone is sufficient to induce sickness behavior, it is not necessary. This is because LPS has been shown to cause sickness behavior in IL-1-knockout mice.[38] Similarly, neither IL-6 nor TNF-α are necessary, as LPS has been shown to cause sickness behavior in IL-6-knockout mice and TNF double receptor knockout mice.[39,40] Thus, the pleiotropic nature of proinflammatory cytokine signaling appears to give rise to a complex redundancy in the mediation of inflammation-induced sickness behavior.

Experiments in this literature have shown that induction of an acute inflammatory response in the periphery with LPS affects not only sickness behaviors but other behaviors that may be deemed representative of depression in humans.[41] Such behaviors, referred to as *anhedonia* and *behavioral despair*, have been developed in animal models of depression since the 1950s.[42] For example, in 1997 Raz Yirmiya reported that chronic pre-treatment with the tricyclic antidepressant, imipramine, attenuated not only the LPS-induced sickness behaviors of decreased food intake, locomotion, and social interaction in rats but also attenuated a decreased preference for a sweet solution, which is a measure of anhedonia.[43] However, additional sickness behavior experiments in rats and mice have not always been as successful. For example, chronic pre-treatment in rats with desipramine (another tricyclic) failed to block an LPS-induced decrease in saccharin consumption.[44] Paroxetine and venlafaxine were also used to block the decrease in saccharin consumption in this same study, but they too were

ineffective. Likewise, a study in mice showed that chronic pre-treatment with venlafaxine and imipramine failed to block an inflammation-induced (both LPS and IL-1β) decrease in sweetened milk intake.[45] In regard to behavioral despair experiments, while there are several very recent studies that have appeared showing that LPS causes an increase in behavioral despair, as measured by tail suspension test (TST) immobility,[46,47,48,49] these studies have not reported on antidepressant usage in the model.

Investigators in this area of research have proposed multiple pathways between the periphery and the brain by which proinflammatory cytokines may operate and induce sickness behaviors generally and/or depressive-like behavior specifically. Some have suggested the existence of active transport mechanisms specific to the cytokines that cross the blood-brain barrier (BBB)[50] or cytokine receptors on the interior wall of blood vessels in the brain that activate secondary messengers that then enter the brain.[51] Others have suggested that cytokines enter the brain at regions where the BBB is weak.[52] Such humoral routes are believed to play a complementary role to neuronal routes,[53] because it has been shown that dense binding sites for IL-1 exist on paraganglia that surround and synapse onto terminals of afferent vagus nerve fibers.[54]

Research into the brain mechanisms that mediate the effect of peripheral inflammation on behavior has also pursued multiple probable avenues. One study found that peripheral inflammation caused a significant increase in synaptic dopamine levels in the nucleus accumbens,[55] which resembles findings in the chronic mild stress (CMS) model of anhedonic depression.[56] A second study found that syntaxin levels in synapses of the nucleus accumbens were decreased by peripheral inflammation in parallel with decreased responding for reward.[57] Investigators in this latter study noted previous research that suggests syntaxin levels are associated with glutamate release,[58] which in turn could affect synaptic dopamine levels.[59] A role for altered glutamatergic function within inflammation-induced depressive-like behavior has recently been investigated in

the TST. Such research has been motivated in part by the finding that levels of indoleamine 2,3 dioxygenase (IDO), an enzyme that metabolizes a precursor of serotonin, tryptophan, are increased by peripheral inflammation, reaching their peak around 24 hours after injection of LPS.[60] However, further investigation in this line of research suggests that serotonin function in the brain is not altered at this time of peak IDO, but the level of kynurenine, a metabolite of tryptophan, is increased.[46] Investigators in this area of research have noted that two metabolites of kynurenine, namely, quinolinic acid and kynurenic acid, will agonize and antagonize glutamatergic N-methyl-D-aspartic acid (NMDA) receptors, respectively.[61] Thus, such findings point to the newly growing research noted above that the glutamatergic system of the brain plays a role in depression. Such findings also offer some of the first experimental evidence that this particular brain system may be substantially altered by systemic inflammation.

Inflammation and Depression in the Context of Cancer

Reactive vs. Endogenous?

Receiving a diagnosis of cancer may result in a cascade of responses such as shock, disbelief, anxiety, anger, and despair that could persist and develop into MDD.[2] Psychosocial factors in cancer patients that may increase the risk for developing MDD after hearing a cancer diagnosis include socioeconomic pressures, pessimistic personality traits, and social isolation.[62] Social isolation, in particular, is a significant risk factor for MDD in the population at large. Previous research has shown that persons with MDD are more likely to report fewer social interactions over the course of the day and to experience less intimacy in those interactions.[63] Another series of studies in women showed that those who lacked a close confidante or had no outside employment were more likely than other women to become depressed after experiencing a major stressful life event.[64,65]

The occurrence of social isolation and/or the occurrence of a stressful life event like a cancer diagnosis may lead one to conclude that cancer patients who develop MDD are manifesting a reactive depression as described above. However, if the presence of cancer in the body is generating a systemic inflammatory response, then there is also reason to believe that the signs and symptoms constituting MDD in the patient are a result of the cancer itself.

In their review of inflammation and depression in cancer patients, Raison and Miller touch upon this distinction in MDD etiology and argue for an "inclusive approach" to screening for MDD in cancer patients and other medically ill patient populations.[1] This argument comes on the heels of an ongoing debate among physicians as to whether or not symptoms shared by an illness and a major depressive episode should be counted toward a diagnosis of MDD. An often referenced compromise to the question has been offered by Cole-Cohen et al.[66] Their suggestion is to use two systems to diagnose MDD in medically ill patients. For clinical purposes, an inclusive approach should be used, which follows the normal criteria procedure in the DSM-IV, because it best protects the patient from risk of undiagnosed MDD. For research purposes, an exclusive approach should be used, which eliminates anorexia and fatigue from the criteria list for a major depressive episode but requires a total of only four of the other criteria to make an MDD diagnosis (depressed mood or anhedonia still being at least one of them).

The exclusive approach aims to achieve specificity but, in the process, makes a dualistic assumption about depression etiology, i.e., that symptoms of depression can only be considered "depression" if they are caused by "depression" and not by some physical pathology outside the brain. However, the forgoing research on cytokine therapy clearly shows that depressive symptoms can be part of a larger sickness syndrome that includes neurovegetative and somatic symptoms--all of which result from elevated levels of systemic inflammation throughout the body.[1] Thus, systemic

inflammation generated by the tumor itself, as is described more fully below, may indeed cause depressive symptoms as part of a larger sickness syndrome.

The Wound That Does Not Heal

In their landmark paper in 2000, Hanahan & Weinberg concluded that a cell needs to acquire six novel capabilities in order to become malignant: (1) autonomous self-signaling for cell proliferation, (2) insensitivity to antigrowth signals, (3) insensitivity to signals for self-termination (known as apoptosis), (4) removal of the normal limit of lifetime cell divisions (known as immortalization), (5) ability to build new blood vessels into itself as a growing tumor mass for nourishment (known as angiogenesis), and (6) ability to invade and metastasize to other tissue.[67] These capabilities are acquired, in part, through changes in the cell's genome. Oncogenes, genes that facilitate these capabilities, become more fully expressed as a result of genome alteration. Tumor suppressor genes, genes that inhibit these capabilities, are expressed less or not at all as a result of genome alteration.

In cancer biology, a genomic alteration has traditionally been understood to occur as a result of exposure to an initiating agent (initiator). For example, a single administration to the skin of the initiator, 7,12-dimethylbenz[a]anthracene (DMBA), a constituent element of coal tar, is known to have no effect on the induction of skin cancer, even after 1 year of observation. This is despite the fact that DMBA is a randomly acting mutagen that causes a variety of genomic alterations in the cells that it comes into contact with. Thus, by itself it is very poor at causing the cell that it has genetically altered to grow into a clinically detectable tumor. However, other promoting agents (promoters) may do a much more efficient job of growing a now cancerous cell into a detectable mass. If a separate agent known as phorbol-12-myristate-13-acetate (PMA) is administered to a cell that DMBA has genetically altered, then papillomas emerge at 4-8 weeks. Thus, PMA acts as a promoting agent for DMBA-altered skin

cells. In time, after the tumor has grown and evolved, it may no longer need the promoter, continuing to grow and progress on its own. In the case of DMBA-PMA-induced skin cancer, new papillomas will regress without repeated administration of PMA. However, after several weeks of PMA administration, the papillomas become independent of the PMA, and some evolve into malignant squamous cell carcinoma.[68]

While much knowledge has been gained over the past 35 years regarding the genomic determinants of neoplastic transformation of normal cells, more recent research findings make it clear that non-neoplastic cells residing in human tumors, notably the stromal cells (i.e., immune cells and fibroblasts), are active essential collaborators with neoplastic epithelial cells in driving tumor progression.[68] These findings stem from the seminal work of physician, Harold F. Dvorak. In 1986, working in the department of pathology at Beth Israel Hospital in Boston, Dvorak published a paper that described striking similarities in the processes that govern cancer progression and those that govern wound healing.[69] These similarities led him to conclude that tumors are like wounds that fail to heal.

As initially described above, there are several mechanisms at work during the inflammatory process that aim to repair damaged tissue in the course of normal wound healing. One of the first of these is the aggregation of platelets at the site of injury. Two important molecules secreted by platelets at this time are platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-β).[68] These molecules attract and activate fibroblasts, which in turn secrete a group of enzymes known as matrix metalloproteinases (MMPs). Once released, the MMPs help break down the ECM, which as noted above, is the structure of proteins that exists between cells in tissue. This allows for structural remodeling of the ECM to facilitate removal of damaged cells and the arrival of new ones. It also releases a variety of other molecules that are tethered to the ECM, which in concert with the other chemotactic proteins and cytokines listed above, attract neutrophils, macrophages, and other immune cells to the site of injury. At the

same time that these immune cells participate in destroying any invasive pathogens and removing cellular debris, they also contribute factors that aid in the rebuilding of vasculature in the area. Such factors include fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF). In parallel with these processes occurring in the inner stroma section of a tissue, undamaged epithelial cells near the surface of the tissue, and on the perimeter of the injury site, become motile and move in to replace damaged surface epithelial cells and complete the wound healing process.

Research is now demonstrating that cancer cells in the body seem to exploit the mechanisms of this inflammatory response in normal wound healing to aid in their own progression.[68,70,71] Many kinds of cancer cells continuously release substantial levels of PDGF, including ovarian cancer,[72] which as noted above participates in turning on the inflammatory response with all of its downstream sequelae. The recruitment and activation of immune cells and fibroblasts into the cancer cell-adjacent stroma allows for the efficient breakdown of the ECM surrounding the tumor by MMPs, replacement of normal epithelial cells with multiplying cancerous cells, and angiogenesis into the growing mass by VEGF and other factors.

Macrophages have been found to be the main source of an active MMP in ovarian cancer known as matrix metalloproteinase 9 (MMP-9).[73,74,75,76] Macrophages have also been found to be one of the stromal cells that produce VEGF.[77] Together, these inflammatory mediators promote tumor invasion and metastasis.

As mentioned above, the number of mediators that constitute the inflammatory response is large, and their orchestration is complex. However, as presented in Figure 1-4,[71] several investigators view the single transcription factor, nuclear factor-kappa B (NF-κB), in both carcinoma and stromal cells of the tumor microenvironment, as the lynchpin for this complex process in cancer progression.[78] This is because NF-κB transcribes the genes for MMP-9, VEGF, TNF-α, IL-1, IL-6, and several other inflammatory mediators.[71]

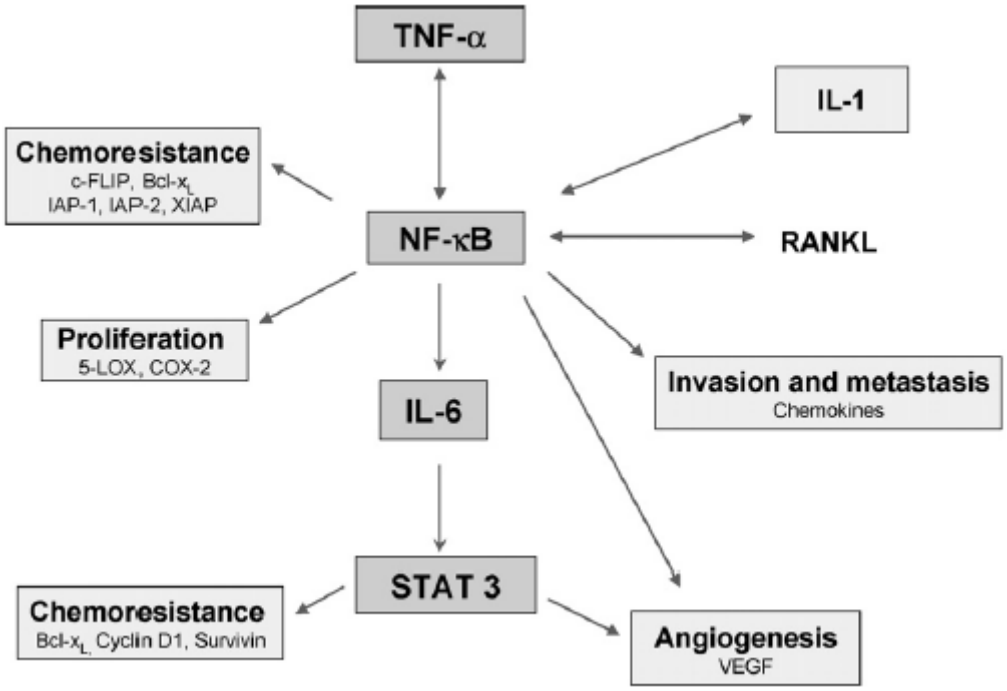

Figure 1-4. Inflammatory networking in the tumor microenvironment where NF-κB regulates the expression of proinflammatory cytokines and several other inflammatory response molecules that are integral to tumor progression.

While NF-κB has been found to be constitutively active in most tumor cell lines, TNF-α, be it from stromal macrophages inside the tumor microenvironment or the tumor cells themselves, potently upregulates activation of NF-κB.[79,80] Not surprisingly, clinical trials are investigating the use of anti-TNF therapies as an adjunct treatment in malignancy.[81] A trial that supplemented standard chemotherapy with etanercept, a recombinant human TNF-α receptor that binds TNF-α and renders it inactive, reported anti-tumor effects exclusively in the group randomized to receive etanercept.[82]

Inflammatory Mediators and Depression in Cancer Patients

As mentioned above, the stressful life event of being diagnosed with cancer may lead one to conclude that patients who develop MDD are manifesting a reactive depression. However, the inflammatory response that is generated by tumor gives reason to believe that the signs and symptoms constituting MDD in a cancer patient could be due, at least in part, to the cancer itself.

In this latter regard, inflammatory mediators within the patient's tumor microenvironment have been found to be positively correlated with symptoms of depression. One report showed that depressed ovarian cancer patients have significantly higher levels of MMP-9 in the tumor microenvironment than non-depressed patients.[83] Another study of ovarian cancer reported that IL-6 levels in the *ascites*, i.e. the malignant effusion that often forms around the tumor, were positively correlated with scores on the vegetative symptom subscale of the Center for Epidemiological Studies-Depression Scale (CES-D), although other facets of depression (e.g., affective symptoms) were not significantly correlated with ascites IL-6.[84] This finding is similar to the report noted above that neurovegetative and somatic symptoms are more pronounced than depressive, anxious, and cognitive symptoms during chronic IFN-α therapy-induced inflammation.[33,34]

Other cancer researchers have focused on depression and IL-6 in patients' systemic circulation. The focus on systemic IL-6 in these human studies is probably due to the fact that this cytokine is expressed to a relatively greater degree than TNF-α or IL-1 in humans, making it a more measurable biomarker of the inflammatory response.[85] To date, eight studies have reported on such relationships. For example, Musselman and colleagues first reported in 2001 that cancer patients (pancreatic, esophageal, or breast cancer) with a diagnosis of MDD had significantly higher levels of IL-6 than cancer patients without MDD.[86] Since that time two other research groups have also found that

cancer patients with a diagnosis of MDD had significantly higher levels of IL-6 than cancer patients without comorbid MDD.[87,88] In contrast, one study found that the total depression score on the CES-D was not significantly correlated with systemic IL-6 but that only the vegetative symptom subscale was related.[84] Also, four other studies examining IL-6 in relation to other self-report depression measures (e.g., Hospital Anxiety and Depression Scale [HADS], Hamilton Rating Scale for Depression [HAM-D], Profile of Mood States [POMS] Depression Scale) have reported either marginal or non-significant findings.[89,90,91,92]

Ovarian Carcinoma: In and of Itself a Cause of Depression?

The forgoing exposition of this chapter has presented evidence that indicates the human body's inflammatory response is capable of inducing the signs and symptoms found in depressive disorders. The evidence, however, is necessarily only correlative in studies that examine disease-associated inflammation and depression in cancer patients. Evidence of a causal relationship between cancer-generated inflammation and depression would be more compelling. Such evidence would also lend valuable information to the inclusive/exclusive debate over how to diagnose MDD in cancer patients, because it would suggest that manifestation of depression in cancer patients is not necessarily just a reaction to the stress of one's diagnosis. Strategies on how to treat MDD in cancer patients would also be better informed.

Limitations inherent to clinical research with human cancer patients preclude rigorous experimental investigation of this question. To test for a causal relationship between tumor and depression, an animal model of cancer-induced depression is needed. Recently, investigators used such a model to examine the effect of carcinogen-induced mammary tumors on the depressive-like behaviors noted above and found that mammary tumors caused increases in both anhedonia and behavioral despair in rats.[93] The

investigators concluded that cautious extrapolation of the results suggests tumors alone are sufficient to trigger changes in emotion in cancer patients.

Given those promising results in an animal model of breast cancer using rats, it was decided that a mouse model of ovarian cancer-induced depression should be used to test for an effect of ovarian tumor on measures of depressive-like behavior. There are several mouse models of ovarian cancer that are used by cancer biologists to examine the processes that govern tumor growth, invasion, and metastasis.[94] While many of these models use an immunocompromised mouse (e.g., the athymic nude mouse) to facilitate successful xenografting of human ovarian cancer cell lines, this type of mouse was deemed less suitable for studying inflammation-induced depression because of both its inhibited immune response and because of marked neuroanatomical and cognitive deficits.[95,96] A more appropriate model for examining both cancer and depression is the ID8 model of ovarian cancer. This model uses the wild-type C57BL/6 mouse and the ID8 ovarian cancer cell line that is syngeneic to the C57BL/6 strain. Thus, not only is the C57BL/6 mouse appropriate, as this mouse has been used in many experiments that model depression as described above,[97] but *in vivo* ID8 tumor growth in this mouse is also well characterized in oncology[98,99,100,101] and typically involves formation of ascites that contains high levels of IL-6 and TNF-α,[102,103,104,105] although systemic levels in the model have not yet been reported in the literature.

As outlined above, animal models of depression have been in use since the 1950s and, more recently, have found their way into experimental investigation of inflammation-induced behavior change, including the study of mammary-cancer induced depressive-like behavior just described. As animal models of depression have evolved, investigators have proposed criteria for how to evaluate the usefulness of a model. One approach asserts that models should be evaluated for three types of validity: (1) construct validity, (2) predictive validity, (3) face validity. Construct validity requires that the behavioral features of the model are theoretically homologous to the features in

depression that are being modeled. Predictive validity requires that established pharmacological antidepressants are effective at altering the homologous behavior in the model. Face validity is determined by issues such as whether antidepressant effects are present only on chronic administration (daily dosing over the course of weeks) vs. acute administration (one dose, typically 30 - 60 minutes before the behavior is measured) and whether the behaviors found in the model are specific to depression.[106]

Early attempts at modeling MDD in animals focused on reproducing the whole syndrome in the animal, but this approach has been abandoned due its impracticality.[107] Researchers realized that there is a considerable heterogeneity of signs and symptoms within the diagnostic category for MDD that can manifest in a given individual. Also, investigators began to assert that the majority of DSM-IV criteria that may constitute MDD cannot be modeled in animals.[108] In this regard, it has been argued that only four of the nine MDD criteria are actually behaviors that can be modeled in animals: markedly diminished interest or pleasure in all, or almost all, activities, significant weight loss or gain, insomnia or hypersomnia, and psychomotor agitation or retardation. [109] The rest of the criteria are self-reported symptoms.

Of the MDD features that are behavioral signs, many animal researchers have come to focus on the core feature of "markedly diminished interest or pleasure," which is required for a diagnosis of MDD if depressed mood is not present.[5] This feature came to be simply called "anhedonia" in animal models of depression. Among the many ways that anhedonia can be operationalized, the most highly utilized method involves characterizing the consumption of highly palatable substances.[110] Paul Willner and colleagues championed measuring anhedonia as a reduction in preference for sucrose intake after being subjected to a series of chronic mild stressors that persist for multiple weeks.[56] This became known as the CMS model of anhedonia. Such stressors might include intermittent loud white noise bursts, stroboscopic illumination, 45-degree cage tilt, wetting the cage bedding, and pairing the mouse with a new unfamiliar cage mate.

Predictive validity is supported by the fact that sucrose consumption by mice in the model is significantly altered by antidepressant treatment.[111] Also, this effect requires chronic administration. Thus, face validity is high in the anhedonia model.

Another behavior that was used to model depression is immobility by rats in a procedure called the forced swim test (FST). This phenomenon was termed "behavioral despair" by Porsolt and colleagues[112] and thought by some to be analogous to the core symptom of depressed mood in MDD.[113] However, this interpretation of the behavior is controversial because, as noted above, many argue that depressed mood is not a behavioral sign but rather a self-reported symptom of MDD that cannot truly be modeled in animals.[108,109] In the procedure, rats are forced to swim in a cylindrical tank from which escape is not possible and typically go from an initial phase of vigorous swimming and pawing at the tank wall ("climbing" in the model's terminology) to a later phase of passive immobile floating. Predictive validity is supported by the fact that immobility is significantly reduced by a broad spectrum of antidepressants, although SSRIs have been found to be less reliable.[114] Face validity is lessened by the fact that these effects are seen after acute administration of the drug, while clinical effect in humans takes chronic administration over multiple weeks.

Porsolt eventually developed the FST for mice,[115] but introduction of the TST for mice by Steru and colleagues[116] offered advantages over the FST while seeming to measure the same construct. In the TST, mice are suspended in the air by their tail for a duration of 6 minutes, and immobility time (vs. time spent struggling to escape) is recorded. Like the FST, immobility in the TST is significantly reduced by a broad spectrum of antidepressants, offering good predictive validity, but, again these effects are obtained after acute administration rather than chronic administration. One advantage of the TST over the FST is the avoidance of a potential confound induced by hypothermic exposure in the FST due to immersion in water at a temperature below that of the body. Also, the TST is more reliable at detecting SSRIs.[114]

What follows in the next chapter is a report of the initial investigation of the methodological issues surrounding ovarian cancer-induced depressive-like behavior in the ID8 model and a formal proposal of hypotheses that were tested in larger experiments that built upon the results of the initial investigation.

CHAPTER 2
INITIAL INVESTIGATION INTO OVARIAN CANCER-INDUCED DEPRESSIVE-LIKE BEHAVIOR IN THE MOUSE

To begin addressing the question of whether ovarian tumor by itself is a cause of depression in cancer patients, several small pilot experiments were conducted that are presented below. The first of these experiments examined the effect of ID8 ovarian tumor on levels of systemic IL-6, sucrose intake, and body weight in female C57BL/6 mice. A second group of experiments tested a modification of the TST for C57BL/6 mice and examined the effect of repeated experiences in the TST on measures of immobility. Further experiments examined the utility of a computer assisted video tracking device for measuring locomotion in the home cage and explored whether any clear difference in depressive-like behavior was found as a result of the estrous cycle in the mice. Finally, remaining questions about methodology based on the results of these pilot experiments were reviewed, and specific hypotheses for larger experiments were articulated.

The Effect of ID8 Ovarian Tumor on Systemic IL-6

In the first pilot experiment, it was hypothesized that ID8 ovarian tumor would induce higher levels of systemic IL-6 in female C57BL/6 mice. Individually-housed mice were randomly assigned to receive an intraperitoneal (i.p.) injection of 5×10^6 viable ID8 ovarian cancer cells in 200 μL of vehicle or to receive a 200 μL i.p. injection of vehicle only and followed for 10 weeks. Tumor-bearing mice ($n = 14$) showed marginally higher systemic levels of IL-6 in comparison to vehicle-injected control mice ($n = 15$) after 10 weeks of tumor incubation, $P = .09$, as detected by an enzyme-linked immunosorbent assay (ELISA). Moreover, as presented in Figure 2-1, tumor size in the peritoneum was significantly correlated with IL-6.

