A ROLE FOR OREXIN NEURON ACTIVITY IN CYTOTOXIC CHEMOTHERAPY-INDUCED FATIGUE

By

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Science is a team sport. Though I take full responsibility and stand alone in defense of this dissertation, I could not and did not conduct this study alone. I was fortunate to collaborate with talented and supportive teams. I appreciate the expert guidance and training I received from my committee, Dr. Daniel Marks, Dr. Lillian Nail, and Dr. Lisa Wood, my dissertation chair. They were generous with advice, discussion, and feedback. In particular, I thank Dan for his guidance with neuroscience and writing. I thank Lisa for being flexible, supportive, and helping me navigate doctoral study and dissertation research. I thank Lillian for her guidance on clinical and career relevance. I thank each of them for helping me obtain funding and covering research expenses.

By chance I came across a journal article by Aaron Grossberg who was completing his doctoral research with Dan Marks. With no prior experience working in an animal model, and already more than half way through clinical research in my doctoral program, I felt compelled to test Aaron's infection model with cytotoxic chemotherapy. Switching research projects mid course is not advised. I wanted to learn more about the brain; I jumped. Thanks for that opportunity, Aaron and Dan.

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Doctoral study and juggling new careers in nursing and in education consumed my weekends for many years. I thank my entire family and my friends who were interested and supportive. I thank my delightful and independent children, Anna and Davis, my husband Dave, and my dog Leo. I appreciated being included in family homework nights while I was in school. I look forward to different family adventures now that I have my weekends back.

Abstract

Background

Fatigue is the most common symptom related to cytotoxic chemotherapeutic treatment of cancer. Peripheral inflammation associated with cytotoxic chemotherapy is likely a causal factor of fatigue. However, the neural mechanisms by which cytotoxic chemotherapy associated inflammation induces fatigue behavior are not known. This lack of knowledge hinders development of interventions to reduce or prevent this disabling symptom. Infection induced fatigue/lethargy in rodents is mediated by suppression of hypothalamic orexin signaling. Orexin is critical for maintaining wakefulness and motivated behavior. Although there are differences between infection and cytotoxic chemotherapy in some symptoms, both induce peripheral inflammation and fatigue. These similarities formed the basis of my hypothesis that cytotoxic chemotherapy induces fatigue by disrupting orexin signaling.

Methods

A cytotoxic chemotherapy cocktail (cyclophosphamide, adriamycin, 5-fluorouracil—CAF) commonly used to treat breast cancer was administered to mice and rats. Fatigue was measured by a decrease in voluntary ambulatory and wheel running activity, measured by telemetry. Reverse transcription real-time PCR was used to measure inflammatory gene expression in the hypothalamus and brainstem. Hypothalamic orexin neuron activity was determined with immunohistochemical examination of nuclear cFos localization and measurement of cerebrospinal fluid levels of orexin-A. Exogenous orexin-A neuropeptide was administered through a

surgically implanted brain catheter.

Results

A single dose of CAF induced fatigue in mice and rats as evidenced by a significant decline in voluntary locomotor activity. CAF induced inflammatory gene expression—IL-1R1 ($p<0.001$), IL-6 ($p<0.01$), TNFα ($p<0.01$), and MCP-1 ($p<0.05$)—in the rodent hypothalamus 6 to 24 hours after treatment, during maximum fatigue/lethargy. CAF decreased orexin neuron activity as reflected by decreased orexin-A in cerebrospinal fluid 16 hours after treatment ($p<0.001$) and loss of normal nuclear cFos localization in orexin neurons 24 hours after treatment ($p<0.05$). Importantly, central administration of 1 ug of orexin-A restored activity in CAF-treated rats ($p<0.05$).

Conclusions

These results demonstrate that cytotoxic chemotherapy induces hypothalamic inflammation and that suppression of hypothalamic orexin neuron signaling has a causal role in cytotoxic chemotherapy-induced fatigue in rodents.