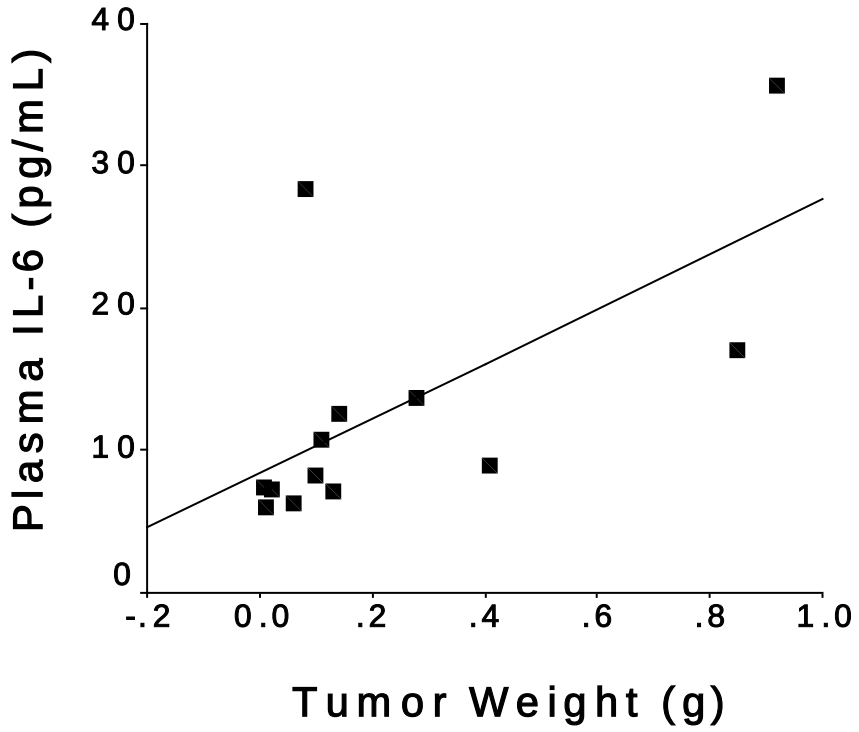

Figure 2-1. IL-6 levels in plasma in relation to tumor weight in mice injected with ID8 ovarian carcinoma, $\beta = .69$, $P = .01$, $N = 13$ (NOTE: Figure shows raw values, but test results are based on ranked data).

ID8 Ovarian Tumor, Sucrose Intake, and Body Weight

It was hypothesized that sucrose intake and body weight would decrease from baseline to 10 weeks post-injection in tumor-bearing mice but not in vehicle-injected mice. Before cancer or vehicle injections were administered to mice in the first pilot experiment, a subgroup of these mice (cancer-injected, $n = 6$; vehicle-injected, $n = 10$) was given 5 days of continuous access to two bottles for adaptation, i.e, one bottle in the cage was filled with a 3% sucrose solution, and the second bottle was filled with water. Then the sucrose was removed for 24 hours before a baseline measurement of 1-hour intake was recorded after the dark phase had begun. Mice were not water deprived before baseline intake was measured, and food was available ad libitum at all times.

After 10 weeks of tumor incubation, mice in both the tumor-bearing group and vehicle-injected control group were again given access to the two bottles for 1 hour after the dark phase had begun.

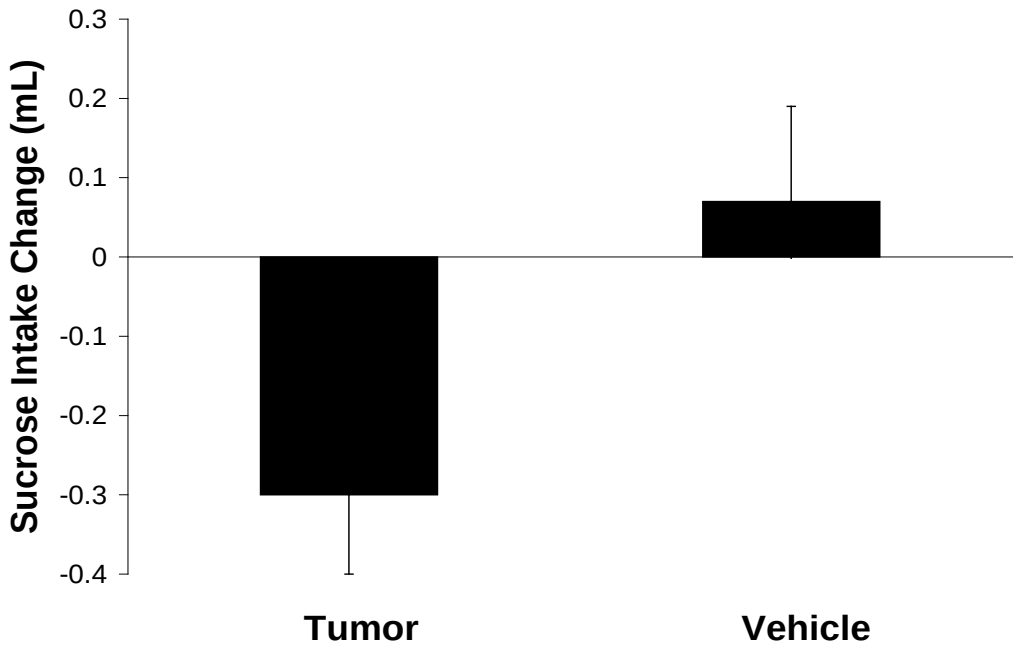

Figure 2-2. Change in sucrose intake from baseline to 10 weeks post-injection in tumor-bearing mice (n = 6) and vehicle-injected mice (n = 10). Difference between groups is marginal,
P = .054.

As presented in Figure 2-2, there was a marginal difference in the sucrose intake change score between tumor-bearing mice and control mice, P = .054. Tumor-bearing mice sucrose intake decreased 0.3 ± .10 mL from baseline to 10 weeks post-injection. Vehicle-injected mice sucrose intake increased 0.07 ± .12 mL.

The food and water aspect of the protocol followed an approach from other studies of anhedonic sucrose intake,[117] as it has been argued that food and water

restriction are proven forms of stress and can be unnecessary confounds when using sucrose intake as a measure of anhedonia.[110] Proponents of this argument assert that consumption of a palatable substance in studies of anhedonia should be based exclusively on the reinforcing nature of the substance itself rather than on its ability to quell basic hunger or thirst from a state of forced deprivation.

Also in this first pilot experiment, there was no significant difference in body weight change scores at the end of the 10-week tumor incubation, $P = .64$. This suggested that the decrease in sucrose intake was not confounded by an anorexic effect but was specific to anhedonia in the mice. This was an important finding, because it suggested that the sucrose paradigm could be used in this model as a measure of anhedonia with an adequate level of face validity. However, it was acknowledged that a larger sample was necessary to test these results and that food intake should be measured directly.

C57BL/6 Mice and the TST

C57BL/6 mice are known to be problematic subjects in the TST because they exhibit higher levels of tail climbing behavior than other mouse strains.[118] If a mouse manages to climb its tail, it then typically stops struggling and confounds the test. Thus, a modification was made of the mouse's connection to the testing apparatus to prevent tail climbing behavior. This modification is presented in Figure 2-3. An adhesive piece of duct tape was applied to a steady vertical metal rod (1.3 cm diameter) such that the end of the tape was flush with the end of the vertical rod. The mouse's tail was then fastened between the tape and the vertical rod such that the base of the tail was also flush with the end of the rod. Given this positioning of the mouse and the diameter of the vertical rod, the mouse was unable to climb its tail or the vertical rod to which it was fastened.

Figure 2-3. C57BL/6 mouse in the TST with modification.

The TST was originally antidepressant-validated as a measure of depressive-like behavior via independent groups that were naive to the experience. However, consideration was given to using a pre-injection/post-injection design in order to demonstrate a decrease in TST immobility over time by tumor-bearing mice while vehicle-injected control mice remained the same. To examine the feasibility of such a design, a second pilot study was conducted where it was hypothesized that healthy individually-caged control mice would show no significant change in TST immobility from baseline to 10 weeks post-injection. Mice (n = 9) were attached to the TST apparatus and video recorded for six minutes. The number of immobile-mobile cycles were counted, and immobility time was scored with a stopwatch to derive the average immobile bout time for each mouse, which was calculated by dividing immobility time by the number of immobile-mobile cycles. After 10 weeks, the same mice were placed in the TST apparatus again, and TST immobility was measured as described.

As presented in Figure 2-4, the mice exhibited a significant increase in TST immobility from baseline to 10 weeks post-injection, $P < .05$. At baseline, the average

immobile bout time was 15.53 ± 2.40 seconds but increased to 24.34 ± 4.03 seconds at 10 weeks post-injection.

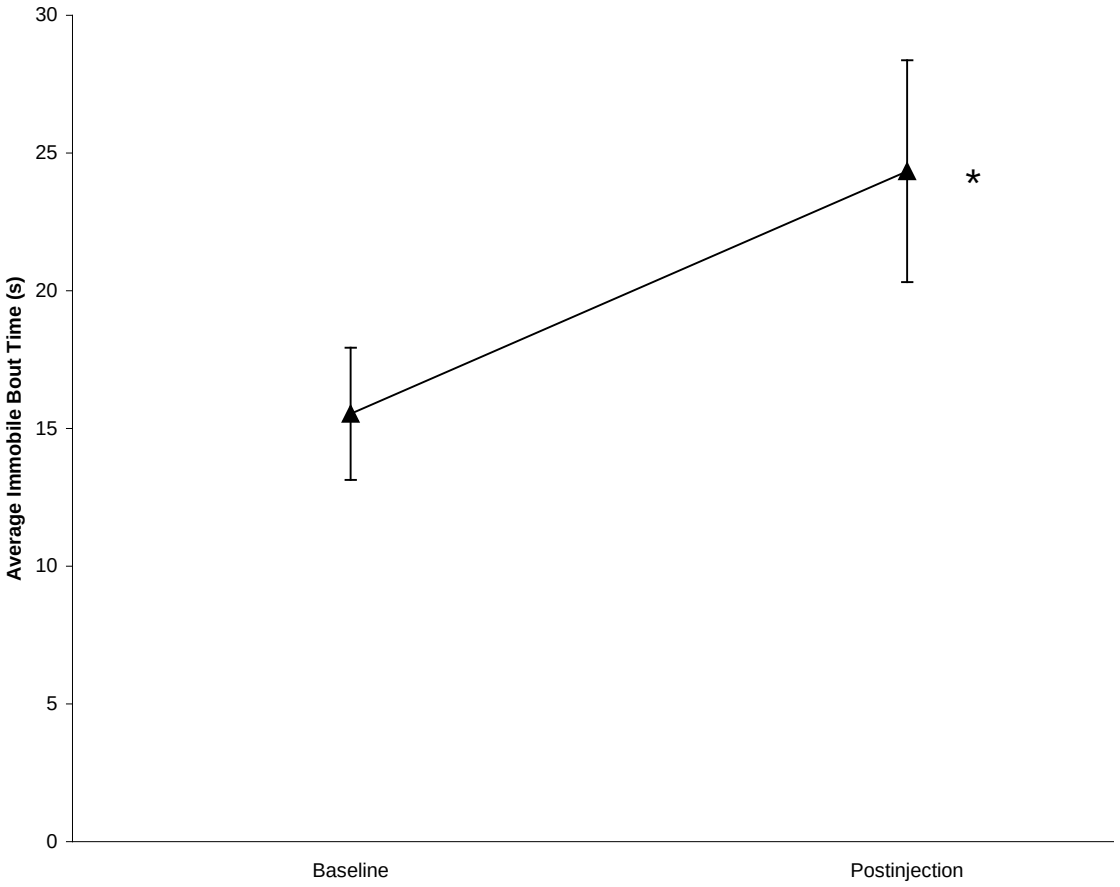

Figure 2-4. Significant change in TST immobility from baseline to 10 weeks post-injection in control mice (n = 9), P < .05.

It seemed possible that the significantly higher level of TST immobility in these controls at 10 weeks post-injection could be attributed to prior experience with the TST procedure rather than simply an effect of time. To test that hypothesis, a separate cohort of individually-housed mice (n = 12) were tested daily on two consecutive days and were found to exhibit a significant increase in TST immobility, P < .05 (data not shown).

Thus, using a standard definition of *learning* in behavioral psychology, that being "a relatively permanent change in behavior that occurs as a function of experience,"[119] one could conclude that the C57BL/6 mice learned there was no escape for them in the TST. However, a review of the TST literature revealed a study that allows attribution of the higher TST immobility seen at 10 weeks post-injection to the social isolation that the female mice underwent as a result of being individually housed. Specifically, the study found that female mice that had been socially isolated (housed alone in individual cages) for 16 weeks or just one week showed a significant difference in the number of immobile bouts manifested during the TST compared to group-housed female mice.[120] Furthermore, treatment of individually housed female mice with the SSRI antidepressant, fluoxetine, blocked the effect, causing the performance of individually-housed mice to be equivalent with group-housed mice that received vehicle only. The implications of this finding are further discussed below in the Summary.

Locomotion and Motor Capacity

As noted above, psychomotor retardation and fatigue are substantially prevalent (40% and 80%, respectively) in persons undergoing cytokine therapy over multiple weeks. Thus, in anticipation of testing for a similar effect of inflammation in tumor-bearing mice, as well as addressing the face validity of an immobility effect that could be found in the TST, mouse locomotor activity in the home cage was measured using an EthoVision® Video Tracking System (Noldus Information Technology, Leesburg, VA). The tracking system software takes the video image from a camera and translates it into monochromatic (grey scale) pixel information where a dark mouse can be distinguished from the lighter background of its home cage bedding (see Figure 2-5). The software then tracks how much distance the mouse travels inside its cage.

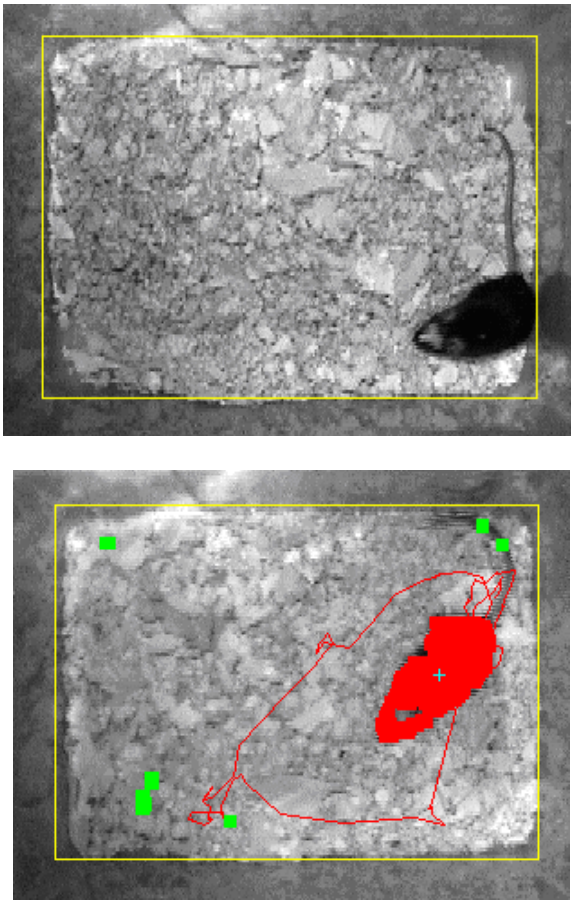

Figure 2-5. Home cage locomotion. The picture on the top shows a dark C57BL/6 mouse sitting inside a defined arena (yellow rectangle) of the digital video tracking system with its lighter bedding background. The picture on the bottom shows a C57BL/6 mouse highlighted in red by the tracking system to designate it as the object to be tracked inside the arena. The red line indicates the track the animal had just taken at the time the picture was captured. Green dots are digital noise in the system. Size of the bottom of the home cage is 16.5 cm x 22.5 cm.

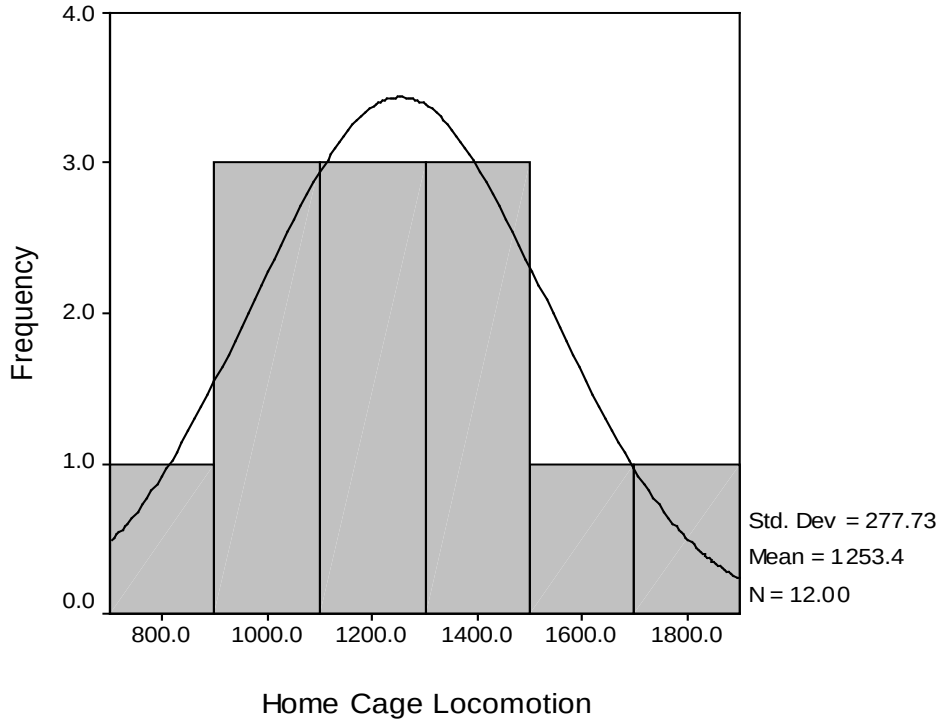

Figure 2-6. Locomotion distance traveled in the home cage for 6 minutes during the dark phase (n = 12).

The data collection using the EthoVision system was collected at the beginning of the dark phase under red light illumination after replacing the normal grid/food-hopper lid with a vented transparent plastic lid. Data from 12 mice was approximately normal, as presented in Figure 2-6.

In order to further determine whether any decrease in locomotion in tumor-bearing animals was attributable to lower motor capacity (and not simply lower motivation), consideration was given to use of a rotarod test. The rotarod test, in which rodents walk on a rotating cylinder as presented in Figure 2-7,[121] is widely used to assess motor capacity in laboratory mice.[122,123] An accelerating rotarod paradigm has been used to test motor impairment in C57BL/6 mice and, depending on the diameter of the cylinder and the speed of acceleration, typically causes healthy mice to fall within 1

minute on the first trial, although performance improves over time with repeated exposure to the test.[124,125] Performance is measured by the duration that an animal stays on the rotating cylinder as a function of rotational speed. Because the mice are compelled to show sufficient motor capacity by walking on the moving rod to keep from falling off, equivalent performance between control mice and tumor-bearing mice in the rotarod test provides an indication that lower home cage locomotion in the tumor-bearing mice is a valid reflection of *psycho*motor retardation or fatigue.

Figure 2-7. Rodents performing in the rotarod test.

Estrous Cycle

A review of the literature finds evidence that the estrous cycle can affect some of the mouse behaviors outlined in this thesis. For example, it is known that food intake in mice can decrease by as much as 25% during the estrus phase when estradiol has just come down from its zenith during proestrus.[126] Similarly, one study found that mice of a hybrid C57BL/6 x 129Sv background engaged in significantly more wheel running during estrus phase in comparison to the other phases.[127] However, a second study of C57BL/6 mice found no differences in wheel running as a function of estrous cycle.[128]

Table 2-1. Phases of the estrous cycle according to cell morphology in vaginal smears

Phase of cycle	Cell Type[a]		
	Leukocytes	Round Epithelial Cells	Cornified Cells
Estrus	0 to +[a]	0 to +	+++ (predominant)
Metestrus	++	++	++
Diestrus	+++ (predominant)	0 to +	0 to +
Proestrus	0 to +	+++ (predominant)	0 to +

a Cell density: 0 = none, + = few, ++ = moderate, +++ = heavy

Following such evidence and suggestion from fellow investigators in the field of sickness behavior research,[129] the estrous cycle was monitored in mice being measured for sucrose intake and TST immobility. To accomplish monitoring, vaginal cytology was carried out for each mouse on each day of sucrose measurement or TST measurement. Phase was determined using the criteria in Table 2-1 that are oriented around the four main phases of the estrous cycle, i.e., proestrus, estrus, metestrus, and diestrus.[130] These phases are based on the number and proportion of three cell types in the vaginal smear:

leukocytes, round epithelial cells, and cornified cells. Cells were always retrieved at the same time of day, about 1 hour before the beginning of the dark period.

There were no differences found in sucrose intake or TST immobility as a function of estrous cycle. Nonetheless, it was deemed prudent to continue monitoring estrous cycle in the study to ensure that it was not a confound or, if it was, to be able to control for it in a satisfactory manner.

Summary of Initial Investigation

Initial Results in Brief

The initial pilot studies suggested that ID8 ovarian tumor was capable of generating an inflammatory response in the C57BL/6 mouse, as evidenced by higher levels of systemic IL-6. Furthermore, the tumor-bearing mice appeared to consume less sucrose without becoming significantly lower in body weight over the course of the experiment, suggesting a specific anhedonic effect of the tumor. Because the C57BL/6 mouse is prone to climbing its tail in the TST, a modification of the mouse's connection to the testing apparatus was developed to ensure this behavior would not confound the study. Additional experimentation found that the individually-housed female control subjects in the study exhibited a significant increase in TST immobility over time, a finding that may be a function of the inherent social isolation that goes with being individually housed. Examination of locomotion using a digital video tracking system was found to be an efficient method for examining potential psychomotor retardation as a function of tumor, and the rotarod test was deemed a useful measure to further differentiate psychomotor retardation from potential deficits in motor capacity. Finally, the estrous cycle was monitored to ensure it had no confounding effect on the primary depressive-like behaviors of interest in the study. All of these preliminary findings were considered for designing the larger experiments executed for this dissertation.

Remaining Issues

Although water intake was also measured in the two bottle experiment reported above, a formal calculation of *sucrose preference* (i.e., the percentage of total fluid consumed by the animal that is sucrose solution) was not made because, after being adapted to the sucrose solution, the vast majority of these mice stopped drinking water completely when given the option for sucrose solution. This finding of little or no water intake has also been reported in a CMS experiment with C57BL/6 mice, where the authors defined the decreased absolute sucrose intake per body weight as anhedonia.[131] However, another research group has dealt with this issue in mice by lessening the adaptation time to hours (rather than days) before a first measurement is taken to ensure intake of both water and sucrose solution.[132] To facilitate a measurable intake of both sucrose solution and water for a calculation of sucrose preference, this latter approach was approximated in a separate cohort of mice of the study where the adaptation period was removed before a single post-injection measurement of sucrose intake and water intake was made.

Body weight in this initial investigation was compared between tumor-bearing mice and control mice without regard to the amount of weight that the disease added to tumor-bearing subjects. Although the amount of tumor and ascites was minimal in the pilot experiment presented above, other investigators have demonstrated that the amount of weight from tumor and/or ascites in this model can be substantial. For example, one study has reported that the average amount of ascites that can be generated in the peritoneal cavity of the mouse was about 7 mL.[102] This amounts to about 7 g of water weight, which is roughly 30% of the normal weight of a healthy age-matched control mouse. Because this is such a large increase, some investigators of this model have used the higher body weight of tumor-bearing mice (compared to controls) as a marker of advanced disease status.[104] Thus, in order to more accurately test for cachexia (i.e., wasting of normal tissue) in the model, it was decided that total disease weight (i.e., the

sum of tumor weight and ascites weight) should be subtracted from post-injection body weight. However, for examination of food intake per body weight and sucrose intake per body weight, it was decided that only ascites weight should be subtracted because, whereas ascites is mostly non-metabolic exudate (i.e., water), tumor tissue is highly metabolic and thus contributes to ingestive behavior.

An interesting result from this initial investigation was the finding that a depressive-like behavior, such as TST immobility, could be significantly increased simply by housing the mice in individual cages for an extended period of time. Although the pilot study of sucrose intake in tumor-bearing mice provided convincing indication that ovarian tumor can induce anhedonic sucrose intake relative to control mice, the question arose as to whether this anhedonic effect would be found in group-housed mice with tumor. Further investigation of the literature revealed no studies that have looked at the effect of social isolation on sucrose intake in female mice. However, there was a study that showed chronic social isolation in female prairie voles produced anhedonia as measured by a decrease in sucrose intake and sucrose preference.[133]

Thus, given the fact, as noted in Chapter 1, that social isolation is a significant risk factor for MDD in cancer patients and in the general population at large, and given the reported effects of social isolation on measures of TST immobility and sucrose-measured anhedonia, it was decided that social isolation should be included as a second experimental factor in a separate cohort of mice in the study when examining the effect of tumor condition on the depressive-like behaviors of TST immobility and sucrose-measured anhedonia.

Although higher levels of IL-6 were found in tumor-bearing mice, examination of other proinflammatory cytokines was not carried out in the preliminary experiments described here. However, as noted in Chapter 1, IL-1 and TNF-α have been implicated along with IL-6 in the effect of systemic inflammation on measures of depression and

sickness behavior. These three cytokines are part of a larger repertoire of proinflammatory cytokines that are typically active during an inflammatory response.

The larger repertoire of proinflammatory cytokines includes cytokines such as IFN-γ and interleukin-17 (IL-17). As mentioned in Chapter 1, the interferons are known for their ability to induce an anti-viral state. During an inflammatory response, IFN-γ is secreted exclusively by Type 1 T cells and natural killer (NK) cells of the immune system.[20] IFN-γ is highly effective at activating macrophages to carry out their inflammatory function and induces the differentiation of $CD8^+$ T cell precursors into full-fledged cytotoxic T-lymphocytes (CTLs) that seek out and destroy virally infected cells. IL-17 is produced by a subset of $CD4^+$ helper T-cells and appears to activate several cell types, including macrophages and fibroblasts, which in turn help produce a milieu of proinflammatory cytokines and chemokines.[134]

The immune system provides inherent regulation to these proinflammatory cytokines via the activity of antiinflammatory cytokines, such as IL-10 and IL-4.[135] These two cytokines are secreted by Type 2 helper T cells. The primary mechanism by which they downregulate proinflammatory cytokines is by acting directly on macrophages, suppressing their inflammatory program.[20] Another antiinflammatory cytokine is transforming growth factor beta (TGF-β). A subset of $CD4^+$ T cells, known as regulatory T cells or "T regs", use TGF-β to downregulate the function of other T cells involved in a proinflammatory response. More provocatively, some cancer types, including ovarian cancer,[136] are known to secrete TGF-β to suppress the cytotoxic actions of CTLs on the tumor.[68]

Given the fact that several mediators are active during an inflammatory response, it was decided that all of the cytokines described here should be examined to provide a more extensive picture of how the inflammatory arm of the immune system is operating in the model.

Hypotheses for Expanded Experiments

Experiment 1

The first expanded experiment examined the effect of tumor burden on anhedonia as measured by sucrose intake. In addition to anhedonia, the effect of tumor burden on the more non-specific behaviors of food intake, decreased locomotion, and body weight change was examined. Finally, the effect of tumor on rotarod performance was examined.

It was hypothesized that animals with tumor would exhibit a significant decrease in sucrose solution intake in comparison to baseline measures and control animal measures. It was also hypothesized that animals with tumor would exhibit a significant decrease in food intake and body weight in comparison to baseline measures and control animal measures. Finally, it was also hypothesized that these same animals would exhibit a significant decrease in home cage locomotion in comparison to baseline measures and control animal measures but that there would be no significant difference in rotarod performance between tumor-bearing animals and control animals.

Experiment 2

The second experiment examined the effects of tumor burden and social housing on TST immobility, a measure of behavioral despair. The effects of tumor burden and social housing on sucrose intake and water intake were also examined in a subgroup of mice in this second experiment. The second experiment also examined the effects of tumor burden and social housing on the more non-specific behaviors of decreased food intake, locomotion, and body weight change. Finally, the effects of tumor burden and social housing on rotarod performance were examined.

It was hypothesized that animals with tumor would exhibit significantly more TST immobility and less home cage locomotion than control animals, but no significant difference in rotarod performance. It was also hypothesized that animals with tumor

would exhibit significantly less sucrose preference, less food intake, and lower body weight than control animals. In regard to housing condition, it was hypothesized that group-housed animals would exhibit significantly less TST immobility and more sucrose preference than individually-housed animals. For home cage locomotion, it was hypothesized that group-housed animals would exhibit significantly less locomotion than individually-housed animals, because previous research has shown that socially isolated mice exhibit a greater amount of motor activity than group-housed mice.[137] For body weight, it was hypothesized that group-housed animals would weigh significantly less than individually-housed animals, because previous research has shown that socially isolated mice of the age used in this experiment weigh more than group-housed mice.[138] For rotarod performance, it was hypothesized that no significant difference would be found between group-housed animals and individually-housed animals. It was also hypothesized that there would be a significant interaction between tumor condition and social housing, which results in individually-housed tumor-bearing mice having significantly more TST immobility, less home cage locomotion, less sucrose preference, and lower body weight in comparison to the other three experimental groupings.

Experiment 3

Using plasma samples from the previous experiments, the third experiment examined the effect of ovarian tumor on systemic levels of IL-6, IL-1, TNF-α, IL-17, IFN-γ, IL-4, IL-10, and TGF-β. It was hypothesized that levels of these cytokines would be significantly higher in tumor bearing animals compared to controls. It was also hypothesized that each cytokine would mediate, in part, the effect of tumor on sickness and depressive-like behavior.

A diagram of the forgoing experiments is presented in Figure 2-8.

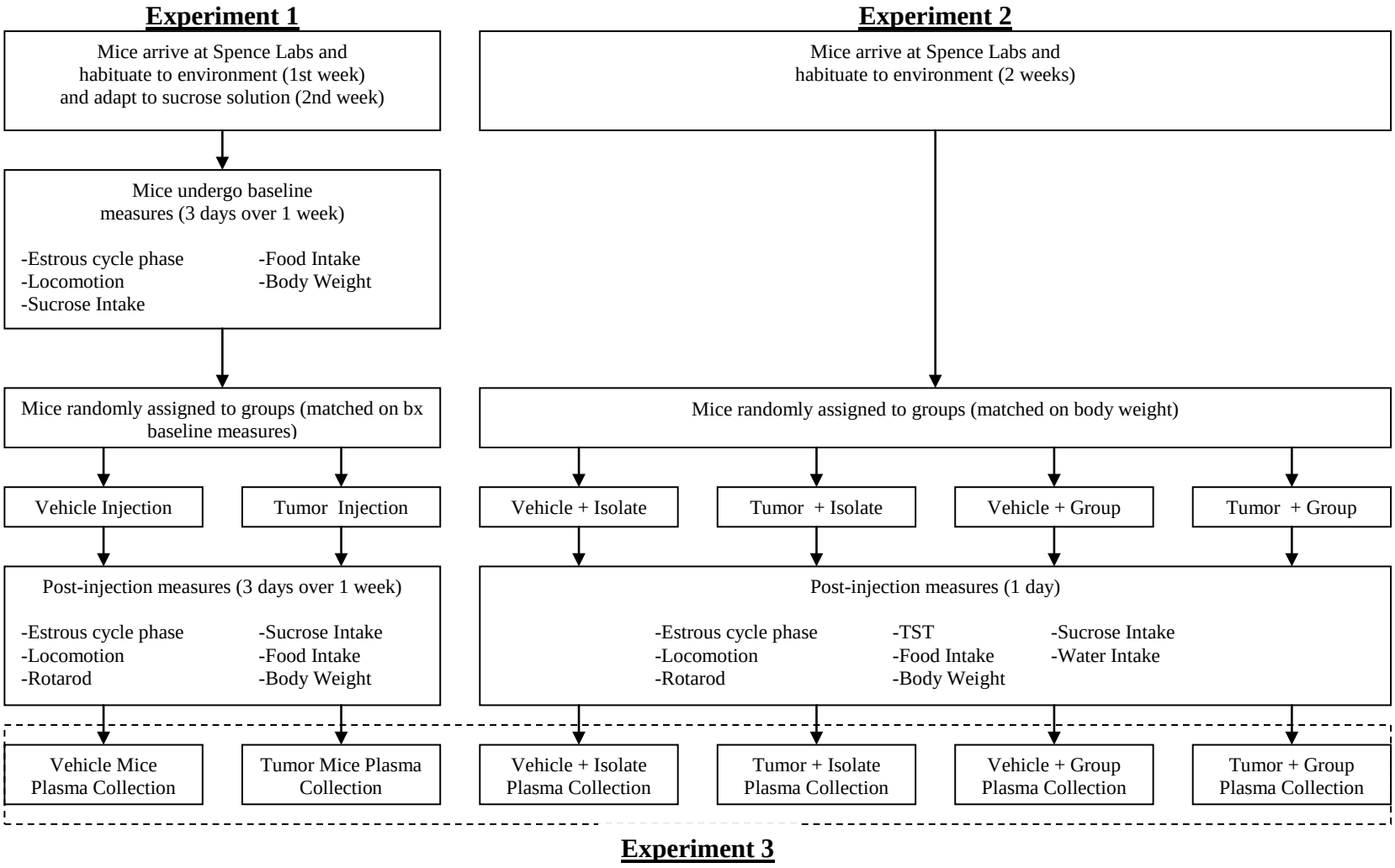

Figure 2-8. Diagram of Expanded Experiments for Dissertation

CHAPTER 3

EXPERIMENT 1: THE EFFECT OF OVARIAN TUMOR ON SUCROSE INTAKE, FOOD INTAKE, BODY WEIGHT, LOCOMOTION, AND ROTAROD PERFORMANCE

Introduction

The goal of Experiment 1 was to examine the effect of tumor burden on anhedonia as measured by sucrose intake. In addition to anhedonia, the effect of tumor burden on the more non-specific behaviors of food intake, decreased locomotion, and body weight change was examined. Finally, the effect of tumor on rotarod performance was examined.

It was hypothesized that animals with tumor would exhibit a significant decrease in sucrose solution intake in comparison to baseline measures and control animal measures. It was also hypothesized that animals with tumor would exhibit a significant decrease in food intake and body weight in comparison to baseline measures and control animal measures. Finally, it was also hypothesized that these same animals would exhibit a significant decrease in home cage locomotion in comparison to baseline measures and control animal measures but that there would be no significant difference in rotarod performance between tumor-bearing animals and control animals.

Method

Subjects

A total of 52 female C57BL/6 mice were obtained from Harlan Laboratories, Inc. (Indianapolis, IN, USA) at 11-12 weeks of age and housed in an animal facility at the Spence Laboratories of Psychology of the University of Iowa. Animals were housed individually in plastic cages with bedding. Food and water were available ad libitum for the duration of the experiment. The temperature was maintained at 22-24^{o}C, and a 12/12-

hour light/dark cycle was followed with lights on at 5:00 AM CST / 6:00 AM CDT. Mice injected with cancer cells that did not develop observable disease at the time of necropsy, i.e., there was no tumor in the peritoneum that could be weighed or the presence of ascites, were not included in the study analysis (n = 5). All procedures were followed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and proceeded as authorized by the University of Iowa Institutional Animal Care and Use Committee (ACURF #0706134).

Procedural Timeline

Mice habituated to their new environment for one week. At the start of their second week, in addition to water the mice were given free continuous access to a 3% sucrose solution for five days for adaptation followed by a two-day withdrawal period. Daily vaginal cytology for determination of estrous cycle phase was begun at the mid point of the second week. At the start of their third week, the mice underwent baseline measurement of estrous cycle phase, body weight, locomotion, food intake, and sucrose intake on three days over the course of the week with two days between each measurement day. Following the week of baseline measurement, the mice were randomly divided into two groups, (1) tumor-bearing mice (n = 34) and (2) control mice (n = 18) and given the injection appropriate to their randomized group status. After tumor became palpable or ascites became visible (8-12 weeks following injection, median = 9), the mice underwent post-injection measurement of estrous cycle phase, body weight, locomotion, food intake, and sucrose intake on three days over the course of a week with two days between each measurement day. Rotarod performance was measured on the first day of the post-injection measurement week. Mice were then euthanized via rapid decapitation for collection of peripheral trunk blood. Tumor and/or ascites was then excised from the peritoneum.

ID8 Ovarian Carcinoma

ID8 murine ovarian carcinoma cells were kindly provided by Dr. Katherine Roby (University of Kansas Medical Center, Kansas City, KS). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), insulin (5 μg/mL), transferrin (5 μg/mL), and sodium selenite (5 ng/mL). Mice randomly assigned to the Tumor group received an i.p. injection of 5×10^6 viable ID8 ovarian cancer cells in 200 μL of vehicle. Mice in the Control group received a 200 μL i.p. injection of vehicle only. After post-injection measurements, tumor was removed from the peritoneum, weighed, flash frozen in a cryovial or in optimal cutting (OCT) media, and stored at -80° C. Similarly, any ascites in the peritoneum was also removed, weighed, and flash frozen before storage at -80° C.

Estrous Cycle

To determine phase of estrous cycle, vaginal cytology was carried out by lavaging the vagina with 10 μL of saline solution using a micropipettor and placing the aspirated fluid on a microscope slide for inspection at 40-80X magnification without the use of the condenser lens. Specific phase was determined by examining the number and proportion of three cell types in the vaginal smear. A predominance of round epithelial cells indicates proestrus; a predominance of cornified cells indicates estrus; a predominance of leukocytes indicates diestrus; an equivalent proportion of all three types indicates metestrus (see Table 2-1).[127] Cells were always retrieved at the same time of day (around 1 hour before the beginning of the dark period).

Sucrose Intake

The sucrose intake of each mouse was determined by first measuring the change in volume of a 3% sucrose solution over one hour after the dark phase had begun and following measurement of home cage locomotion (and rotarod test performance on first night) (i.e., approximately 1-3 hours into the dark phase). A Kimax® glass graduated

cylinder with fitted sipper-tube held the sucrose solution and was placed into the cage on measurement days. The recorded volume of solution was then divided by the mouse's body weight minus ascites weight. Sucrose intake is reported as milliliters of sucrose solution per 100 grams of body weight (mL/100g - BW).

Food Intake

The food intake of each mouse was determined by first measuring the change in weight of chow in the mouse's food-hopper over a 24-hour period. Chow was weighed at the beginning of a measurement day and 24 hours later at the end of the measurement day. Care was taken to include any chow that had fallen through the hopper onto the cage bottom. The recorded weight of food was then divided by the mouse's body weight minus ascites weight. Food intake is reported as milligrams of chow per gram of body weight (mg/g - BW).

Body Weight

A portable weight scale (Ohaus Corporation, Pine Brook, NJ, USA), was used to measure the weight of each mouse at the beginning of each measurement day. Total disease weight (i.e., the sum of tumor weight and ascites weight) was subtracted from post-injection body weight for comparison with baseline body weight. Weight is reported in grams (g).

Locomotion

The locomotion of each mouse in its home cage was recorded using the EthoVision® Video Tracking System (Noldus Information Technology, Leesburg, VA). The tracking system software takes the video image from a camera and translates it into monochromatic (grey scale) pixel information where a dark mouse can be distinguished from the lighter background of its home cage bedding. The software then tracks the distance the mouse travels inside its cage. The software was calibrated to measure

distance in centimeters (cm). The duration of each recording was six minutes. Each recording began after replacing the metal-grid/food-hopper lid of the cage with a transparent vented plastic lid. All recordings took place within the first three hours of the dark phase under red light illumination on measurement days. Locomotion is reported as total distance traveled during the six minute period.

Rotarod Performance

Post-injection motor capacity was determined by measuring performance in the rotarod test. Each mouse was placed on a plexiglass cylinder with diameter of 3.5 cm that was rotating at a speed of 4 rpm that accelerated uniformly to 17 rpm over 1 minute. The cylinder was scored to reduce slipping. The fall height was 27 cm. On the first day of post-injection measurement, three trials were conducted for each mouse separated by a 30-second rest period. The amount of time each mouse stayed on the rotating cylinder was scored with a stop watch. In line with previous research, a mouse that stayed on the rotating cylinder up to 90 seconds in a given trial was removed from the cylinder and given this amount of time as its score for that trial.[123] The three trial times for a given mouse were averaged to derive the rotarod time for that mouse. Rotarod time is reported in seconds (s).

Statistical Analyses

The effect of the estrous cycle on sucrose intake, food intake, and locomotion was examined in baseline scores with a repeated measures ANOVA where the average estrus phase score was compared to the average non-estrus phase score. This same effect was examined in post-injection measurements with a 2 x 2 mixed model ANOVA where the between-subjects factor was tumor condition (control mice vs. tumor-bearing mice) and the within-subjects factor was phase (estrus vs. non-estrus). Estrous cycle was treated as a covariate when found to have a significant effect. Sucrose intake, food intake, body weight, and locomotion were each examined with a 2 x 2 mixed model ANOVA where

the between-subjects factor was tumor condition (control mice vs. tumor-bearing mice) and the within-subjects factor was time (average baseline vs. average post-injection). Significant interaction effects were followed with an independent groups *t* test to examine the simple effect at the time of post-injection. Rotarod performance was examined with an independent groups *t* test. All statistical procedures were performed using SPSS software. Group parameters are reported as mean ± standard error of the mean (SEM). *P* values < .05 were considered statistically significant.

Results

ID8 Ovarian Tumor

Of the 34 mice injected with ID8 ovarian carcinoma, 29 mice exhibited visible tumor and/or ascites in the peritoneum at necropsy. Mean tumor weight was .70 ± .17 g, and mean ascites weight was 2.69 ± .99 g. Control mice exhibited no sign of disease in the peritoneum.

Estrous Cycle

There was a significant effect of estrous cycle on locomotion at baseline, $F(1,50) = 9.159$, $P < .01$, as mice in estrus phase moved a greater distance than mice in non-estrus phases, 1239.88 ± 32.09 vs. 1150.13 ± 34.45 cm. Thus, baseline estrous cycle was treated as a covariate for analysis of locomotion. There was no significant effect of estrous cycle on baseline measures of food intake, $F(1,50) = .774$, $P = .38$, or sucrose intake at 1 hour, $F(1,50) = 2.773$, $P = .11$. Similarly, post-injection examination of estrous cycle showed no significant effect on any of these measures, *P* values > .32.

Sucrose Intake

To test for an effect of ovarian tumor on anhedonia, sucrose intake was measured. As presented in Figure 3-1, there were significant main effects of tumor condition, $F(1,45) = 4.003$, $P < .05$, and time, $F(1,45) = 18.869$, $P < .001$, on sucrose intake at 1

hour. These effects were part of a significant interaction between tumor condition and time, $F(1,45) = 5.092$, $P < .05$. At the end of the experiment, tumor-bearing mice consumed significantly less sucrose solution than control mice over 1 hour, .027 ± .003 vs. .041 ± .004 mL/100g - BW, $P < .05$.

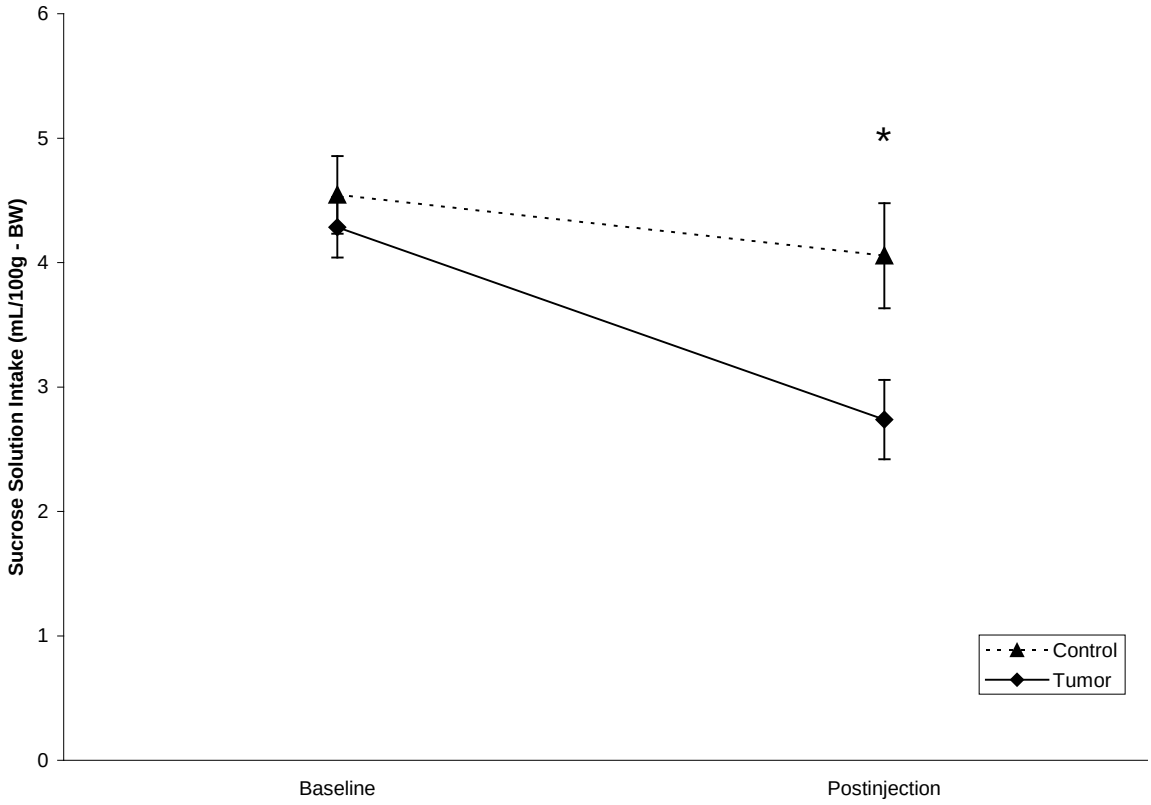

Figure 3-1. 1-hour sucrose intake between experimental groups. Mean ± SEM 1-hour sucrose intake for control and tumor mice at baseline and 8-12 weeks post-injection. Tumor-bearing mice (diamond) ($n = 29$) consumed significantly less than control mice (triangle) ($n = 18$) at the end of the experiment, *$P < .05$.

Food Intake and Body Weight

To further examine the specificity of the effect of ovarian tumor on sucrose intake, food intake and body weight were measured to test for an effect of ovarian tumor

on anorexia. Figure 3-2 shows that there was a significant main effect of time on food intake, $F(1,45) = 23.498$, $P < .001$, as both experimental groups ate less food per body weight at the end of the experiment than at baseline. Tumor-bearing mice exhibited a decrease of 14% from 180 ± 5 to 155 ± 8 mg/g - BW. Control mice exhibited a slightly larger decrease of 22% from 186 ± 7 to 145 ± 10 mg/g - BW. Neither the main effect of tumor condition, $F(1,45) = .029$, $P = .87$, nor the interaction, $F(1,45) = 1.385$, $P = .25$, was significant.