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List of Abbreviations

5-FU	5-fluorouracil
5HT	Serotonin (5-hydroxytryptamine)
ACTH	Adrenocorticotopic hormone
ATP	Adenosine triphosphate
AgRP	Agouti-related peptide
AP	Area postrema
BBB	Blood-brain barrier
CA	Cyclophosphamide-Adriamycin
CAF	Cyclophosphamide-Adriamycin-5-fluorouracil
CARD	Caspase-recruitment domain
CCL-2	Chemokine CL-2=Monocyte chemoattractant protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
CVO	Circumventricular organ
DA	Dopamine
DMH	Dorsomedial nucleus of the hypothalamus
DNA	Deoxyribonucleic Acid
DR	Dorsal raphe nucleus
DVC	Dorsal vagal complex
GABA	γ-aminobutyric acid
HPA	Hypothalamic-pituitary-adrenal
ICE	IL-1β converting enzyme
icv	Intracerebroventricular
IDO	Indoleamine 2,3-dioxygenase
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IHC	Immunohistochemistry
I-κB	Inhibitor of NFκB
IL-1β	Interleukin-1beta
IL-1RI	Interleukin-1 receptor Type I
IL-1ra	Interleukin-1 receptor antagonist
IL-6	Interleukin-6

IL-8	Interleukin-8
ip	Intraperitoneal
IR	Immunoreactive
IRAK	Interleukin-1 receptor-associated kinase
iv	intravenous
JAK	Janus kinase
JNK	cJun NH_2-terminal kinase
LC	Locus coeruleus
LDT	Laterodorsal tegmental nucleus
LHA	Lateral hypothalamic area
LMA	Locomotor activity
LPS	Lipopolysaccharide
MAP	Mitogen activated protein
MCP-1	Monocyte chemoattractant protein
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor-kappa B
NE	Norepinephrine
NLR	Nod-like receptro
NS	Normal saline
NTS	Nucleus of the solitary tract
OVLT	Organum vasculosum of the lamina terminalis
Ox	Orexin
OX1R	Type 1 orexin receptor
OX2R	Type 2 orexin receptor
Ox-A	Orexin-A
Ox-B	Orexin-B
PCR	Polymerase chain reaction
PFA	Perifornical area of the hypothalamus
PPT	Pedunculopontine nucleus
PVN	Paraventricular nucleus of the hypothalamus
qRT-PCR	Quantitative reverse transcriptase PCR
REM	Rapid eye movement
RT-PCR	Reverse transcriptase PCR
SCN	Suprachiasmatic nucleus of the hypothalamus

SFO	Subfornical organ
STAT1	Signal transducer and activator of transcription 1
TMN	Tuberomammillary nucleus of the
TNFα	Tumor necrosis factor-α
VLPO	Ventrolateral preoptic nucleus of the hypothalamus
VMH	Ventromedial nucleus of the hypothalamus
VTA	Ventral tegmental area
VWRA	Voluntary wheel running activity
WT	Wild-type

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Chapter 1: Introduction

How cytotoxic chemotherapy causes fatigue is not known. Fatigue is the most common symptom with cytotoxic chemotherapeutic treatment for cancer (Reilly et al., 2013; Shi et al., 2011). Acute fatigue occurs in most people after each dose of chemotherapy (Berger, 1998; Berger & Farr, 1999; de Jong et al., 2004; Schwartz et al., 2000). In addition, fatigue can persist weeks, months, or years after chemotherapy treatment is completed (Ahles et al., 2002; Berger et al., 2012; Geinitz et al., 2004; Knobel et al., 2001; Minton & Stone, 2008; Ruffer et al., 2003). Fatigue negatively impacts physical and social function, return to work, and quality of life (Curt et al., 2000; Shi et al., 2011; Spelten et al., 2003). The lack of knowledge about how cytotoxic chemotherapy causes fatigue hinders development of interventions to prevent or treat this disabling symptom.

Fatigue is Associated with Peripheral Inflammation

Symptoms associated with cytotoxic chemotherapy such as anorexia, cachexia, sleep impairment, and fatigue are similar to symptoms following infection and injury (Cleeland et al., 2003; Kelley et al., 2003). These symptoms, called “sickness behaviors”, conserve energy and promote recovery from infection in the short term (Hart, 1988; Konsman et al., 2002). Research demonstrates that infection induces peripheral and central inflammation that alters neural signaling in the brain hypothalamus, resulting in fatigue and other sickness behaviors (Dantzer et al., 2008; Hart, 1988; Konsman et al., 2002). Prolonged activation of this innate immune response to infection and injury can interfere with recovery (Dantzer & Kelley, 2007).