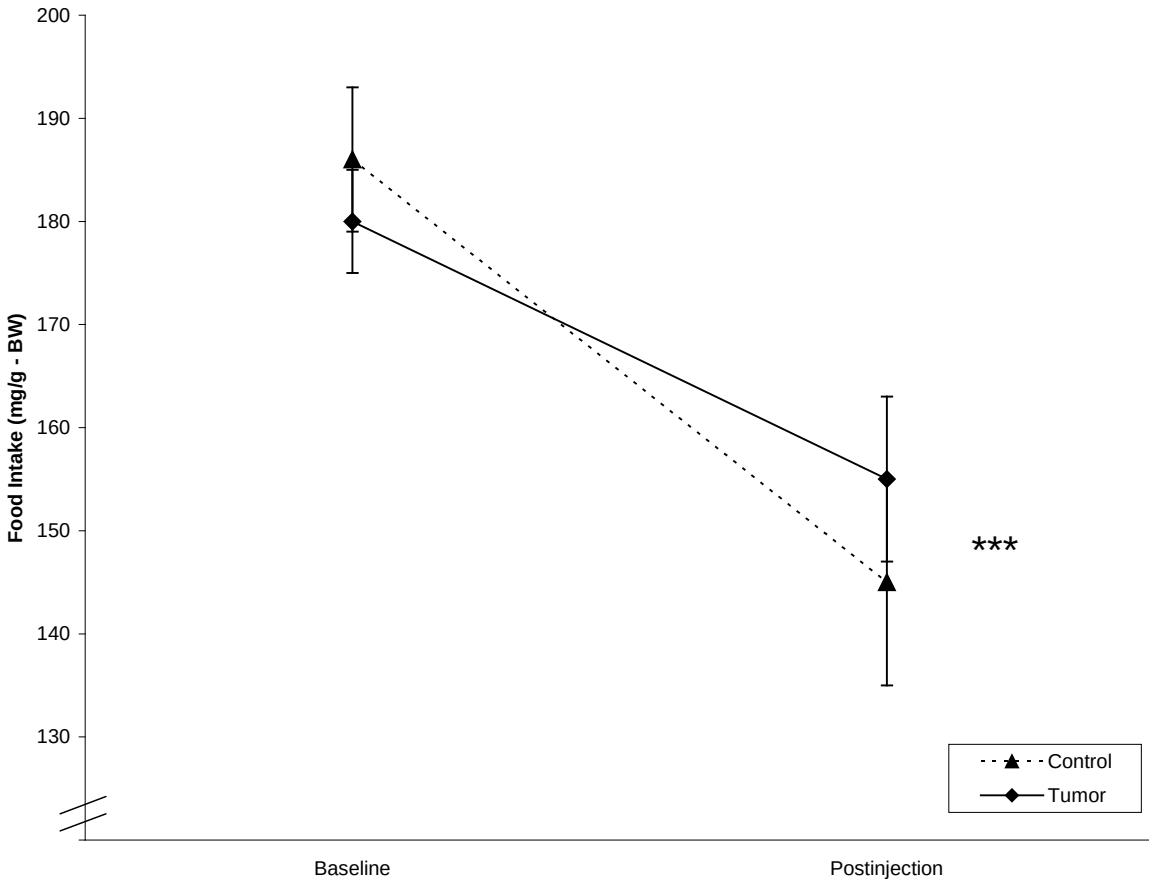

Figure 3-2. Food intake between experimental groups. Mean ± SEM 24-hour food intake per body weight for control ($n = 18$) and tumor mice ($n = 29$) at baseline and 8-12 weeks post-injection. Chow consumed per body weight decreased significantly from baseline to post-injection,
***$P < .001$.

Although there was also a significant main effect of time on body weight, $F(1,45) = 31.043$, $P < .001$, as presented in Figure 3-3 this effect was smaller in tumor-bearing mice as the interaction effect between time and tumor condition was significant, $F(1,45) = 5.880$, $P < .05$. Tumor-bearing mice weighed significantly less than control mice at the end of the experiment, 22.98 ± .99 vs. 25.27 ± .83 g, $P < .05$. The main effect of tumor condition on body weight was marginal, $F(1,45) = 3.177$, $P < .09$.

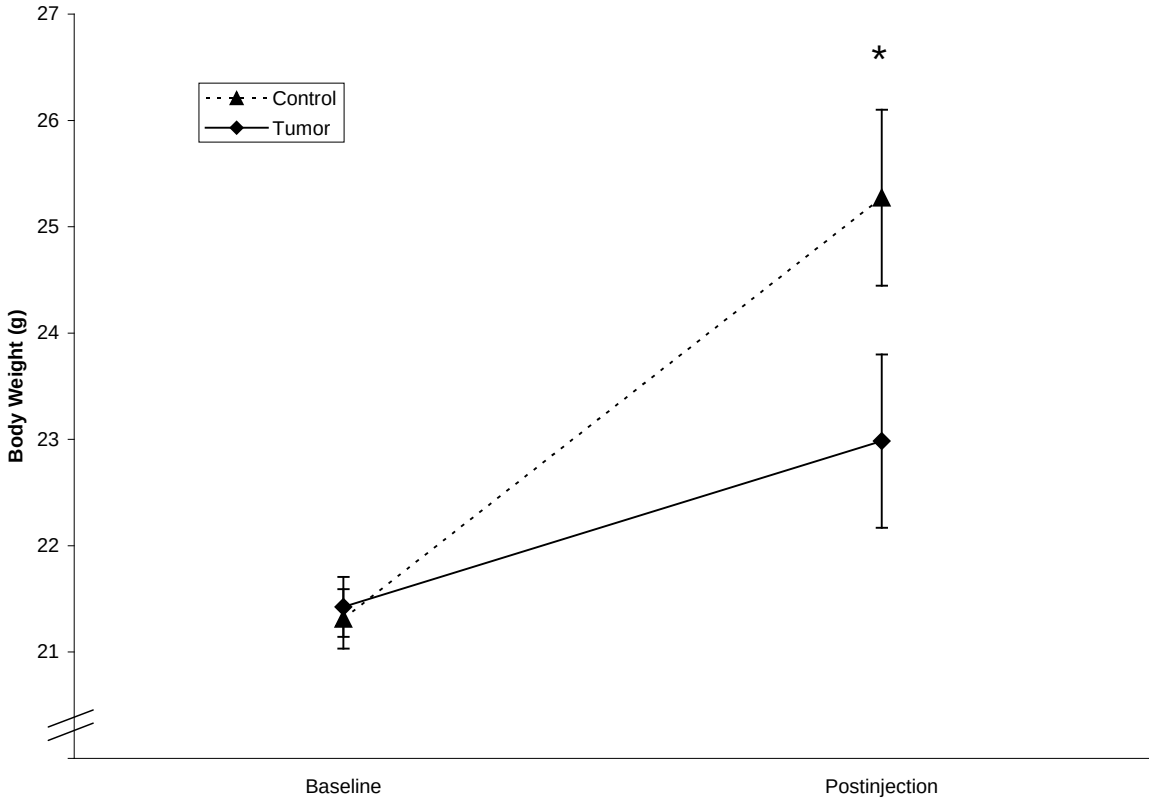

Figure 3-3. Body weight between experimental groups. Mean ± SEM body weight in grams for control and tumor mice at baseline and 8-12 weeks post-injection. Tumor-bearing mice (diamond) ($n = 29$) weighed significantly less than control mice (triangle) ($n = 18$) at the end of the experiment, *$P < .05$.

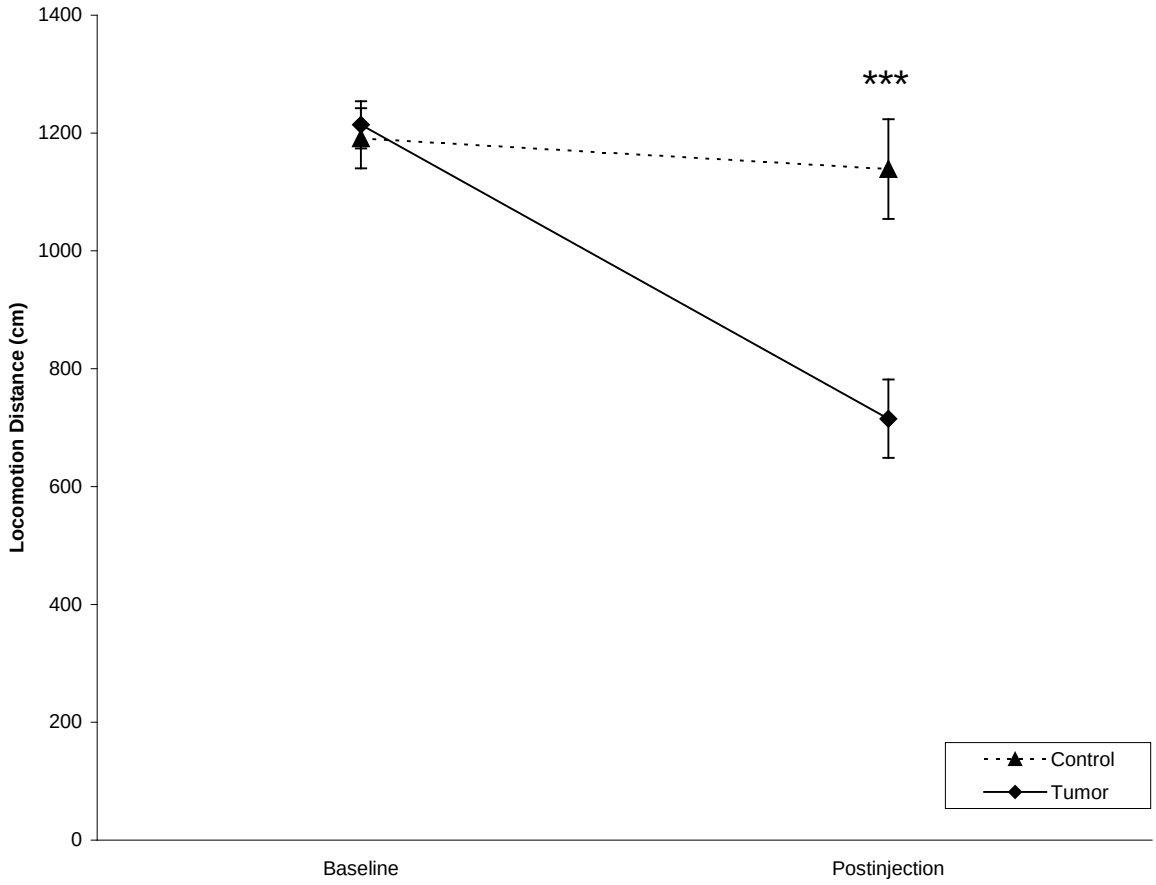

Figure 3-4. Locomotion between experimental groups. Mean ± SEM locomotion distance for control and tumor mice at baseline and 8-12 weeks post-injection. Tumor-bearing mice (diamond) (n = 29) moved significantly less than control mice (triangle) (n = 18) at the end of the experiment, ***P < .001.

Locomotion and Motor Capacity

To test for an effect of ovarian tumor on the sickness behavior of decreased motor activity, locomotion in the home cage was measured. As presented in Figure 3-4, there were significant main effects of tumor condition, $F(1,45) = 9.580$, $P < .01$, and time, $F(1,45) = 20.525$, $P < .001$, on locomotion, adjusting for baseline estrous cycle phase. These effects were qualified by a significant interaction between time and tumor condition, $F(1,45) = 13.491$, $P < .001$. Tumor-bearing mice moved significantly less than

control mice at the end of the experiment, 715.19 ± 66.62 vs. 1138.97 ± 84.56 cm, $P < .001$.

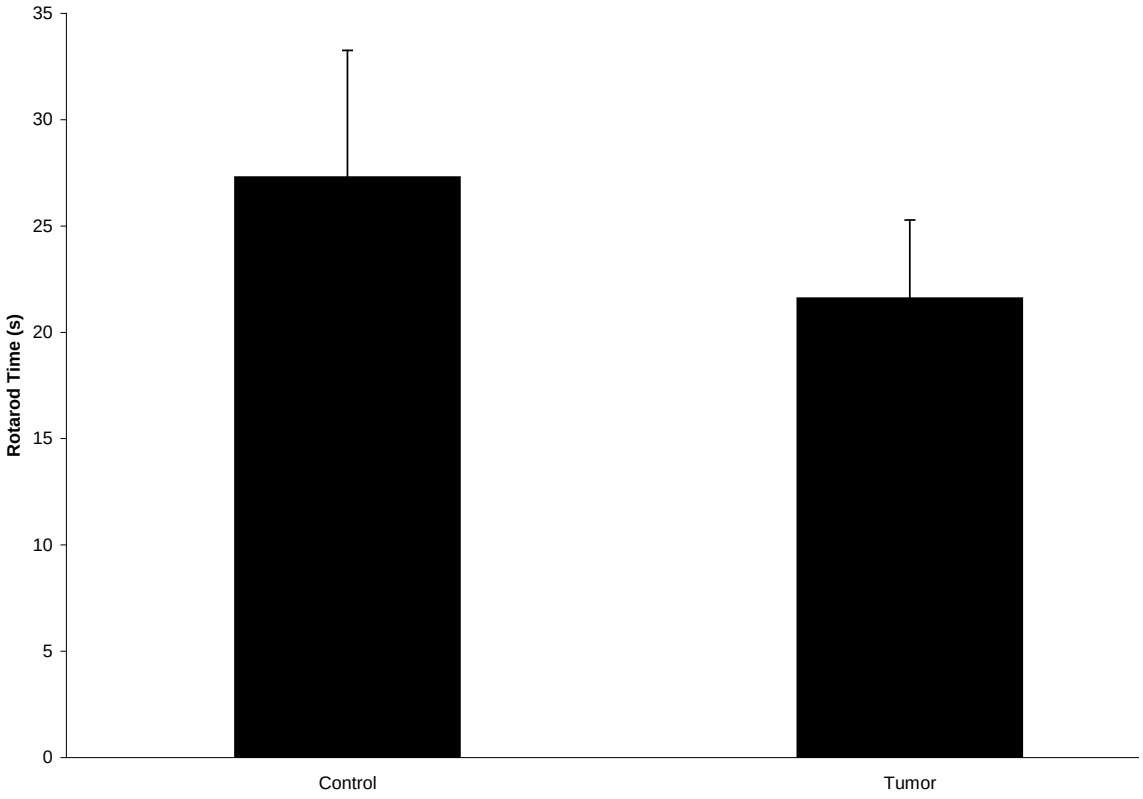

Figure 3-5. Rotarod performance between experimental groups. Mean ± SEM accelerating rotarod time for control mice (n = 17) and tumor-bearing mice (n = 29) at 8-12 weeks post-injection. The difference is non-significant, P = .39.

To further determine whether the decreased locomotion in tumor-bearing mice was attributable to a decrease in motor capacity, rotarod performance was measured at the end of the experiment. Despite the significant difference in home cage locomotion between groups at the end of the experiment, the amount of time that tumor-bearing mice stayed on the accelerating rotarod was not significantly different from control mice, $t(44) = 0.860$, P = .39. This is presented in Figure 3-5. Tumor-bearing mice stayed on the

accelerating rotarod for 21.61 ± 3.67 s. Control mice stayed on slightly longer for 27.29 ± 5.96 s.

Discussion

Sucrose intake, a conventional measure of anhedonia in mouse models of depression, was significantly lower in mice with ovarian tumor than in control mice. This was despite the fact that both tumor-bearing mice and control mice exhibited no significant difference in appetite at the end of the experiment. However, tumor-bearing mice did have significantly less body weight than control mice at the end of the experiment. Tumor-bearing mice also exhibited significantly less locomotion than control mice at the end of the experiment, but both groups showed an equivalent level of motor capacity in the rotarod test. Effects of the estrous cycle were tested on sucrose intake, food intake, and locomotion before all other analyses were begun. Only locomotion at baseline was found to be significantly affected and, thus, was adjusted for estrus phase.

The sucrose intake finding from this experiment is consistent with the previously reported finding that mammary tumors caused a decrease in sucrose intake in rats,[93] and extends that finding into a mouse model with a different type of cancer. The sucrose intake finding is also consistent with a previous report that showed experimentally-induced heart failure, another condition marked by systemic inflammation, caused anhedonia.[139] The behavior of decreased locomotion found in this experiment is consistent with reports in the sickness behavior literature as a whole.[61]

Although tumor-bearing mice exhibited the same level of appetite as control mice at the end of the experiment, the tumor-bearing mice weighed less than the control mice. This can be attributed to the metabolic needs of the tumor. It has been known since the 1930s that, in comparison to normal cells, most types of cancer cells exhibit an increased rate of glucose uptake and metabolism via the glycolysis pathway, a phenomenon known

as the *Warburg Effect*.[140,141] Further research has since shown that a second major pathway for glucose metabolism, the pentose phosphate pathway, is also increased in neoplastic cells.[142,143] Thus, the additional energy requirements of tumor may not allow excess energy from food consumption to be converted to fat mass for subsequent higher body weight.

The forgoing results of this experiment provide evidence of a causal relationship between ovarian tumor and anhedonia. Anhedonia is a core feature of depressive disorders and is found in 95% of all patients diagnosed with MDD.[144] The results here suggest that pathology in the periphery is capable of inducing this core feature of depression in persons with ovarian cancer. Furthermore, the results lend valuable information to the issue of inclusive/exclusive criteria for diagnosing MDD in cancer patients, as the results provide additional support for the notion that depression in cancer patients is not necessarily just a reaction to the stress of one's diagnosis but can result from pathophysiology in the body outside the brain.

CHAPTER 4

EXPERIMENT 2: THE EFFECTS OF OVARIAN TUMOR AND SOCIAL HOUSING ON TST IMMOBILITY, LOCOMOTION, ROTAROD PERFORMANCE, SUCROSE PREFERENCE, FOOD INTAKE, AND BODY WEIGHT

Introduction

The goal of Experiment 2 was to examine the effects of tumor burden and social housing on TST immobility, a measure of behavioral despair. The effects of tumor burden and social housing on sucrose intake and water intake were also examined in a subgroup of mice in Experiment 2. Also examined were the effects of tumor burden and social housing on the more non-specific behaviors of decreased food intake, locomotion, and body weight change. Finally, the effects of tumor burden and social housing on rotarod performance were examined.

It was hypothesized that animals with tumor would exhibit significantly more TST immobility and less home cage locomotion than control animals but no significant difference in rotarod performance. It was also hypothesized that animals with tumor would exhibit significantly less sucrose preference, less food intake, and lower body weight than control animals. In regard to housing condition, it was hypothesized that group-housed animals would exhibit significantly less TST immobility and more sucrose preference than individually-housed animals. For home cage locomotion, it was hypothesized that group-housed animals would exhibit significantly less locomotion than individually-housed animals, because previous research has shown that socially isolated mice exhibit a greater amount of motor activity than group-housed mice.[137] For body weight, it was hypothesized that group-housed animals would weigh significantly less than individually-housed animals, because previous research has shown that socially isolated mice of the age used in this experiment weigh more than group-housed mice.[138]

For rotarod performance, it was hypothesized that no significant difference would be found between group-housed animals and individually-housed animals. It was also hypothesized that there would be a significant interaction between tumor condition and social housing, which results in individually-housed tumor-bearing mice having significantly more TST immobility, less home cage locomotion, less sucrose preference, and lower body weight in comparison to the other three experimental groupings.

Method

Subjects

A total of 73 female C57BL/6 mice were obtained from Harlan Laboratories, Inc. (Indianapolis, IN, USA) at 11-12 weeks of age and housed in an animal facility at the Spence Laboratories of Psychology of the University of Iowa. After their arrival, animals were randomly chosen to be housed individually or in groups of 4-5 in plastic cages with bedding. Food and water were available ad libitum for the duration of the experiment. The temperature was maintained at 22-24^{o}C, and a 12/12-hour light/dark cycle was followed with lights on at 5:00 AM CST / 6:00 AM CDT. All procedures were followed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and proceeded as authorized by the University of Iowa Institutional Animal Care and Use Committee (ACURF #0706134).

Procedural Timeline

Mice in each housing condition (individual vs. group) were allowed to habituate to their new environment for two weeks, upon which baseline measurement of body weight was completed. The mice in each housing condition were then randomly chosen to be injected with ovarian carcinoma or vehicle only, resulting in four experimental groups: (1) grouped tumor-bearing mice (n = 14), (2) grouped control mice (n = 15), (3) individual tumor-bearing mice (n = 23), (4) individual control mice (n = 21). After tumor

became palpable or ascites became visible (6-12 weeks following injection, median = 9), the mice underwent post-injection measurement of estrous cycle phase, body weight, locomotion, rotarod performance, TST immobility, food intake, sucrose intake, and water intake. Mice were then euthanized via rapid decapitation for collection of peripheral trunk blood. Tumor and/or ascites was then excised from the peritoneum.

ID8 Ovarian Carcinoma

The same methods from Experiment 1 (see Chapter 3) for culturing ID8 murine ovarian carcinoma cells, injecting them into mice, and collecting tumor and ascites at the end of the incubation period were used in the present experiment.

Estrous Cycle

The same methods from Experiment 1 (see Chapter 3) for determining the phase of estrous cycle in mice were used in the present experiment.

TST Immobility

The TST was utilized with a modification of the mouse's connection to the testing apparatus to prevent tail climbing behavior (see Figure 2-3). An adhesive piece of duct tape was applied to a steady vertical metal rod (1.3 cm diameter) such that the end of the tape was flush with the end of the vertical rod. The mouse's tail was then fastened between the tape and the vertical rod such that the base of the tail was also flush with the end of the rod. Given this positioning of the mouse and the diameter of the vertical rod, the mouse was unable to climb its tail or the vertical rod to which it was fastened. Mice were then video recorded for six minutes. The number of immobile-mobile cycles were counted by a trained observer blind to experimental condition. Immobility time was scored with a stopwatch to derive the average immobile bout time for each mouse, which was calculated by dividing immobility time by the number of immobile-mobile cycles. Average immobile bout time is reported as seconds per bout (s / bout).

Locomotion and Rotarod Performance

The same methods from Experiment 1 (see Chapter 3) for measuring locomotion in the home cage and motor capacity in the rotarod test were used in the present experiment.

Sucrose Preference

Sucrose preference in a subgroup of mice (n = 37) was calculated according to the following formula: % preference = (sucrose solution intake) / (sucrose solution intake + water intake). Sucrose solution intake and water intake were determined by measuring their change in volume over a three hour period after the dark phase had begun and following measurement of home cage locomotion, rotarod test performance, and TST immobility (i.e., approximately 2-4 hours into the dark phase). Kimax® glass graduated cylinders with fitted sipper-tubes held the sucrose solution and water. Sucrose solution concentration was 3%.

Food Intake and Body Weight

The same methods from Experiment 1 (see Chapter 3) to measure the food intake and body weight were used in the present experiment. However, note that food intake could not be measured in group-housed mice.

Statistical Analyses

The effect of the estrous cycle on TST immobile-mobile cycles, TST average immobile bout time, locomotion, sucrose preference, sucrose intake, water intake, and food intake was examined with an independent groups t test (estrus phase vs. non-estrus phase). TST immobile-mobile cycles, TST average immobile bout time, locomotion, rotarod performance, sucrose preference, sucrose intake, water intake, and body weight were each examined with a 2 x 2 univariate ANOVA where the first factor was housing condition (grouped mice vs. individual mice) and the second factor was tumor condition

(tumor-bearing mice vs. control mice). Follow up tests were completed with Tukey's HSD test for multiple comparisons to examine simple effects. Food intake in individually housed mice was examined with an independent groups *t* test. All statistical procedures were performed using SPSS software. Group parameters are reported as mean ± SEM. *P* values < .05 were considered statistically significant.

Results

ID8 Ovarian Tumor

All 37 mice injected with ID8 ovarian carcinoma exhibited visible tumor and/or ascites in the peritoneum at necropsy at the time of post-injection measurement. There was no significant difference in tumor weight between individually-housed mice, .66 ± .13 g, and group-housed mice, .99 ± .39 g, $t(35) = .977$, $P = .34$. Similarly, there was no significant difference in ascites weight between individually-housed mice, 3.76 ± 1.21 g, and group-housed mice, 2.75 ± 1.15 g, $t(35) = .977$, $P = .58$. Control mice exhibited no sign of disease in the peritoneum.

Estrous Cycle

There was no significant effect of estrous cycle on TST immobile-mobile cycles, $t(71) = .084$, $P = .93$, TST average immobile bout time, $t(71) = .357$, $P = .72$, locomotion, $t(71) = .882$, $P = .38$, sucrose preference, $t(35) = .850$, $P = .40$, sucrose intake, $t(37) = .286$, $P = .78$, water intake, $t(37) = 1.259$, $P = .22$, or food intake, $t(42) = 1.083$, $P = .28$.

TST Immobility

As presented in Figure 4-1, there was no significant effect of tumor condition on the number of immobile-mobile cycles, $F(1,69) = .384$, $P = .54$. However, there was a significant main effect of housing condition on the number of immobile-mobile cycles, $F(1,69) = 4.285$, $P < .05$. Individually-housed mice exhibited fewer immobile-mobile cycles over the course of the test than group-housed mice, 15.52 ± .91 vs. 18.50 ± 1.12

cycles. The interaction effect between tumor condition and housing condition on number of immobile-mobile cycles was not significant, $F(1,69) = .014$, $P = .91$.

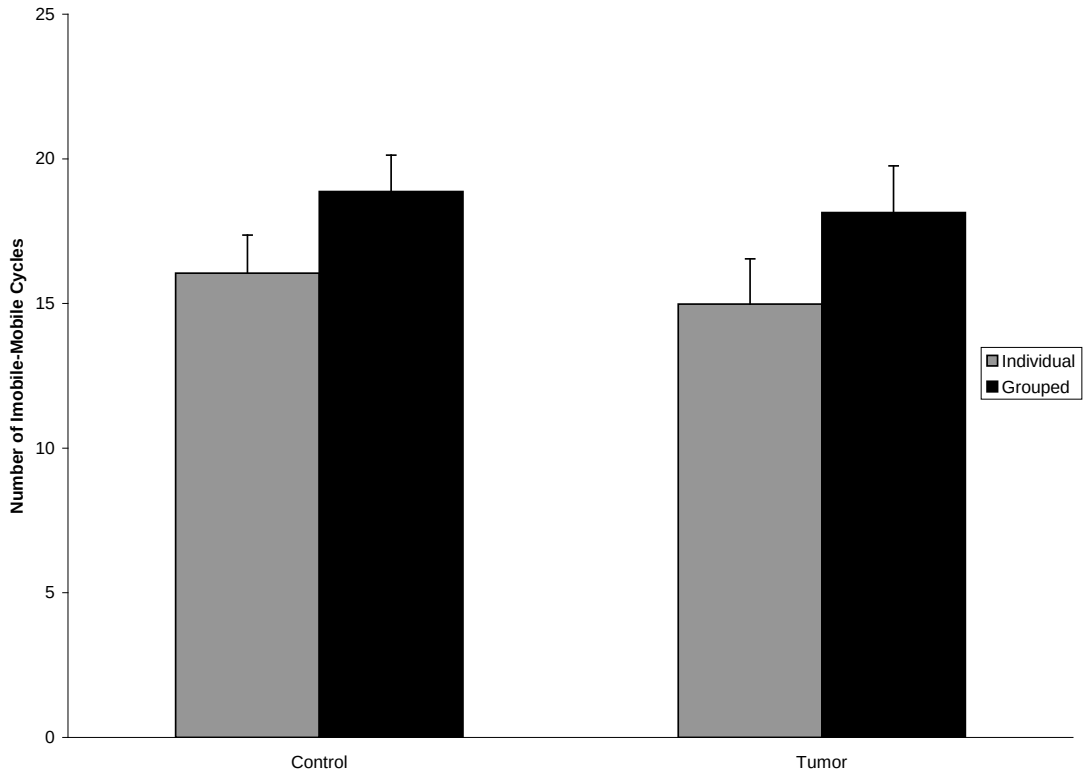

Figure 4-1. Cycles of immobility-mobility among experimental groups in the TST. Mean ± SEM number of immobile-mobile cycles during a 6 minute test in tumor-bearing mice that were individually-housed ($n = 23$) or group-housed ($n = 14$) and control mice that were individually-housed ($n = 21$) or group-housed ($n = 15$). Individually-housed mice exhibited significantly fewer cycles than group-housed mice, $P < .05$.

Figure 4-2 shows that there was not a significant main effect of tumor condition on average immobile bout time, $F(1,69) = .118$, $P = .73$, but that housing condition did have a significant main effect on the average immobile bout time, $F(1,69) = 5.027$, $P < .05$. The average immobile bout time for an individually-housed mouse was longer than that for a group-housed mouse, 16.98 ± 1.03 vs. 13.32 ± 1.27 s / bout. The interaction effect between tumor condition and housing condition on average immobile bout time

was not significant, $F(1,69) = .001$, $P = .97$.

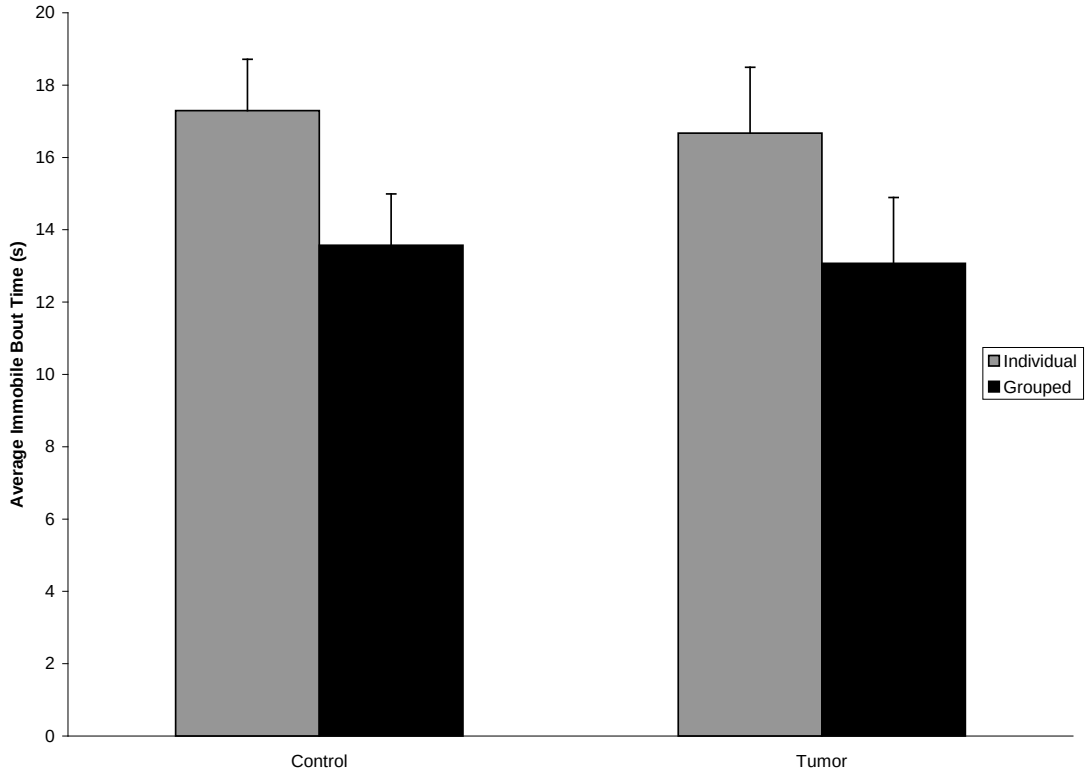

Figure 4-2. Average immobile bout time among experimental groups in the TST. Mean ± SEM immobile bout time during a 6 minute test in tumor-bearing mice that were individually-housed ($n = 23$) or group-housed ($n = 14$) and control mice that were individually-housed ($n = 21$) or group-housed ($n = 15$). Individually-housed mice exhibited significantly longer immobile bout times than group-housed mice, $P < .05$.

Locomotion and Motor Capacity

To test for an effect of ovarian tumor on general motor activity and to further examine the specificity of the effect of social housing on TST immobility, locomotion in the home cage was measured. As presented in Figure 4-3, there was a significant main effect of tumor condition, $F(1,69) = 27.192$, $P < .001$, and housing condition, $F(1,69) =$

3.831, $P < .05$, on locomotion. Tumor-bearing mice moved less than control mice, 857.07 ± 61.71 vs. 1311.50 ± 61.54 cm. Group-housed mice moved less than individually-housed mice, although the difference was relatively small, 998.99 ± 67.65 vs. 1169.57 ± 54.94 cm. The interaction effect between tumor condition and housing condition was not significant, $F(1,69) = .190$, $P = .66$.

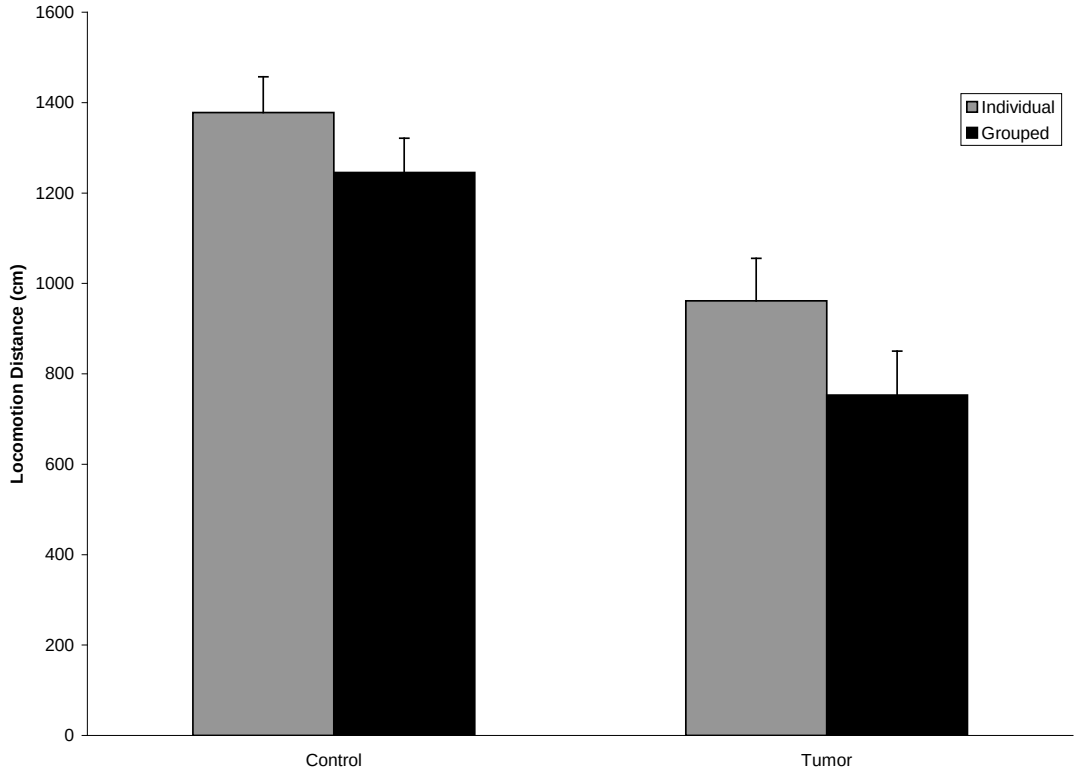

Figure 4-3. Locomotion among experimental groups. Mean ± SEM locomotion distance in tumor-bearing mice that were individually-housed ($n = 23$) or group-housed ($n = 14$) and control mice that were individually-housed ($n = 21$) or group-housed ($n = 15$). Tumor-bearing mice moved significantly less than control mice, $P < .001$, and group-housed mice moved significantly less than individually-housed mice, $P < .05$.

To determine whether less locomotion was attributable to a deficit in motor capacity, rotarod performance was measured. As presented in Figure 4-4, there were no

significant main effects of tumor condition, $F(1,69) = 1.531$, $P = .22$, or housing condition, $F(1,69) = 1.467$, $P = .23$, on rotarod time. Although group-housed control mice appeared to stay on the rotarod for a longer time than the other mice, the interaction effect between tumor condition and housing condition did not reach statistical significance, $F(1,69) = 2.184$, $P = .14$.

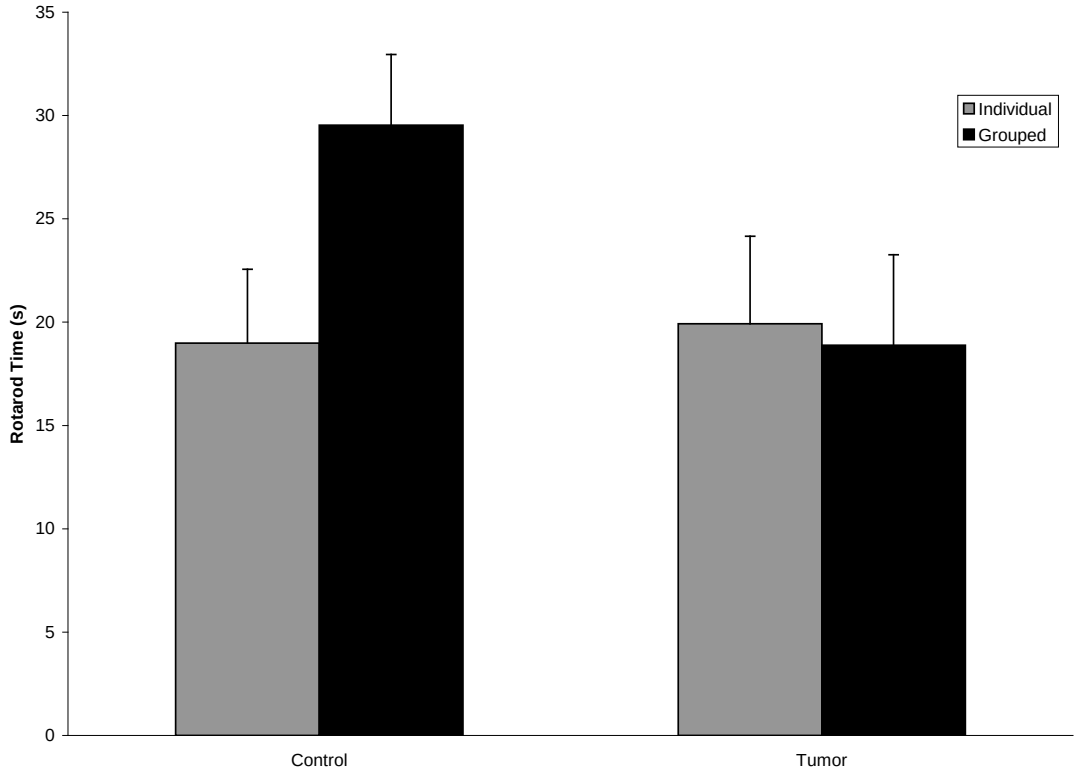

Figure 4-4. Rotarod performance among experimental groups. Mean ± SEM accelerating rotarod time in tumor-bearing mice that were individually-housed ($n = 23$) or group-housed ($n = 14$) and control mice that were individually-housed ($n = 21$) or group-housed ($n = 15$).

Sucrose Preference

To test for effects of ovarian tumor and social housing on anhedonia, sucrose preference was measured in a subgroup of mice ($n = 37$). The sucrose preference of each

mouse was calculated according to the following formula: % preference = (sucrose solution intake) / (sucrose solution intake + water intake). As presented in Figure 4-5, there were significant main effects of tumor condition, $F(1,33) = 4.292$, $P < .05$, and housing condition, $F(1,33) = 5.011$, $P < .05$, on sucrose preference over 3 hours, which were qualified by a significant interaction between tumor condition and housing condition, $F(1,33) = 4.793$, $P < .05$. Follow up tests showed that individually-housed tumor-bearing mice consumed a significantly lower percentage of sucrose solution, 56.54 ± 8.27, than individually-housed control mice, 93.33 ± 8.71, group-housed control mice, 93.75 ± 8.27, and group-housed tumor-bearing mice, 94.77 ± 9.24, all P values < .05.

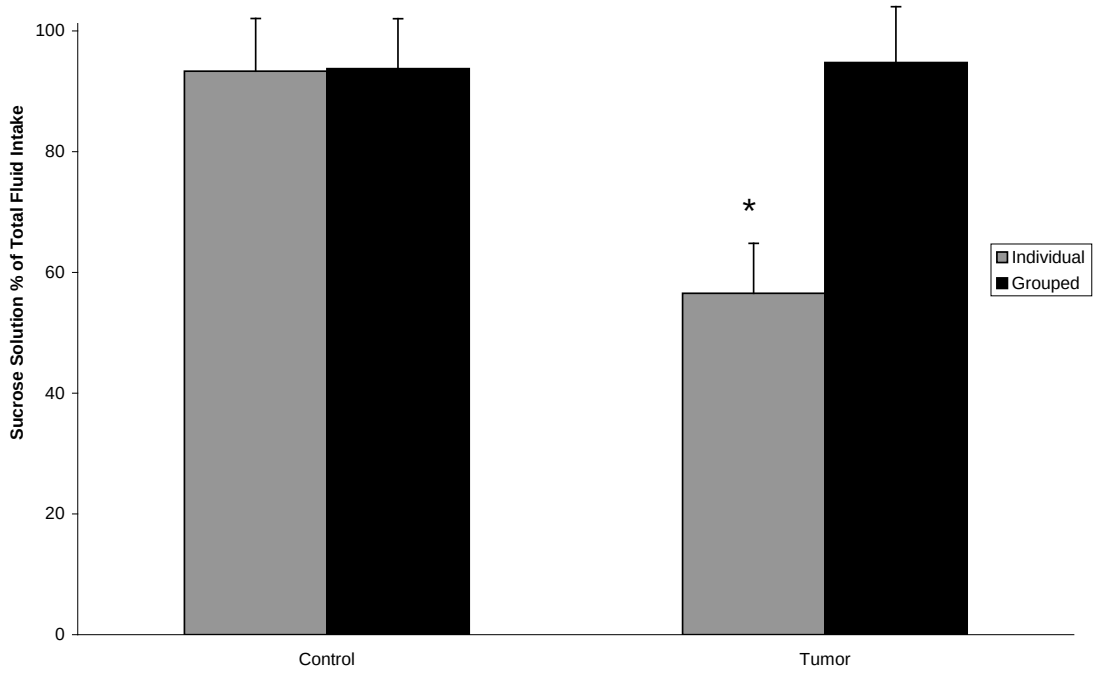

Figure 4-5. Sucrose preference among experimental groups. Mean ± SEM sucrose preference over 3 hours in tumor-bearing mice that were individually-housed ($n = 10$) or group-housed ($n = 8$) and control mice that were individually-housed ($n = 9$) or group-housed ($n = 10$). Sucrose preference = (sucrose solution intake) / (sucrose solution intake + water intake). Individually-housed tumor-bearing mice exhibited a significantly lower preference for sucrose in comparison to all other groups, *$P < .05$.

As Figure 4-6 shows, the effect on sucrose preference was driven primarily by a significant main effect of tumor condition, $F(1,35) = 3.973$, $P < .05$, and housing condition, $F(1,35) = 4.380$, $P < .05$, on sucrose intake. Tumor-bearing mice consumed less sucrose solution than control mice, 5.31 ± .97 vs. 8.08 ± .99 mL/100g - BW. Similarly, individually-housed mice consumed less sucrose solution than group-housed mice, 5.24 ± .97 vs. 8.15 ± .99 mL/100g - BW. The interaction effect between tumor condition and housing condition was not significant, $F(1,35) = .120$, $P = .73$.

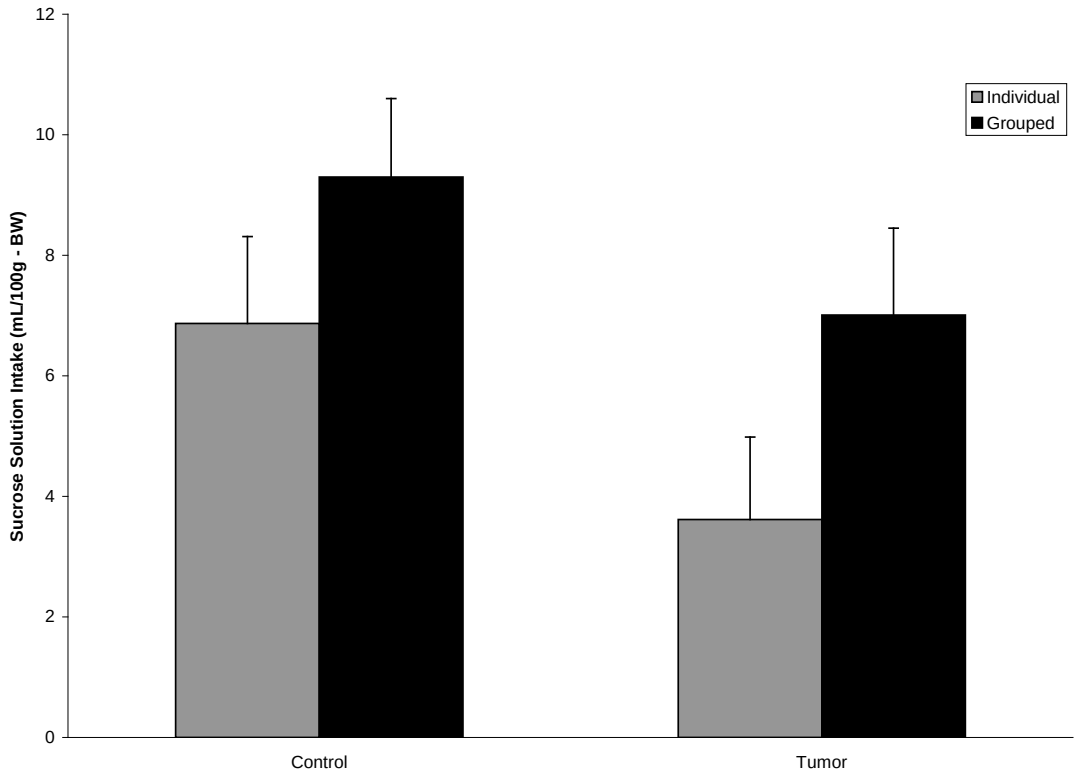

Figure 4-6. Sucrose intake among experimental groups. Mean ± SEM sucrose solution intake over 3 hours in tumor-bearing mice that were individually-housed ($n = 11$) or group-housed ($n = 9$) and control mice that were individually-housed ($n = 9$) or group-housed ($n = 11$). Tumor-bearing mice consumed significantly less sucrose solution than control mice, $P < .05$, and individually-housed mice consumed significantly less sucrose solution than group-housed mice, $P < .05$.

There was no significant main effect of tumor condition, $F(1,35) = .041$, $P = .84$, or housing condition, $F(1,35) = .454$, $P = .51$, on water intake, which is presented in Figure 4-7. Similarly, there was no significant interaction effect between tumor condition and housing condition on water intake, $F(1,35) = 1.302$, $P = .26$.

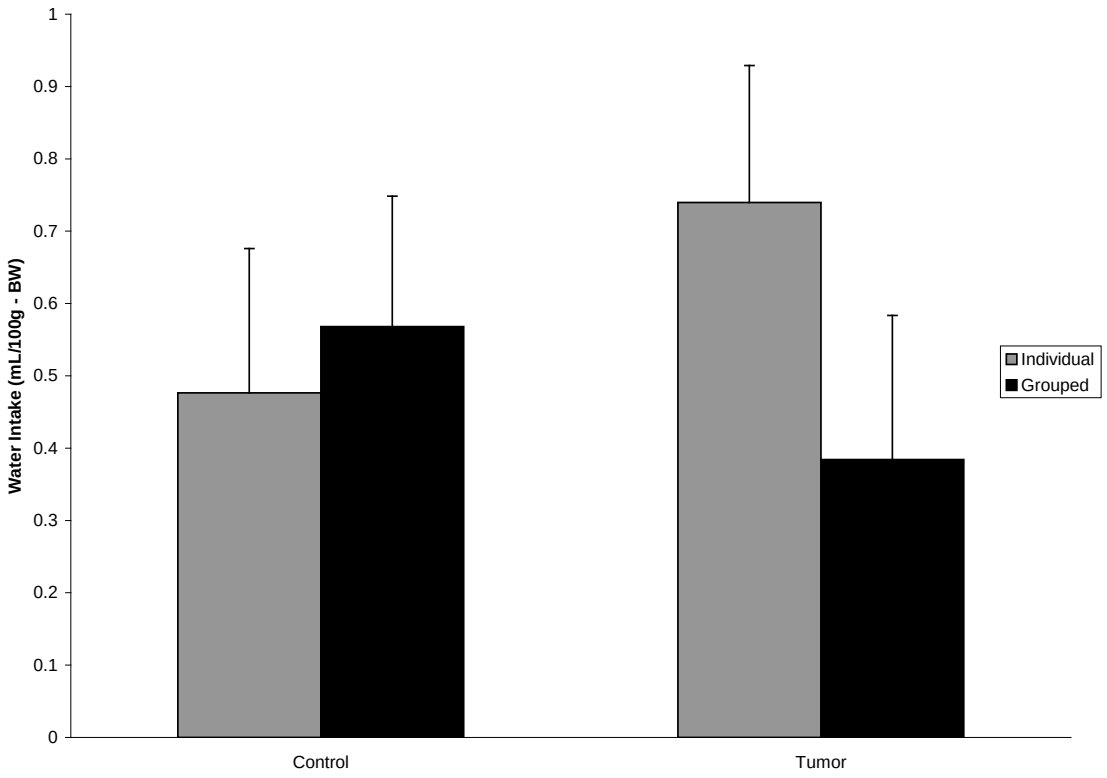

Figure 4-7. Water intake among experimental groups. Mean ± SEM water intake over 3 hours in tumor-bearing mice that were individually-housed ($n = 11$) or group-housed ($n = 9$) and control mice that were individually-housed ($n = 9$) or group-housed ($n = 11$).