Similar to infection, peripheral inflammation induced by cytotoxic chemotherapy

may underlie cytotoxic chemotherapy-induced fatigue (Figure 1.1) (Cleeland et al., 2003; Jager et al., 2008; Wood et al., 2006). Cytotoxic chemotherapy induces inflammatory cytokine production in cell cultures and when administered to rodents (Elsea et al., 2008; Sauter et al., 2011; Wong et al., 2012; Wood et al., 2006b). In clinical studies, elevated blood levels of inflammatory cytokines correlate with fatigue in cancer patients exposed to cytotoxic chemotherapy (Liu et al., 2012; Schubert et al., 2007). Clinical therapy with the cytokine interferon-α induces fatigue in people who previously lacked fatigue (Capuron et al., 2002), providing direct evidence of the association between inflammatory cytokines and fatigue symptoms.

Neural Signaling of Fatigue Behavior

Infection-induced fatigue is associated with suppressed hypothalamic orexin neuron function (Gaykema & Goehler, 2009; Grossberg et al., 2011). In addition, replacement of the orexin-A neuropeptide after inflammatory challenge reversed fatigue (Grossberg et al., 2011). Catecholaminergic projections from the brainstem to the hypothalamus appear to have a role in infection-induced fatigue behavior (Gaykema & Goehler, 2011). This evidence suggests disruption of orexin signaling as an etiologic mechanism of cytotoxic chemotherapy-induced fatigue.

Orexin neuron signaling promotes arousal and wakefulness (Anaclet et al., 2009; Carter et al., 2013; Kantor et al., 2013; Saper et al., 2005). The absence of orexin signaling is associated with narcolepsy, a disorder of disrupted wakefulness and disrupted sleep (Chemelli et al., 1999; Lin et al., 1999; Mochizuki et al., 2004; Nakamura et al., 2011). Suppression—in contrast to absence—of orexin neuron