Food Intake and Body Weight

To further examine the specificity of the effects on sucrose preference and sucrose intake, food intake and body weight were measured to test for an effect of ovarian tumor on anorexia (note that 24 hour food intake could not be measured in group-housed mice). Figure 4-8 shows that there was no significant difference in food intake per body weight

between tumor-bearing mice and control mice, $t(42) = .486$, $P = .63$. Control mice consumed 146.34 ± 7.22 mg/g - BW. Tumor-bearing mice consumed slightly more, 152.85 ± 10.97 mg/g - BW.

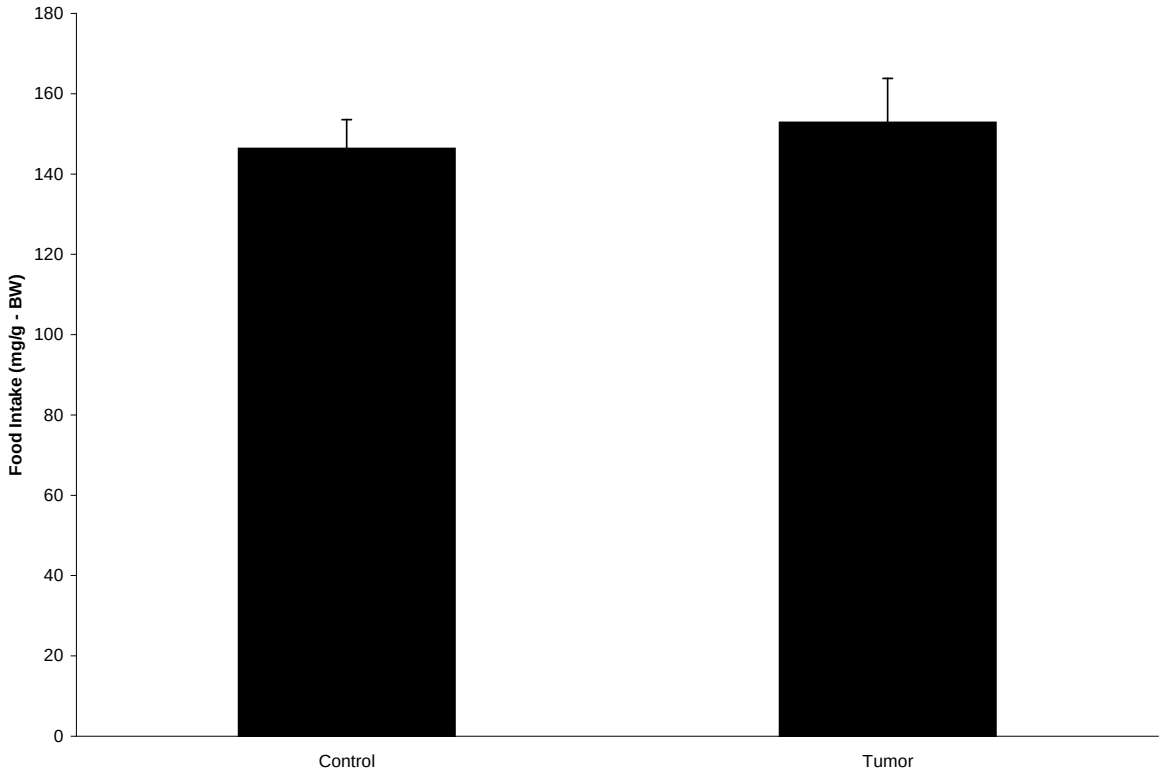

Figure 4-8. Mean ± SEM 24-food intake (mg) per gram of body weight (g-BW) between control mice ($n = 21$) and tumor-bearing mice ($n = 23$). Note that individual food intake could not be measured in group-housed mice sharing the same food source.

Although there was no significant difference in food intake between tumor bearing mice and control mice, there was a significant main effect of tumor condition on body weight, $F(1,69) = 9.758$, $P < .01$ as presented in Figure 4-9. Tumor-bearing mice weighed less than control mice, 23.72 ± .48 vs. 25.83 ± .47. Although group-housed control mice appeared to approach the lower weight of tumor-bearing mice, neither the main effect of housing condition, $F(1,35) = 2.258$, $P = .14$, nor the interaction effect

between tumor condition and housing condition, $F(1,35) = 2.628$, $P = .11$, reached statistical significance.

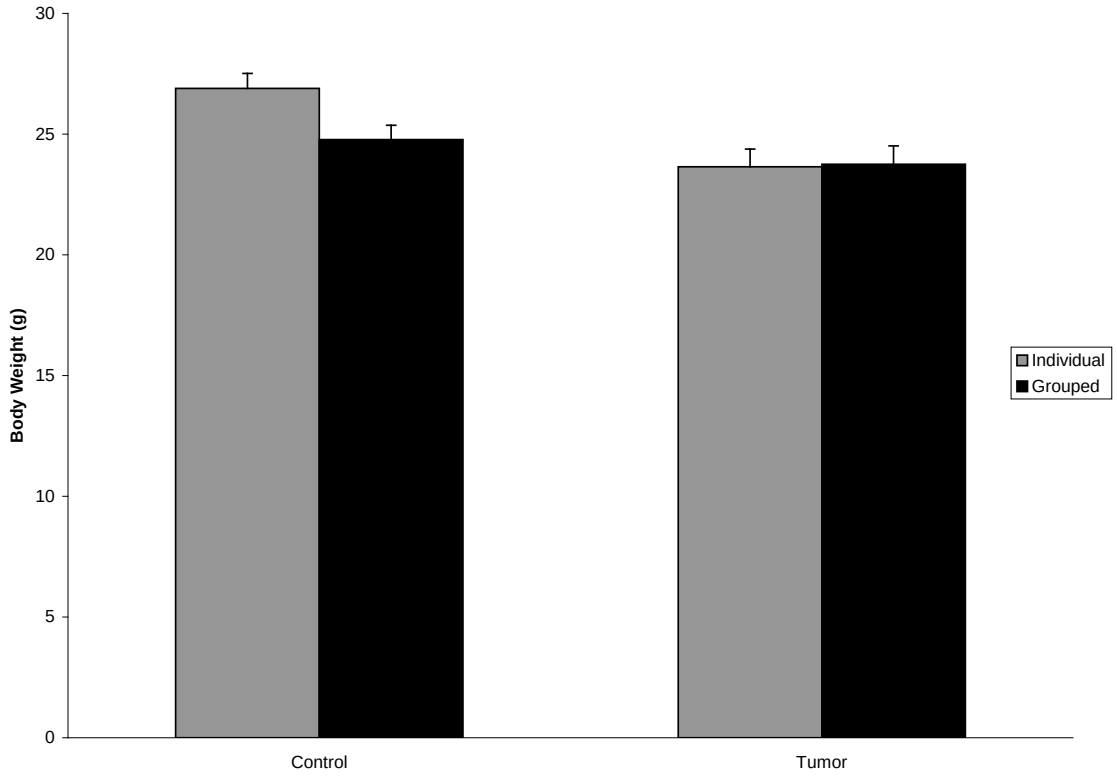

Figure 4-9. Body weight among experimental groups. Mean ± SEM body weight in grams in tumor-bearing mice that were individually-housed ($n = 23$) or group-housed ($n = 14$) and control mice that were individually-housed ($n = 21$) or group-housed ($n = 15$). Tumor-bearing mice weighed significantly less than control mice, $P < .01$.

Discussion

In this experiment, TST immobility, a behavioral measure similar to the behavioral despair/immobility of the FST, was found to differ significantly only between mice that were group-housed and mice that were individually-housed. Individually-housed mice displayed significantly fewer immobile-mobile cycles than group-housed mice, and the immobile bout portion of those cycles was significantly longer for

individually-housed mice compared to group-housed mice. In contrast, individually-housed mice displayed slightly more motor activity than group-housed mice when measured for home cage locomotion. For mice in the tumor group and control group, no significant difference in TST immobility was found. This was despite the fact that tumor-bearing mice exhibited significantly less locomotion in the home cage than control animals. For mice in which sucrose and water intake was measured at the end of the experiment, individually-housed tumor-bearing mice showed a significantly smaller preference for sucrose solution than their tumor-bearing counterparts in group-housed cages and control mice in both housing conditions. Furthermore, the preference level for these latter three conditions was equivalent, although sucrose intake was lower in individually-housed mice compared to group-housed and was lower in tumor-bearing mice compared to controls. Appetite was not significantly different between individually-housed tumor-bearing mice and control mice at the end of the experiment, suggesting once again that anorexia does not confound the sucrose intake results in this model. As was also found in Experiment 1, body weight was significantly lower in tumor-bearing mice than in control mice. Finally, effects of the estrous cycle were tested on TST immobility, locomotion, sucrose intake, and food intake before all other analyses were begun, but no significant effects were found.

The effect of group housing on TST immobility in this experiment is consistent with a previous report that showed a significant difference in TST immobility between female socially isolated C57BL/6J mice and group-housed female mice.[120] The effect of tumor condition on sucrose intake in this experiment replicates the results of Experiment 1 and extends those results by showing a significant difference in sucrose preference between individually-housed tumor-bearing mice and individually-housed control mice. The group-housing component of the experiment also extends the findings from Experiment 1, showing that social housing with other cage mates provides some protection to tumor-bearing mice from anhedonia. This is in line with a previous study

that reported pair-housed female prairie voles were protected from the sucrose-measured anhedonia that was seen in socially isolated prairie voles.[133] Also, the effect of group housing on locomotion is consistent with a previous report that showed socially isolated mice exhibit a slightly greater amount of locomotion compared to group-housed mice.[137]

Despite the robust effect of ovarian tumor on anhedonia, the hypothesis that ovarian tumor would cause significantly more TST immobility was not supported in this experiment. This may be explained in part by a set of reports that suggest anhedonia is more sensitive to peripheral inflammation than TST immobility. Specifically, one study reported that an i.p. injection of 100 ng of IL-1 into mice caused a significant reduction of sweetened milk intake.[145] However, that study's research group showed in a subsequent report that this same dose of IL-1 did not significantly increase TST immobility but rather that a larger dose was needed.[47]

Also, even though ovarian tumor did not cause a significant increase in TST immobility, tumor-bearing mice exhibited significantly less locomotor activity in the home cage. Previous experiments have shown that inflammation-induced TST immobility and locomotion are not always correlated. For example, a study showed that aged mice (80-96 weeks) exhibited normal locomotor activity by 72 hours following i.p. injection of LPS but that TST immobility was still significantly elevated compared to vehicle-injected controls.[49] Similar results were found in a report that showed younger mice (10-14 weeks) exhibited normal locomotor activity by 24 hours after peripheral LPS injection but still manifested significantly higher levels of TST immobility compared to vehicle-injected controls.[46] Thus, it has been suggested that inflammation-induced changes in locomotor activity and TST immobility are mediated by partially different systems in the brain.[48]

The results of this experiment provide additional evidence that suggests ovarian tumor can be a cause of depression in ovarian cancer patients, as the results indicate an increase of anhedonia in tumor-bearing subjects. However, the lack of any significant

effect of tumor on behavioral despair in the TST suggests that the nine signs and symptoms that may constitute MDD in ovarian cancer patients may not be equally affected by the tumor in and of itself. Thus, this finding gives experimental support to the correlative finding in ovarian cancer patients that only the vegetative dimension of depression is significantly correlated with systemic levels of inflammation as indexed by IL-6.[84] That result in ovarian cancer patients was elucidated with the CES-D measure of depression,[146] which has been shown to have a four-factor structure consisting of depressed affect symptoms, positive affect symptoms, interpersonal discord symptoms, and vegetative symptoms.[147] The vegetative symptoms that a respondent can endorse include items such as feeling like everything one does is an effort, feeling like one cannot get going, having restless sleep, and having trouble keeping one's mind on what one is doing. In contrast, the depressed affect symptoms that a respondent can endorse include feeling that one cannot shake off the blues, feeling depressed, feeling lonely, feeling sad, and having crying spells. This latter dimension of the CES-D was not significantly correlated with systemic levels of IL-6 in ovarian cancer patients.

The protective effect of group housing on tumor-induced anhedonia, as measured by sucrose preference in this experiment, provides new experimental evidence that suggests social factors may be highly relevant in the physiological contribution of tumor to MDD in ovarian cancer patients. Tumor-bearing mice in the group-housed condition exhibited essentially the same amount of high sucrose preference as control mice in both the group-housed and individually-housed conditions. Measures of sucrose intake alone suggested that individually-housed mice as a whole were anhedonic in comparison to group-housed mice. As noted previously, social isolation is a significant psychosocial risk factor for MDD in cancer patients.[62] The results here suggest the social isolation risk factor does not simply contribute to a reactive depression but may sensitize the mechanisms by which the tumor itself contributes to depression, at least in the case of anhedonia.

Although speculative, one potential mechanism by which social isolation exerts its depressive-like effect may be through a substantial decrease in estrogen levels. Female mice that have undergone near complete loss of estrogen production by ovariectomy exhibit significantly greater levels of behavioral despair in the FST, and this effect can be reversed by administration of 17β-estradiol.[148] Furthermore, other research in animals has shown that hypothalamic-pituitary-gonadal axis function and consequent estrogen synthesis is suppressed by ongoing stress,[149] an effect that is known in humans as functional hypothalamic amenorrhea.[150] Thus, the prolonged social isolation experienced by female mice in this study, which is likened to chronic stress in the many studies that examine males,[120] may have substantially reduced estrogen in a manner that synergized with inflammatory mediators from the tumor to bring about the largest deficit of sucrose intake seen among the groups in the individually-housed tumor-bearing subjects.

In summary, the results of this experiment provide additional evidence of a causal relationship between ovarian tumor and anhedonia and, to the best of our knowledge, the first experimental evidence that social isolation may increase the magnitude of this effect. Although social isolation was found to cause a significant increase in TST immobility, ovarian tumor did not cause an increase in this measure of behavioral despair. Together, results from both measures of depressive-like behavior suggests that the signs and symptoms of MDD in ovarian cancer patients may not be equally affected by the tumor in and of itself.

CHAPTER 5

EXPERIMENT 3: SYSTEMIC PROINFLAMMATORY AND ANTIINFLAMMATORY CYTOKINES IN MICE WITH OVARIAN TUMOR

Introduction

The goal of Experiment 3 was to examine the effect of ovarian tumor on systemic levels of IL-6, IL-1, TNF-α, IL-17, IFN-γ, IL-4, IL-10, and TGF-β in plasma samples from the previous experiments. It was hypothesized that levels of these cytokines would be significantly higher in tumor-bearing animals compared to controls. It was also hypothesized that each cytokine would mediate, in part, the effect of tumor on sickness and depressive-like behavior.

Method

Subjects

A total of 92 samples were examined for proinflammatory and antiinflammatory cytokines. Thirty-five of these samples were from Experiment 1 (*n* = 18 for tumor-bearing animals; *n* = 17 for control animals). The remaining 57 samples were from Experiment 2 (*n* = 19 for individual-housed tumor-bearing animals; *n* = 17 for individually-housed control animals; *n* = 11 for group-housed tumor-bearing animals; n = 10 for group-housed control animals).

Procedure

After mice were euthanized via rapid decapitation, peripheral trunk blood was collected into heparinzed tubes and centrifuged at 2000 x *g* for 10 minutes. Plasma was then drawn off the sample and stored in 0.5 mL polypropylene tubes at -80^{o} C. For assay, samples were thawed and processed using a 7-plex multiplex kit for simultaneous

measurement of IL-6, IL-1 (soluble form, IL-1β), TNF-α, IFN-γ, IL-17, IL-4, IL-10 (Millipore, St. Charles, MO, USA, #MPXMCYTO-70K), and a singleplex kit for measurement of TGF-β (#TGFB-64K-01). In brief, standards, blanks, controls, and samples were mixed with 5.6 μm polystyrene microspheres, termed *beads,* that are internally color-coded with two fluorescent dyes according to a specific concentration that is matched to a specific capture antibody that is coated on the outside of the bead. Once mixed together in a 96-well filter-bottom microplate, the microplate was attached to an orbital shaker set at 600 rpm for overnight incubation (16-18 hours) at 4^{o} C to facilitate capture of cytokines in the standard, control, and sample wells by the capture antibody-coated beads. Following this incubation, wells were washed and a biotinylated detection antibody was introduced to the wells to attach to cytokines that had been immobilized on the beads during the overnight incubation. Following a 1 hour incubation on an orbital shaker, the reporter molecule, Streptavidin-PE, was added to the wells to incubate for 30 minutes so as to bind to the detection antibodies. The wells were then washed and analyzed using a Luminex® 200™ dual laser detection system. The beads in each well were aspirated and passed through a first laser that excites the internal dyes of a given bead to determine which cytokine is bound to it. A second laser excites the PE bound to the cytokine via the detection antibody to determine the quantity of cytokine on the bead. The fluorescence intensities and known concentrations for each cytokine in the standards of the assay provide a regression curve that is used to convert the fluorescence intensity of a given cytokine in a given sample to an observed concentration for that sample. Background fluorescence intensity from blank wells, i.e., wells filled only with assay media with 0 pg/mL of cytokine, was subtracted from fluorescence intensity values from all other wells before deriving regression curve and observed concentrations. Samples for which the fluorescence intensity was equivalent to that of the blank wells (22% of samples) were assigned a value of 0 pg/mL because their fluorescence intensity in the assay indicated there was no measurable level of cytokine.

For the cytokines used in this study, the reported inter-assay variability ranges from 6.3 to 10.1 percent.

Statistical Analyses

All cytokine distributions were examined for outliers and non-normality. Square root transformations were applied to normalize the data before analysis. The effect of ovarian tumor on systemic levels of IL-6, IL-1β, TNF-α, IFN-γ, IL-17, IL-4, IL-10, and TGF-β in subjects from Experiment 1 was examined with an independent groups *t* test. For subjects from Experiment 2, each cytokine was examined with a 2 x 2 univariate ANOVA where the first factor was housing condition (grouped mice vs. individual mice) and the second factor was tumor condition (tumor-bearing mice vs. control mice). Locomotion data and sucrose intake data in both Experiment 1 and Experiment 2 (individually-housed subjects only) were pooled together to test for mediation of the effect of tumor on these behaviors via cytokines. In the case of sucrose intake, data were converted into z scores before pooling. Cytokines were selected for mediation analysis if there was evidence that tumor had an effect on them and they correlated with the behavior of interest. The multiple mediator model developed by Preacher and Hayes was used for this analysis.[151] This mediation approach provides the advantage of comparing the relative strength of multiple potential mediators without the parameter bias that is had in a single mediator model due to omission of other mediators. Also, in addition to individual significance testing of indirect paths in the mediator model via the Sobel test, the approach also allows testing of all mediators together. All statistical procedures were performed using SPSS software. Group parameters are reported as mean ± SEM. *P* values < .05 were considered statistically significant.

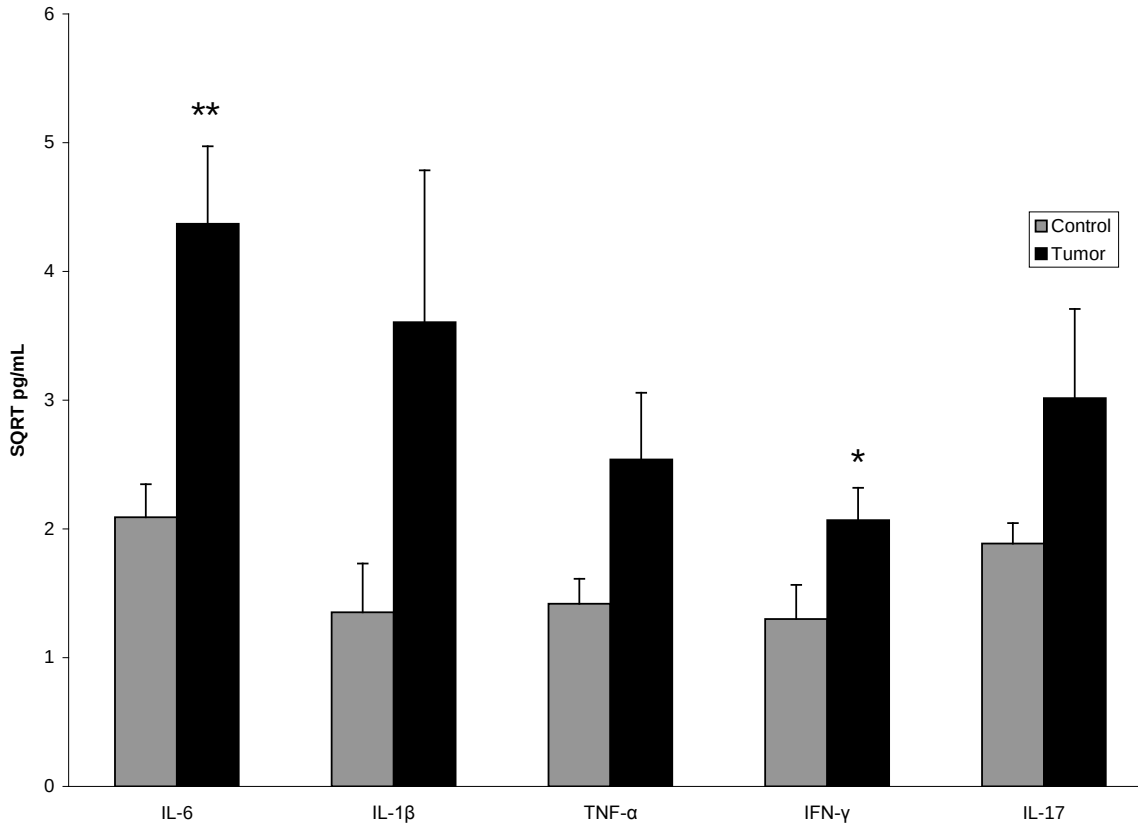

Figure 5-1. Systemic proinflammatory cytokine levels in mice from Experiment 1. Mean ± SEM square root transformed (SQRT) pg/mL of cytokine in plasma of tumor-bearing mice (n = 18) and control mice (n = 17) at 8-12 weeks post-injection. Tumor-bearing mice exhibited significantly higher levels of IL-6 and IFN- γ than control mice, *P < .05, **P < .01. Difference between groups was marginal for TNF-α, P = .06, and IL-1β, P = .08.

Results

Cytokine Response of Subjects in Experiment 1

As presented in Figure 5-1, mice bearing ovarian tumors exhibited higher systemic levels of all proinflammatory cytokines examined in Experiment 1. Significantly higher levels in tumor-bearing animals were observed for IL-6, $t(33) = 3.418$, $P < .01$, and IFN-γ, $t(33) = 2.097$, $P < .05$, and marginally higher levels were observed for TNF-α, $t(33) = 1.982$, $P = .06$, and IL-1β, $t(33) = 1.776$, $P = .08$. The difference in IL-17 between groups was not significant, $t(33) = 1.546$, $P = .13$.

Figure 5-2 shows that the Type 2 antiinflammatory cytokines IL-4 and IL-10 were significantly higher in tumor-bearing mice than in control mice, $t(33) = 2.064$, $P < .05$ and $t(33) = 3.610$, $P < .001$, respectively. In contrast, there was no significant difference in systemic levels of TGF-β between tumor-bearing mice and control mice, $t(33) = .385$, $P = .70$.

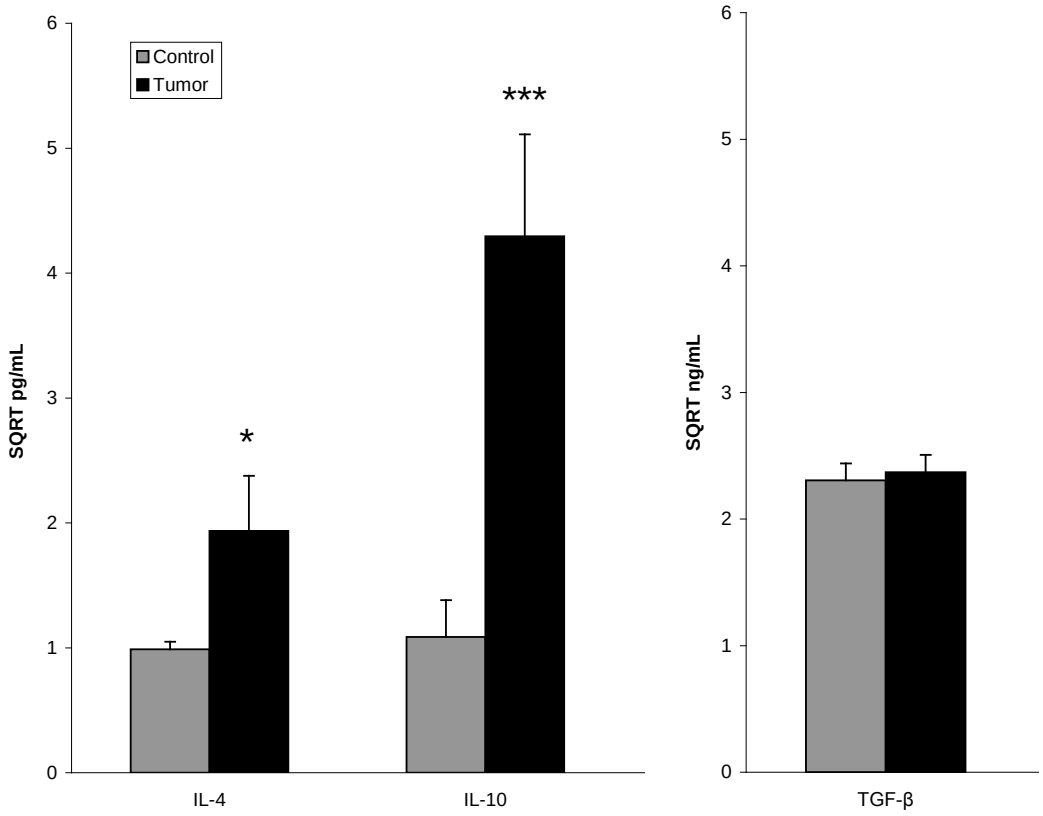

Figure 5-2. Systemic antiinflammatory cytokine levels in mice from Experiment 1. Mean ± SEM square root transformed (SQRT) pg/mL (IL-4, IL-10) or ng/mL (TGF-β) of cytokine in plasma of tumor-bearing mice ($n = 18$) and control mice ($n = 17$) at 8-12 weeks post-injection. Tumor-bearing mice exhibited significantly higher levels of IL-4 and IL-10 than control mice, *$P < .05$, ***$P < .001$.

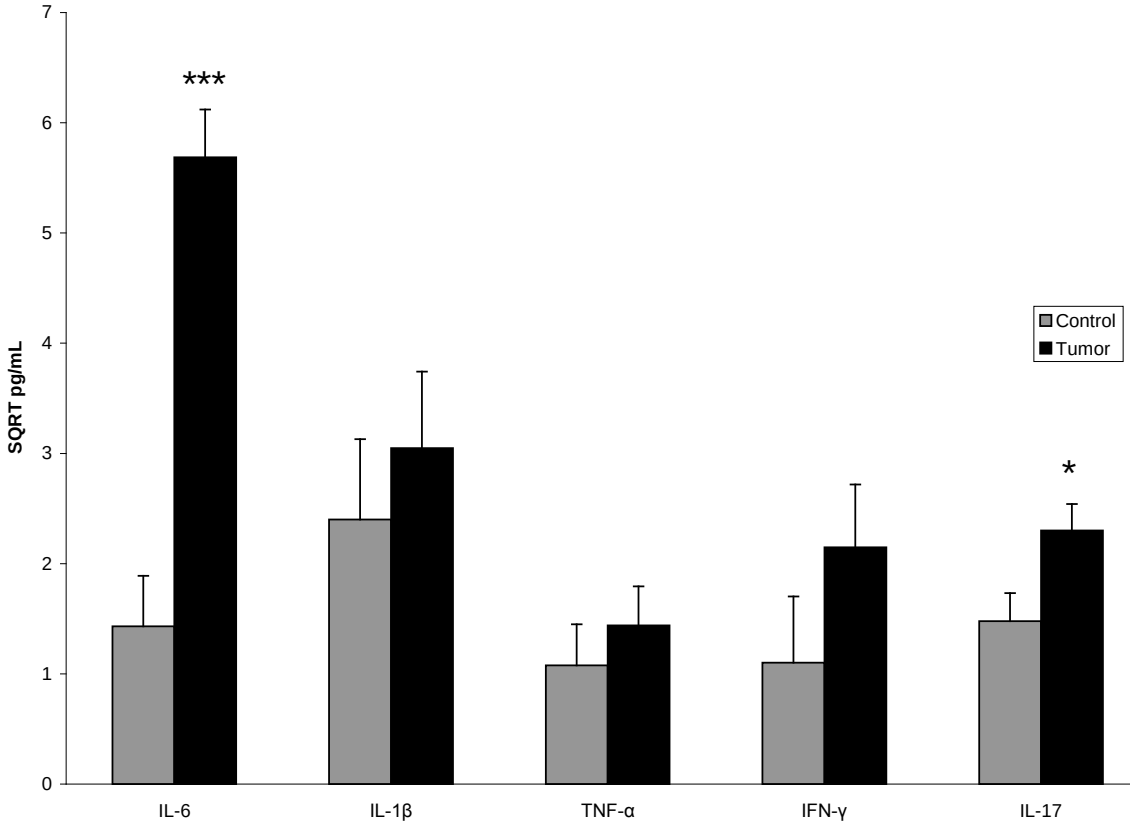

Figure 5-3. Systemic proinflammatory cytokine levels in mice from Experiment 2. Mean ± SEM square root transformed (SQRT) pg/mL of cytokine in plasma of tumor-bearing mice (n = 30) and control mice (n = 27) at 6-12 weeks post-injection. Tumor-bearing mice exhibited significantly higher levels of IL-6 and IL-17 than control mice, *P < .05, ***P <.001.

Cytokine Response of Subjects in Experiment 2

As presented in Figure 5-3, systemic proinflammatory cytokine levels in Experiment 2 followed a similar pattern to that found in Experiment 1. Univariate ANOVAs showed that these levels were significantly higher in tumor-bearing mice for IL-6, $F(1,53) = 45.586$, $P < .001$, and IL-17, $F(1,53) = 5.473$, $P < .05$. The main effect of tumor condition on other proinflammatory cytokines in Experiment 2 was not significant, IFN-γ, $F(1,53) = 1.594$, $P = .21$, TNF-α, $F(1,53) = .657$, $P = .42$, and IL-1β, $F(1,53) = .597$, $P = .44$. The main effect of housing condition on each proinflammatory cytokine

was not significant, all *P* values > .15. Similarly, the interaction effect between tumor condition and housing condition on each of the proinflammatory cytokines was not significant, all *P* values > .24.

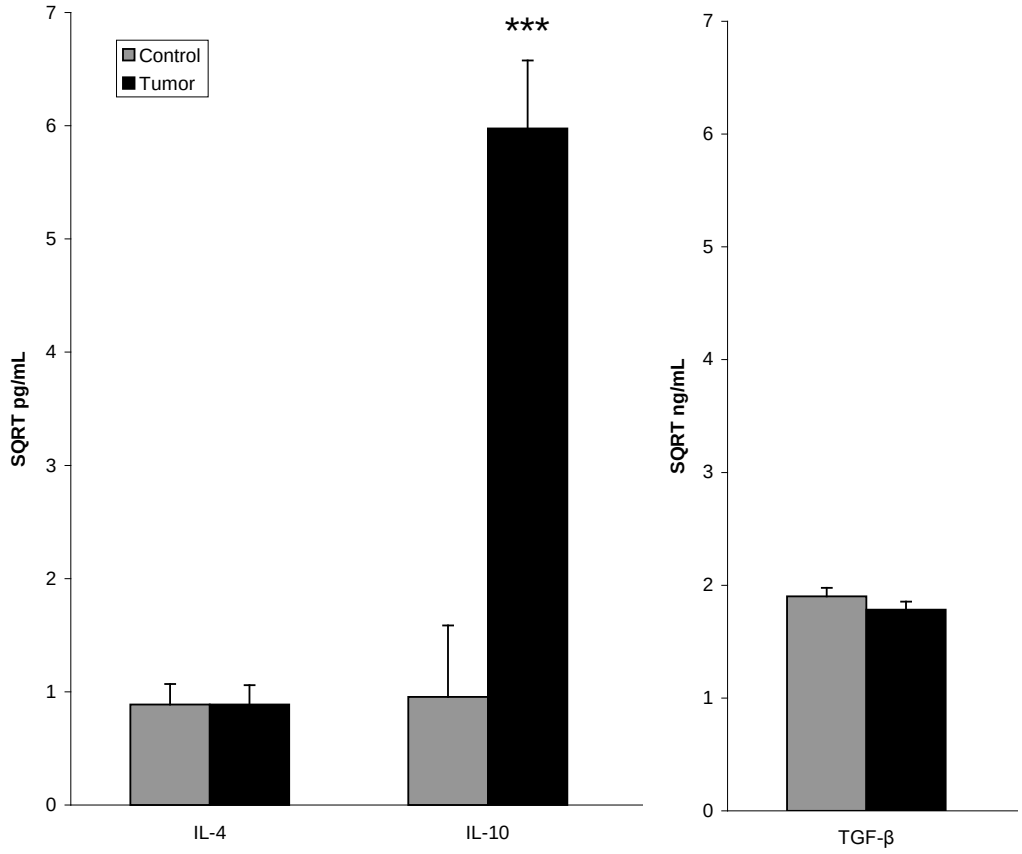

Figure 5-4. Systemic antiinflammatory cytokine levels in mice from Experiment 2. Mean ± SEM square root transformed (SQRT) pg/mL (IL-4, IL-10) or ng/mL (TGF-β) of cytokine in plasma of tumor-bearing mice (n = 30) and control mice (n = 27) at 6-12 weeks post-injection. Tumor-bearing mice exhibited significantly higher levels of IL-10 than control mice, ***P < .001.

As presented in Figure 5-4, IL-10 was again significantly higher in tumor-bearing mice than in control mice, $F(1,53) = 33.298$, $P < .001$. In contrast, IL-4 was not significantly different between tumor-bearing mice and control mice in Experiment 2,

$F(1,53) = .028$, $P = .89$. TGF-β continued to show no significant difference between groups, $F(1,53) = 1.266$, $P = .27$. The main effect of housing condition on each antiinflammatory cytokine was not significant, all P values > .19. Similarly, the interaction effect between tumor condition and housing condition was not significant for any of the antiinflammatory cytokines, all P values > .25.

Mediation Analysis of Cytokines in the Effect between Tumor and Behavior

Given the reliable effect of ovarian tumor on locomotion and sucrose intake from Experiment 1 and Experiment 2, data on these measures in individually-housed subjects were pooled together for mediation analysis ($n = 82$ for locomotion; $n = 66$ for sucrose intake). Of the seven cytokines presented above in which tumor had a significant or marginal effect in Experiment 1 or 2, four of these cytokines showed evidence of a zero-order correlation with locomotion. These cytokines were IL-6, TNF-α, IL-4, and IL-10, and are presented in Table 5-1.

Table 5-1. Zero-order correlations between locomotion and cytokines

Cytokine	*r*	*P*
IL-1	-.12	.28
IL-6	**-.55**	**<.001**
TNF-α	**-.38**	**<.001**
IFN-γ	-.14	.21
IL-17	-.09	.44
IL-4	**-.20**	**.08**
IL-10	**-.41**	**<.001**

cytokines in bold have $P < .10$

Thus, these cytokines were entered into a multiple mediator model with tumor condition as the independent variable and locomotion as the dependent variable to test for a mediation effect as presented in Figure 5-5.

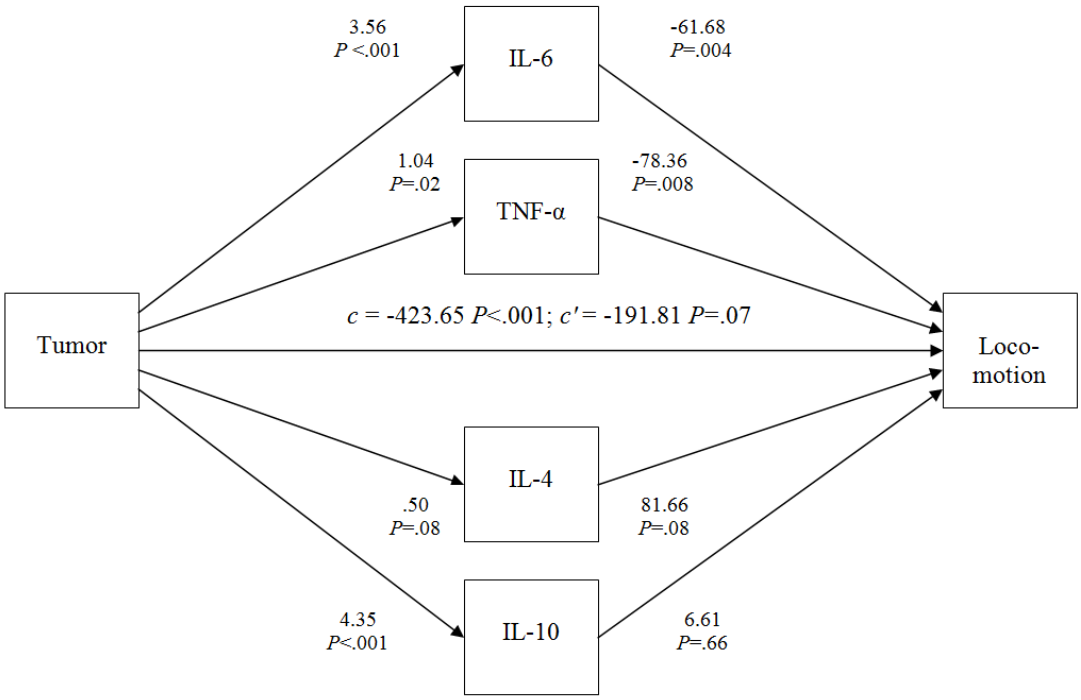

Figure 5-5. Multiple mediator model for the effect of tumor on locomotion (n = 82). Unstandardized regression coefficients and significance values are noted for each path. Total effect between tumor and locomotion is delineated by the c coefficient. Direct effect between tumor and locomotion, adjusting for mediators in model, is delineated by c' coefficient.

As presented in Table 5-2, the indirect effect of tumor on locomotion through IL-6 was significant, Z = -2.84, P = .004. TNF-α showed evidence of a marginal effect, Z = -1.79, P = .07. IL-4 and IL-10 were not significant mediators, P = .20 and P = .65, respectively.

Table 5-2. Indirect effects of tumor on locomotion through proposed cytokines

Cytokine	*ab* coefficient	*SE*	*Z*	*P*
IL-6	**-219.86**	**77.35**	**-2.84**	**.004**
TNF-α	-81.54	45.63	-1.79	.07
IL-4	40.76	31.62	1.29	.20
IL-10	28.80	64.30	.45	.65
All Together	**-231.83**	**78.87**	**-2.94**	**.003**

indirect effects in bold are significant

For sucrose intake, three of the seven cytokines affected by tumor showed evidence of a correlation with this measure of behavior. As presented in Table 5-3, these cytokines were IL-6, TNF- α, and IL-10.

Table 5-3. Zero-order correlations between sucrose intake and cytokines

Cytokine	*r*	*P*
IL-1	-.05	.68
IL-6	**-.33**	**.008**
TNF-α	**-.26**	**.03**
IFN-γ	-.08	.53
IL-17	-.04	.74
IL-4	-.15	.23
IL-10	**-.35**	**.004**

cytokines in bold have $P < .10$

Thus, these cytokines were entered into a multiple mediator model with tumor condition as the independent variable and sucrose intake as the dependent variable to test for a mediation effect as presented in Figure 5-6.

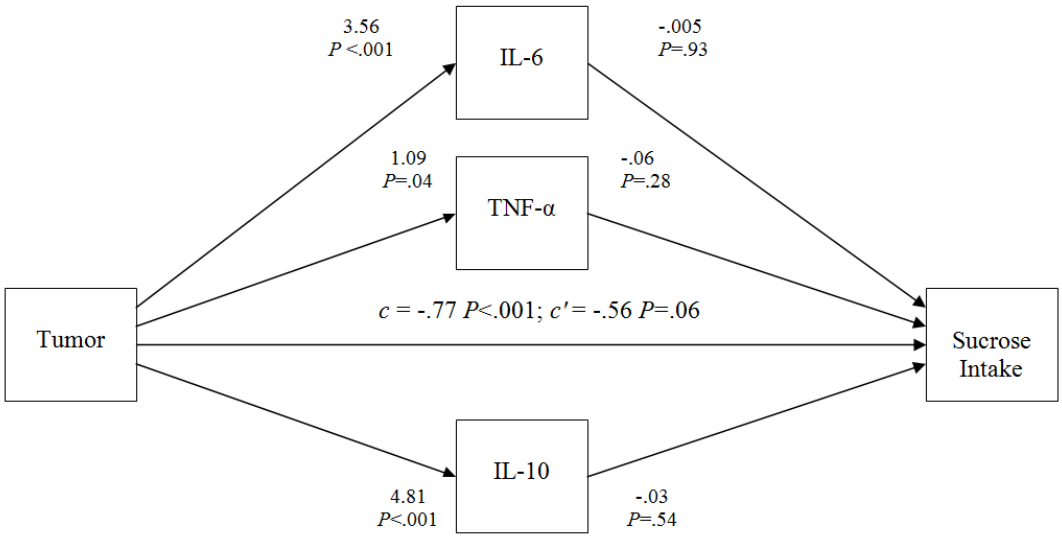

Figure 5-6. Multiple mediator model for the effect of tumor on sucrose intake (n = 66). Unstandardized regression coefficients and significance values are noted for each path. Total effect between tumor and locomotion is delineated by the c coefficient. Direct effect between tumor and locomotion, adjusting for mediators in model, is delineated by c' coefficient.

As presented in Table 5-4, mediation analysis found that none of the cytokines entered into the model were significant mediators of sucrose intake among individually-housed mice from Experiments 1 and 2, all P values > .32.

Table 5-4. Indirect effects of tumor on sucrose intake through proposed cytokines

Cytokine	*ab* coefficient	*SE*	*Z*	*P*
IL-6	-.02	.20	-.09	.93
TNF-α	-.07	.07	-1.00	.32
IL-10	-.12	.19	-.64	.52
All Together	-.21	.20	-1.05	.29

Discussion

In this experiment, both proinflammatory and antiinflammatory cytokines were found to be significantly higher in tumor-bearing mice than in control mice. These effects were largest for IL-6 and IL-10, followed by IFN-γ, IL-17, and IL-4. IL-1β and TNF-α showed marginally elevated levels in Experiment 1, but TGF-β was not found to differ significantly between tumor-bearing subjects and control subjects in either Experiment 1 or 2. There was no significant effect of housing condition on systemic levels of any of the cytokines examined. Mediation analysis showed that systemic IL-6 was a significant mediator of the effect between tumor and locomotion and that the indirect effect through systemic TNF-α was marginal. In contrast, mediation analysis found that none of the cytokines examined in this study were significant mediators of the effect between tumor and sucrose intake.

To the best of our knowledge, this is the first study to report that systemic levels of proinflammatory and antiinflammatory cytokines are elevated in the ID8 ovarian cancer model compared to healthy controls. These findings are consistent with previous reports that IL-6 and TNF-α are found in high levels in the ascites of mice in this model.[102,103] The findings are also consistent with studies that report elevated systemic levels of proinflammatory and antiinflammatory cytokines in ovarian cancer patients.

For example, systemic levels of the proinflammatory cytokines IL-6, IL-8, and VEGF have been found to be higher in ovarian cancer patients compared to healthy controls.[152] Also, the antiinflammatory cytokine IL-10 has been found to be higher in the serum of ovarian cancer patients compared to healthy controls.[153]

The finding that systemic IL-6 was a significant mediator of the relationship between tumor and locomotion but that TNF-α only weakly mediated the effect is consistent with previous studies in the sickness behavior literature. Multiple studies now suggest that endogenous systemic IL-6 from an inflammatory response, in contrast to the other often studied proinflammatory cytokines, IL-1β and TNF-α, is the dominant peripheral mediator of inflammation-induced sickness behavior,[40,154,155,156,157,158] while IL-1β and TNF-α appear to exert their effects more in the central nervous system.[159,160] Examination of the kinetics and peak levels of systemic IL-6, IL-1β, and TNF-α following i.p. injection of LPS in mice has shown that TNF-α peaks first (1-2 hours after injection), followed by IL-6 (3 hours after injection), and then IL-1β (4-hours after injection).[161] However, the systemic levels of IL-6 and TNF-α are substantially higher than IL-1β at these times (ng/mL vs. pg/mL), and, thus, may help explain why these two cytokines of the prototypical three were the more significant peripheral mediators in the present study.

The lack of any significant mediation by cytokines in the effect between tumor and sucrose intake was unanticipated because, in comparison to control mice, tumor-bearing mice in both Experiment 1 and Experiment 2 had significantly lower levels of sucrose intake and significantly higher levels of proinflammatory and antiinflammatory cytokines. This finding suggests at least two possibilities: (1) aside from the cytokines examined in this study, there is a more directly acting peripheral factor(s) of mediation in the causal pathway from tumor to lower sucrose intake; (2) the effect of ovarian tumor-generated inflammatory cytokines on anhedonia is mediated in a non-linear dynamic manner. In fact, there is evidence that both of these possibilities may have been at work

in the present experiment.[162,163] Specifically, a recently developed model of chronic inflammation-induced depressive-like behavior has shown that inoculation of mice with *Bacillus Calmette-Guerin*, an attenuated form of the bacterium *Mycobacterium bovis*, causes a significant decrease in sucrose preference for 2 days after injection. This decrease in sucrose preference is accompanied by a significant decrease in body weight, but both measures normalize by 1 week post-injection. From 1 week to 3 weeks post-injection, systemic IL-6 levels also begin to exhibit a steady decrease toward normal levels, but while this happens there is a reappearance of lower sucrose preference by 3 weeks post-injection (this time in the absence of lower body weight). In contrast to systemic IL-6, a different factor in the periphery becomes significantly elevated by 3 weeks post-injection when the anhedonia reappears. This factor is kynurenine, which as explained in Chapter 1, is produced in greater levels during an inflammatory response as a result of elevated IDO activity. IDO is expressed to a high degree by macrophages and dendritic cells during an inflammatory response, primarily as a result of stimulation by the proinflammatory cytokines, IFN-γ and TNF-α.[164,165,166,167] Investigators of inflammation-induced depressive-like behavior are now beginning to focus more intently on this factor because it has been shown to easily cross the BBB via the large neutral amino acid transporter system[168] and because metabolites of kynurenine, quinolinic acid and kynurenic acid, will agonize and antagonize glutamatergic NMDA receptors, respectively, and thus may represent more proximate mechanisms of inflammation-induced depression that are downstream of cytokines generated during a systemic inflammatory response.[61] This hypothesis is supported by the finding that peripheral administration of kynurenine dose-dependently induces depressive-like behavior in the FST and TST but does not cause change in locomotor activity.[46]

Another downstream factor in the brain of systemic proinflammatory cytokines that may affect depressive-like behavior is suggested by the finding that the HPA axis is often hyperactive in persons with MDD.[169] As noted in Chapter 1, proinflammatory

cytokines such as IL-6 stimulate the HPA axis, and clinical studies have shown that the end product of the HPA axis, the glucocorticoid cortisol, is often elevated in persons with MDD.[170] However, experimental research in animals has not shown an increase in anhedonic behavior as a result of glucocorticoid administration but, rather, has shown a decrease.[171] Also, unlike the high incidence of MDD in patients undergoing proinflammatory cytokine therapy, evidence is lacking that glucocorticoid therapy in patients induces MDD. Despite these findings, the initial hormone in the HPA axis cascade, corticotropin-releasing hormone (CRH), which also is often elevated in patients with MDD (in the cerebral spinal fluid and limbic area of the brain), has been shown to cause an increase in measures of sickness behavior and depressive-like behavior in laboratory animals.[172] Thus, CRH may represent another more proximate mechanism in the brain by which inflammation in the periphery induces symptoms of depression.