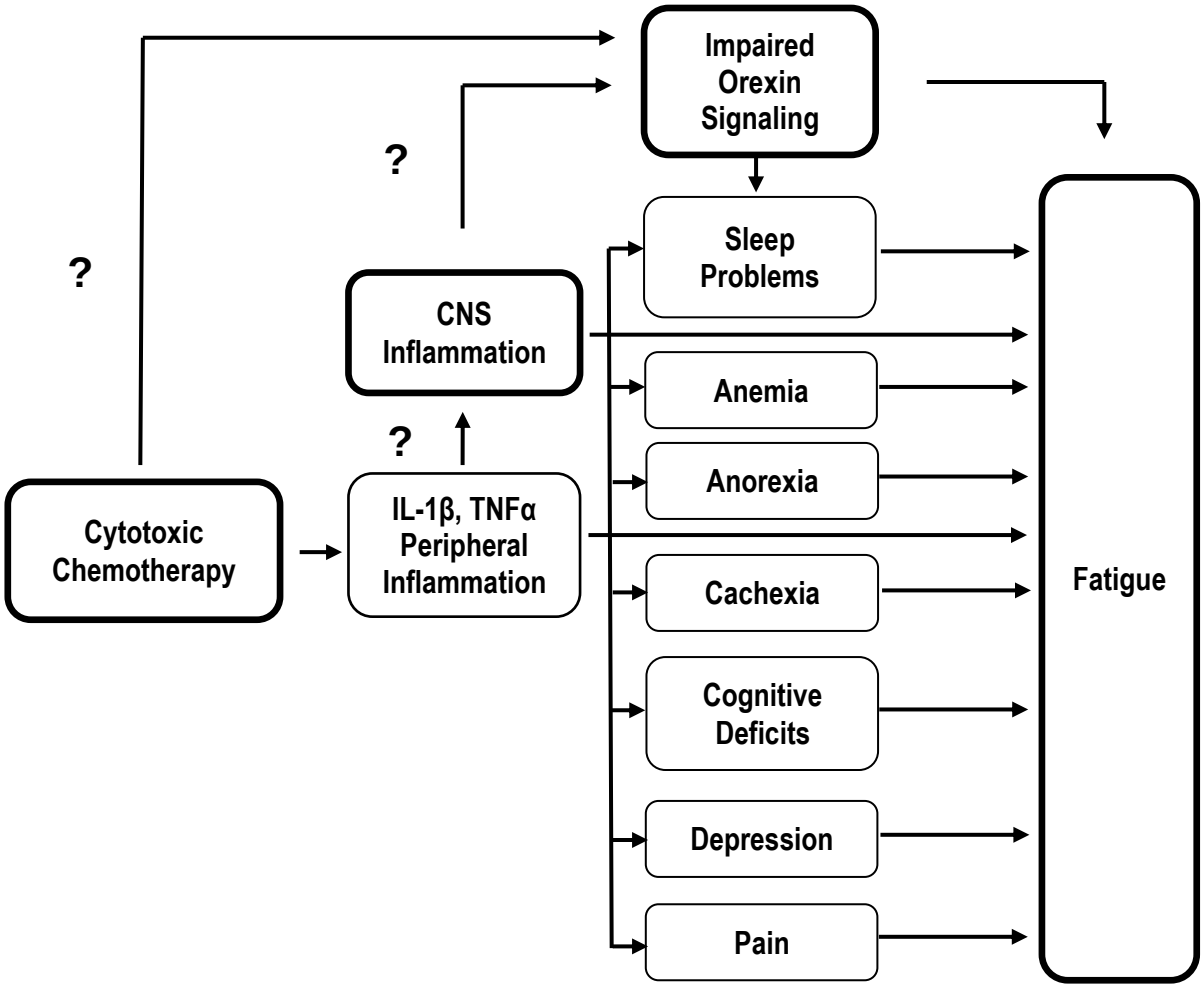

Figure 1.1. Conceptual model of cytotoxic chemotherapy-induced fatigue. Inflammation is an underlying mechanism of impaired orexin signaling and of cancer treatment related symptoms, all possible contributors to fatigue. This study examines the effect of chemotherapy on central inflammation, impaired orexin signaling, and fatigue. CNS-Central nervous system, IL-interleukin, TNF-Tumor necrosis factor. (Modified from Wood, L.J., Nail, L.M., Gilster, A., Winters, K.A., & Elsea, C.R. (2006). Cancer chemotherapy-related symptoms: evidence to suggest a role for proinflammatory cytokines. Oncology nursing forum, 33, 535-54.)

function is likely to disrupt wakefulness. Disrupted wakefulness is likely to be observed or perceived as fatigue.

Peripheral inflammation induced by cytotoxic chemotherapy must eventually act on the central nervous system to cause fatigue behavior. There are few studies of the effects of cytotoxic chemotherapy on central nervous system inflammation or neural signaling (Dantzer et al., 2012; Seigers & Fardell, 2011). Research using lipopolysaccharide in an infection model indicates that orexin neuron dysfunction is associated with fatigue. It is not known if cytotoxic chemotherapy disrupts orexin neuron function. In addition, it is not known if cytotoxic chemotherapy induces inflammation in the hypothalamus or brainstem, brain regions implicated in fatigue behavior. My research addresses these gaps.

Conceptual Model Framing this Study

The purpose of this research was to test if cytotoxic chemotherapy induces inflammatory gene expression in the hypothalamus or brainstem and to test if single or multiple doses of chemotherapy suppress orexin neuron activity in rodents. If orexin neuron activity is suppressed, then an additional purpose was to test if orexin administration reverses chemotherapy-induced fatigue. Fatigue outcomes were measured using mouse models of chemotherapy-induced fatigue modified for rats (Mahoney et al., 2013; Ray et al., 2011; Wood et al., 2006b). The components of Figure 1.1 examined in this study are the effects of peripherally administered cytotoxic chemotherapy on central inflammation, orexin neuron function, and fatigue.