In summary, this experiment has demonstrated that ovarian tumor induces significantly higher levels of proinflammatory and antiinflammatory cytokines that are implicated in the link between a chronic medical condition like ovarian cancer and symptoms of depression. Furthermore, it has demonstrated that IL-6 and, to a smaller extent, TNF-α mediate part of the effect between tumor and locomotion but not sucrose intake. These findings may be useful to elucidating the more immediate mechanisms that underlie specific symptoms of inflammation-induced MDD such as psychomotor retardation and fatigue as well as the core symptom of anhedonia.

CHAPTER 6

GENERAL DISCUSSION

Summary of Principal Findings

The principal findings of this dissertation were as follows: (1) Ovarian tumor caused a significant reduction in sucrose intake relative to baseline level and control subject levels (Experiment 1--Chapter 3). Despite the fact that tumor-bearing subjects weighed significantly less than controls at the end of the experiment, there was no significant difference in appetite between tumor-bearing subjects and control subjects. Thus, the difference in sucrose consumption appeared to be a specifically anhedonic effect. (2) The effect of ovarian tumor on sucrose intake and sucrose preference was moderated depending on whether the tumor-bearing subjects were group-housed or socially isolated (Experiment 2--Chapter 4). Subjects that were group-housed consumed significantly more sucrose solution than individually-housed subjects, and this gave rise to a significant interaction in sucrose preference such that individually-housed tumor-bearing subjects exhibited a lower preference for sucrose solution than group-housed tumor-bearing subjects and the control subjects in each housing condition. (3) Ovarian tumor did not cause a significant difference in TST immobility compared to control subjects (Experiment 2--Chapter 4). This result was found despite the fact that tumor-bearing subjects moved significantly less during measures of home cage locomotion, suggesting a functional dissociation between the mechanisms that mediate TST immobility and those that mediate spontaneous motor activity in ovarian tumor-induced behavioral change. (4) Ovarian tumor caused higher levels of systemic proinflammatory and antiinflammatory cytokines compared to control subjects (Experiment 3--Chapter 5). These effects were most robust for IL-6 and IL-10, followed by IFN-γ, IL-17, and IL-4. IL-1β and TNF-α showed marginally elevated levels in Experiment 1, but TGF-β was not found to differ significantly between tumor-bearing subjects and control subjects.

Mediation analysis showed that IL-6 and, to a smaller extent, TNF-α mediated part of the effect between tumor and locomotion but not anhedonia.

The Breadth of Depressive Symptomatology

The hypothesis that subjects with tumor would exhibit significantly more TST immobility was not supported in this study. At first glance, it may seem curious that ovarian tumor would cause a systemic inflammatory response, an increase in anhedonia as indicated by a decrease in sucrose intake, and a psychomotor change as exhibited by a decrease in home cage locomotion but no decrease in mobility during the TST. Review of several animal studies in Chapter 1 provided examples of experiments where the induction of a systemic inflammatory response was followed by decreased sucrose intake, reduction in locomotion, and decreased mobility in the TST. However, as noted in Chapter 4, there is some indication that the "dose of inflammation" in the periphery may need to be higher to affect TST performance than that which is needed to affect anhedonia.[47,145] Also, there is evidence that decreased spontaneous locomotion and TST immobility can occur independently of one another following a systemic inflammatory response.[46,49] Thus, it has been suggested that inflammation-induced changes in locomotor activity and TST immobility are mediated by partially different systems in the brain.[48]

This heterogeneity of results in animal models of inflammation-induced behavior change is not dissimilar to the heterogeneity found in the human clinical literature. Recall from Chapter 1 the findings from Andrew Miller and colleagues that show IFN-α causes neurovegetative and somatic symptoms to occur early during treatment while the depressive, anxious, and cognitive symptoms occur later. Furthermore, the specific neurovegetative features of fatigue and appetite loss were not remedied by treatment with an SSRI. Both of these results led the investigators to conclude that the effects of

proinflammatory cytokines on different symptom dimensions are ultimately mediated by different downstream mechanisms.

Heterogeneity is intrinsic to MDD in general. This is a reflection of the current descriptive approach used to classify mental disorders. The evolution of this approach can be traced back to the pioneering work of 19th century German physician, Emil Kraeplin. Kraeplin, who was one of the first persons to scientifically categorize mood and other mental disorders,[173] was a member of Wilhem Wundt's experimental psychology laboratory from 1882 to 1884. During this time, Kraeplin began work on a nosological classification scheme that went through multiple versions over a period of more than four decades. His fifth edition made radical changes and introduced a much-simplified schema based on his accumulating observations of the links between initial symptoms and eventual outcomes in his psychiatric patients.[174] These changes ultimately gave rise to a listing of 11 relatively mild disorder categories and two serious groups of functional mental disorders in his sixth edition. These two latter categories were termed *dementia praecox* (which would subsequently be renamed *schizophrenia* by Bleuler in 1911) and *manic-depressive psychosis* (which is *bipolar disorder* in today's DSM-IV taxonomy).

Kraeplin's work is considered foundational to the classification system used by the APA today to define mental disorders.[175] Beginning with the third edition of APA's *Diagnostic and Statistical Manual* (DSM-III) in 1980, the classification system used more explicit criteria to define a mental disorder and attempted to remain neutral with respect to etiology of the disorder, a significant deviation from the approach used since the first version of the DSM (DSM-I) published in 1952. For example, the DSM-I used the term *reaction* throughout its contents to explain how a given mental disorder came about. At the time, formulators of the DSM-I believed that all mental disorders were caused by reactions of the personality to various possible factors.[5] Today, however, the DSM-IV, published in 1994 and with minor text revisions in 2000, continues to

emphasize the descriptive, less etiologically-focused approach begun by Kraeplin and adopted by the DSM-III.

The Endophenotype Perspective

Researchers have argued that the current DSM-IV classification for MDD described above likely reflects multiple pathogeneses leading to varying constellations of signs and symptoms.[176] Subsequently, some investigators of MDD find the current heterogeneity problematic to the search for etiological processes in MDD and have proposed the establishment of *endophenotypes* of MDD that attempt to separate the signs and symptoms of the larger syndrome into smaller key components that uniquely constitute MDD and show evidence of having a specific biologically plausible mechanism.[177]

One proposed endophenotype with a specific biologically plausible mechanism is the Anhedonia Endophenotype. Among all of the many features that may be shared by multiple mental disorders, it is noteworthy that anhedonia is a feature relatively specific to the category for depression.[177,178] A large growing body of clinical and experimental research has now demonstrated that anhedonia is associated with functional alterations of dopaminergic neurons emanating from the ventral tegmental area (VTA) and terminating in the limbic system. This system of neurons is known as the mesolimbic dopamine pathway and is seen as an essential component of how the perception of pleasure or reward is mediated by the brain. It has been shown that medication-free, severely depressed patients experience greater reward than healthy controls after ingesting the psychostimulant drug, d-amphetamine.[179] This drug enters presynpatic dopaminergic terminals, provokes dopamine release from vesicles into the cytoplasm, and then facilitates the movement of dopamine from the cytoplasm into the synaptic cleft via a reverse action of the extracellular membrane-bound dopamine reuptake transporter, thus, increasing synaptic levels of dopamine.[11] Other research has shown that patients with

MDD who have been successfully treated with an SSRI (fluoxetine, citalopram, or paroxetine) show a decrease in perceived pleasure of varying activities after administration of sulpiride, a drug that antagonizes D2-like receptors in the mesolimbic dopamine system.[180]

A second proposed endophenotype with a specific biologically plausible mechanism is the Psychomotor Slowing Endophenotype. Psychomotor function is another feature of MDD that well-designed studies have shown differentiates MDD patients from other psychiatric groups, save Parkinson's disease.[177,181] Dopaminergic neurons emanating from an area adjacent to the VTA, known as the nigrostriatal dopamine tract, are critical to how the brain mediates psychomotor function and are damaged in Parkinson's disease.[11] Research using a reaction time task to measure psychomotor function in depressed patients has shown that the performance of patients with MDD is significantly different from that of healthy controls but on par with patients with Parkinson's disease.[182] Further analysis of this result revealed that the effect was driven by patients in the MDD group with a diagnosis of *melancholic* MDD, of which the essential defining feature is anhedonia.

Endophenotypes in Ovarian Cancer?

As noted in Chapter 1, the median prevalence rate for MDD in cancer patients is estimated at 15%. This is about five times the rate for the general population. If one includes other depressive disorders that manifest in cancer patients, in which only some of the symptoms for MDD are present, then the rate in cancer patients is about ten times higher than that found in the general population. However, as just noted above, a diagnosis of MDD or other depressive disorder lacks specificity because of the heterogeneity of signs and symptoms that may constitute the syndrome. A striking example of this is the case of "depression without depression" in which a person is diagnosed with MDD because of the presence of anhedonia and other symptoms but does

not manifest depressed mood.[183] A recent study showed that, among physically ill patients in a hospital setting, 20% of major depressive episode cases present with anhedonia but without depressed mood.[184]

The results of the experiments in this dissertation could be interpreted to reflect "depression without depression," because the core MDD feature of anhedonia was reliably induced by ovarian tumor but behavioral despair in the TST was not. This is not unlike the finding in ovarian cancer patients that IL-6 levels in ascites and systemic circulation do not correlate with depressed mood but do correlate with the more vegetative symptoms of MDD (e.g., feeling like everything one does is an effort, feeling like one cannot get going).[84] Given that the similar finding of psychomotor slowing in the present study and the anhedonia finding both have a common biologically plausible mechanism in the dopaminergic system as described above, it is reasonable to conclude that ovarian tumor contributes to an anhedonic depression endophenotype and psychomotor slowing/vegetative depression endophenotype in ovarian cancer patients.

Dopamine's Connection to Inflammation

It has been proposed that the neurovegetative symptoms and anhedonia that arise during inflammation-induced MDD are mediated by alteration in the dopaminergic systems of the brain.[185] As mentioned in Chapter 1, there is evidence that peripheral inflammation causes a significant increase in synaptic dopamine levels in the nucleus accumbens and a significant decrease in glutamate-related syntaxin levels when anhedonia is present.[55,57] Together, these results are consistent with the notion that tonic dopamine expression in the nucleus accumbens is controlled by afferent glutamatergic neurons synapsing onto dopaminergic neurons.[186] More recent evidence indicates that glutamatergic neurons are controlled by levels of IDO that rise during an inflammatory response. As described in the previous chapter, IDO is expressed to a high degree by macrophages and dendritic cells during an inflammatory response, primarily as a result of

stimulation by the proinflammatory cytokines, IFN-γ and TNF-α. IDO increases the level of kynurenine in the body, which breaks down into kynurenic acid or quinolinic acid that agonizes and antagonizes glutamatergic NMDA receptors, respectively.

It is pertinent to note that the recent exploration by investigators into kynurenine and the glutamatergic system in inflammation-induced depression has resulted in part from earlier research into potential serotonin mechanisms. As noted in Chapter 1, the monoamine hypothesis of affective disorders suggests that serotonin, norepinephrine, and dopamine may play a role in depressed mood and other symptoms of depressive disorders. The role for serotonin and norepinephrine is especially supported by the fact that the majority of antidepressant drugs developed for clinical use (i.e., tricyclics and SSRIs) primarily affect the highly integrated serotonin and norepinephrine systems.[11] Thus, the earlier finding that IDO from an inflammatory response degrades the precursor of serotonin, tryptophan,[187] naturally led to the hypothesis that inflammation causes depression via a depletion of tryptophan and subsequent lack of serotonin. Certainly, research has shown that among patients undergoing cytokine therapy, patients who develop MDD also exhibit significantly lower levels of systemic tryptophan than those who do not develop MDD.[188] However, further research into this question has shown a number of findings that suggest serotonin systems in the brain are not affected by IDO degradation of tryptophan. Robert Dantzer and colleagues have recently shown that systemic tryptophan levels decrease in mice following acute or chronic inflammation in conjunction with increases in depressive-like behavior, consistent with the above finding in humans.[46,164] Yet, the decrease in systemic tryptophan was not followed by reduced tryptophan levels in the brain nor a reduction in brain serotonin levels. Furthermore, direct inhibition of IDO did not alter serotonergic neurotransmission (measured by serotonin turnover rate), even though such inhibition did block depressive-like behavior. Such findings, along with the finding that patients who develop MDD not only exhibit significantly lower levels of systemic tryptophan but also significantly *higher* levels of

kynurenine than those who do not develop MDD following cytokine therapy, led the investigators to test the effect of peripherally-administered kynurenine on depressive-like behavior in mice. Kynurenine dose-dependently induced depressive-like behavior in the FST and TST. Thus, this rigorous line of research led the investigators to conclude that the mechanism of inflammation-induced depression lies more with kynurenine and the downstream glutamatergic effects of its metabolites rather than the closely integrated serotonin-norepinephrine systems of the brain.

Additional studies have shown that inhibition of glutamatergic neurons in the greater striatum with kynurenic acid, an NMDA receptor antagonist, will cause a significant decrease in dopamine levels.[189,190] Given this finding, the converse logic would suggest that the increased levels of dopamine found in the nucleus accumbens as a result of peripheral inflammation or CMS would *not* be associated with glutamatergic inhibition by kynurenic acid but, rather, would be associated with excitation by the other metabolite of kynurenine, quinolinic acid. In fact, this has recently been shown to be the case in CMS. Animals that underwent CMS for 6 weeks not only had increased peripheral levels of kynurenine, similar to what has been found in animals with inflammation-induced depressive-like behavior, but within the striatum, kynurenine was metabolized preferentially toward quinolinic acid rather than kynurenic acid.[191] This result provides further explanation of how peripheral administration of kynurenine would dose-dependently induce depressive-like behavior as it has been shown to do.[46]

Anhedonia, Psychomotor Retardation, and Dopamine

Several researchers have postulated a specific "dopamine hypothesis of depression."[192] Some investigators assert there may be diminished dopaminergic neurotransmission in MDD and that this deficit is responsible in part for the anhedonia, psychomotor changes, and cognitive changes that can characterize the depressive syndrome.[193] However, a close assessment of the findings that examine the dopamine

hypothesis of depression could lead one to conclude that there is "too much" dopaminergic tone in the system.

Before reviewing such findings, it is necessary to understand basic dopamine neurobiology. Dopaminergic neurotransmission is regulated in part by two major types of receptors for dopamine.[11] Activation of D1-like receptors (i.e., D1 and D5) is known to cause an increase in the rate of cyclic adenosine monophosphate (cAMP) production inside the cell, which results in an increase of dopamine synthesis inside the cell. Activation of D2-like receptors (i.e., D2, D3, D4) causes a decrease in cAMP activity with subsequent decreased production of dopamine. D2-like receptor activation can also increase the opening of potassium ion channels on the cell membrane, thus facilitating a hyperpolarization of the neuron and decreasing its excitability. These inhibitory D2-like receptors reside on both the presynaptic cell as an autoreceptor and on the postsynaptic cell.[193] At the terminal end of the mesolimbic dopamine pathway, i.e., the nucleus accumbens, there is evidence that dopamine release occurs in two forms.[186] One form, called *phasic*, is a large burst of dopamine that is released during behavioral response to rewarding stimuli. The second form, called *tonic*, is a constant low level secretion of dopamine that is thought to be modulated by glutamatergic afferents synapsing onto the dopaminergic neurons of the nucleus accumbens. Phasic dopamine is thought to be mostly cleared from the synaptic cleft by reuptake through dopamine reuptake transporter proteins. Tonic dopamine is thought to be primarily removed from the synaptic cleft by the enzymatic action of catechol-o-methyl transferase (COMT) and, as mentioned in Chapter 1, MAO. The resulting final metabolite of these enzymatic processes is homovanillic acid (HVA).

In the early 1990s, Paul Willner and colleagues conducted a series of experiments using the CMS-induced model of anhedonia in rats to examine the effect of chronic stress on dopamine in the nucleus accumbens.[56] They showed that by three weeks of CMS, the animals exhibited anhedonia and *increased* levels of dopamine and its metabolites

throughout the limbic area of the brain. Further experiments showed that in vivo dopamine release in the nucleus accumbens from electrical stimulation of the mesolimbic dopamine pathway was significantly *higher* in CMS animals than controls. They also found at this time that CMS decreased the sensitivity of inhibitory D2-like autoreceptors. Together, the two results made logical sense. An "inhibition of inhibition" of the dopaminergic neuron would result in more excitation of the dopaminergic neuron, causing more dopamine to be synthesized and released when stimulated. By seven weeks of CMS, the problem only got worse as they found that the inhibitory D2-like receptors were actually decreasing in number.

Thus, Willner and colleagues concluded that the neurobiological mechanism of anhedonia had less to do with the amount of dopamine being expressed in the mesolimbic pathway and more to do with a subsensitivity of D2-like receptors to the high level of dopamine in the system. In accord with this interpretation, further experiments by other research groups have come to support a critical role for D2-like receptors in anhedonia. For example, chronic treatment for three weeks with antidepressants of the tricyclic class, SSRI class, and MAOI class has been shown to cause an increase in mRNA of a D2-like receptor (i.e., D3) in the nucleus accumbens of animals.[194] The tricyclic, imipramine, has been shown to block the loss of D2-like receptors in rats that occurs as a result of CMS.[195] In the human clinical literature, the drug pramipexole, a D2-D3 receptor agonist used for the treatment of Parkinson's disease, has shown remarkable results in depression. Specifically, in one study 68% of in-patients with treatment-resistant MDD responded to the drug with a >50% reduction in symptoms on the Montgomery-Asberg Depressive Rating Scale (MADRS).[196] In a double-blind placebo-controlled trial of 175 patients with MDD, pramipexole showed significant reduction of depression symptoms in comparison to the placebo group as measured by the MADRS.[197] Also, as noted above, severely depressed patients experience greater reward than healthy controls after

ingesting d-amphetamine, an effect that is positively correlated with the extent to which the drug binds to D2-like receptors in the nucleus accumbens.[179,198,199]

The role of D2-like receptors in psychomotor retardation was already defined before interest in the receptor for anhedonia took on full form. Quinpirole is a standard D2/D3 agonist used in experimental animal research that causes increased locomotion.[11] Thus, it is logical that a decrease in D2 function in anhedonia would correlate with increases of psychomotor retardation. Complete inhibition of D2 function can result in complete catalepsy, as exemplified by high doses of the D2-like receptor antagonist, haloperidol, a first generation antipsychotic drug used in the treatment of schizophrenia.

The role of D1-like receptors in relation to alterations in D2-like receptors in anhedonia has been examined to a lesser extent. One study showed that CMS caused only a slight increase in D1-like receptor number in rats in striatal areas outside the nucleus accumbens.[195] Furthermore, D1-like receptor number was not significantly altered by antidepressant treatment in accord with the increase in D2-like receptor expression and reduction of anhedonia. This finding is consistent with the view that D1-like receptors are less dynamic than other elements of dopamine neurobiology.[193]

Although they have received less attention, at least one study suggests that hyperfunction of D1-like receptors should not be ruled out as part of the neurobiological mechanism mediating anhedonia. Animals were trained in Skinner boxes to respond for food pellet reward and then divided into groups that would receive either quinpirole, the D2-like receptor agonist noted above, or SKF38393, a D1-like receptor agonist before measuring the groups' responses for reinforcement at three different doses of their respective drug. The authors then demonstrated that animals receiving quinpirole exhibited no change in sensitivity to reinforcement over the range of different doses, but that animals in the SKF38393 group showed a significant reduction in sensitivity to reward when the highest dose was administered. The authors conclude that sensitivity to reward is affected more by D1-like receptors than D2-like receptors because activation of

the former at high levels decreased the subjects' sensitivity to natural food reinforcement.[200] This conclusion is consistent with the high levels of D1-stimulating dopamine and fewer D2-like receptors to block it that Willner found in the early 1990s in subjects with CMS-induced anhedonia.

Together, the foregoing results in this section suggest that there may be "too much" dopaminergic tone underlying anhedonia and psychomotor retardation. As described in the last section, in the case of inflammation-induced anhedonia and psychomotor retardation, this elevated dopaminergic tone may result from altered glutamatergic regulation of tonic dopamine levels brought about by increased kynurenine production and specific metabolism to quinolinic acid in the striatum.

Conclusion

Summary

This project has investigated the question of whether ovarian carcinoma can be a cause of depression symptoms in ovarian cancer patients. Evidence from the project supports the conclusion that ovarian tumors generate a systemic inflammatory response in the body and can contribute in an endogenous manner to the anhedonia and psychomotor slowing and/or fatigue that may characterize the psychological state of patients with ovarian neoplastic disease. Thus, the evidence supports a biopsychosocial (and, thus, non-dualistic) position that pathology in the periphery is capable of inducing "real" depression in patients with cancer or other disease marked by chronic systemic inflammation.

Limitations and Future Directions

Although the findings presented here demonstrate experimental evidence of a causal relationship between ovarian tumor and depression, it is acknowledged that animal models of depression are necessarily limited in their inference because depressive

disorders are constituted by a heterogeneous cluster of symptoms that are diagnosable only in humans. Thus, MDD or other depressive disorders cannot be demonstrated in animals. The project has only set out to show that ovarian cancer can induce one or more behaviors in the mouse that are deemed uniquely representative of depressive disorders in humans based on previous demonstrations of construct, predictive, and face validity as described in Chapter 1. It is also acknowledged that the modification utilized in the TST for this study has not previously been afforded predictive validity with antidepressants. It is possible that the modification affected depressive-like behavior in a way that may have altered the difference or lack thereof between groups on this measure. Future experiments may benefit from using the FST to test for an effect of ovarian tumor on behavioral despair with special care taken to prevent hypothermic exposure in the mouse (e.g., warm-up the water). This would confirm that the behavioral despair facet of depressive-like behavior in mice is not affected by ovarian tumor and would lend further credibility to the notion that ovarian tumor contributes more to an anhedonic endophenotype and psychomotor/vegetative endophenotype than a depressed mood endophenotype. Future experiments may also try using an intracranial self stimulation (ICSS) method to measure anhedonia in the model in which the mouse's rate of operant responding for electrical brain stimulation reward is measured before and after ovarian tumor grafting. Although traditionally used in rats, researchers have begun to advocate protocols for ICSS in mice.[201] Demonstration of both decreased sucrose intake and decreased responding during ICSS in the model would provide converging lines of evidence that ovarian tumor causes anhedonia.

Future investigation of the specific mediators between ovarian tumor and the depressive-like behaviors demonstrated in this model may help further define which aspects of the inflammatory response and which systems in the brain underlie tumor-induced depression in ovarian cancer patients. The investigation of specific inflammatory mediators may be hampered by the finding that particular antiinflammatory

agents not only attenuate depressive-like behavior but also attenuate tumor growth, which would be a good problem. Nonetheless, clinically-used antagonists of proinflammatory cytokines are available and could be tested in the model. For example, tocilizumab is a humanized IL-6 receptor antibody that has been used to treat several types of cancer and was approved by the FDA in 2009 for the treatment of rheumatoid arthritis (RA).[202] The IL-1 receptor antagonist agent, anakinra, is another drug used to treat RA that could have antidepressant effects in the model.[203] As mentioned in Chapter 1, etanercept is a recombinant human TNF-α receptor that binds TNF-α and renders it inactive. This and other anti-TNF agents, such as inflixibmab and adalimumab, are all good candidate antiinflammatory drugs for testing in the model.

Some have argued that targeting inflammatory mediators is a less suitable approach to treating inflammation-induced depression than targeting brain mechanisms because such blockade may compromise the body's resistance to infectious pathogens.[61] In line with this position, a panel of conventional antidepressant drugs should be tested in the model for efficacy and to lend further validity to the model. Experiments that test the effect of different classes of antidepressants on depressive-like behavior in the model may find that predictive validity is afforded at a higher rate to drugs whose mechanism of action is focused on dopaminergic systems. For example, nomifensine and amineptine both antagonize the dopamine re-uptake transporter.[204,205] Also, there is some evidence that the atypical antidepressant bupropion specifically affects dopamine.[206,207] MAOIs should be tested in this respect as well.

Examination of other neurobiological mediators may benefit from an approach that focuses on the connection between dopaminergic and glutamatergic systems or on specific D2-like receptors on dopaminergic neurons. In this respect, drugs like pramipexole, which as noted in the last section is a D2-D3 receptor agonist used for the treatment of Parkinson's disease that also shows antidepressant activity, may yield highly favorable results in the effort to block anhedonic behavior or low locomotion in the home

cage. Also, drugs that block the action of quinolinic acid in the striatum, such as the NMDA receptor antagonist, MK-801,[208] may also attenuate anhedonic behavior in the model and restore normal spontaneous locomotor activity.

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