Hypothesis and Specific Aims

Cytotoxic chemotherapy is similar to infection in that both induce peripheral

inflammation and fatigue. The purpose of this study was to test my hypothesis that cytotoxic chemotherapy induces fatigue by disrupting orexin signaling. Since infection induces hypothalamic inflammation, a second hypothesis tested is that cytotoxic chemotherapy induces inflammatory gene expression in the hypothalamus and/or brainstem during acute fatigue. I tested these hypotheses with the following specific aims:

Specific Aim 1. Determine if peripherally administered cytotoxic chemotherapeutic drugs induce inflammatory gene expression in the hypothalamus or brainstem.

Inflammatory gene expression in the hypothalamus mediates the febrile, anorectic, and cachectic sickness behavior responses to infection. Inflammatory signaling in the brainstem may be associated with LPS-induced fatigue behavior (Gaykema & Goehler, 2011). Relative gene expression of the inflammatory cytokines IL-1β, IL-1 receptor type I (IL-1RI), interleukin-6 (IL-6), TNFα, and monocyte chemoattractant protein-1 (MCP-1) in the rodent hypothalamus and brainstem were determined using quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) after peripheral cytotoxic chemotherapy treatment.

Specific Aim 2. Determine if peripherally administered cytotoxic chemotherapeutic drugs suppress hypothalamic orexin neuron activity during acute fatigue.

Inflammation-induced fatigue is associated with suppressed signaling of hypothalamic orexin neurons. It is not known if cytotoxic chemotherapy affects orexin neuron activity. The effects of cytotoxic chemotherapy on rodent orexin neuron activity during acute fatigue were examined with measurement of

cerebrospinal fluid (CSF) levels of the orexin-A neuropeptide and quantification of cFos nuclear expression in orexin neurons.

Specific Aim 3. Determine if orexin replacement reverses cytotoxic chemotherapy-induced fatigue.

The neurotransmitter orexin-A was centrally administered 24 hours after cytotoxic chemotherapy treatment during acute fatigue. Ambulatory activity was measured by telemetry to determine if orexin replacement lessens this fatigue.

Specific Aim 4. Determine if administration of multiple cycles of cytotoxic chemotherapeutic drugs is associated with diminished hypothalamic orexin neuron numbers or activity.

Voluntary wheel running activity (VWRA) in mice is reduced immediately following cytotoxic chemotherapy administration and returns to baseline approximately 1 week after the first dose. The return to baseline activity takes longer after subsequent doses of cytotoxic chemotherapy. Since activity remains persistently decreased for a number of weeks following the 4^{th} dose, mice were sacrificed 3 weeks following the 4^{th} dose of cytotoxic chemotherapy during persistent fatigue. The percentage of orexin neurons with cFos expression and the total number of hypothalamic orexin neurons were determined using immunohistochemistry.

Significance to Nursing

Fatigue is the most common symptom associated with cytotoxic chemotherapy treatment for cancer. It is not known how cytotoxic chemotherapy causes fatigue. There is increasing evidence that chemotherapy-induced inflammation is an

underlying causal mechanism (Bower & Lamkin, 2013; Cleeland et al., 2003; Dantzer et al., 2012; Ryan et al., 2007; Saligan & Kim, 2012; Wood & Weymann, 2013). But, the neural mechanisms by which cytotoxic chemotherapy associated inflammation induces fatigue behavior are not known. Suppressed orexin signaling is associated with infection-induced fatigue in rodents (Grossberg et al., 2011). Since cytotoxic chemotherapy induces inflammation similar to infection, it is plausible that cytotoxic chemotherapy disrupts orexin signaling, resulting in fatigue.

Cytotoxic chemotherapy-induced fatigue is an untreated problem. Understanding etiologic mechanisms of this fatigue will inform development of successful strategies to prevent or treat this debilitating symptom and improve functional recovery from cancer. More than 65% of the 1.5 million people diagnosed with invasive cancer each year will live beyond 5 years after their diagnosis (American-Cancer-Society, 2010; CDC, 2011) — making the treatment of fatigue symptoms an important component of functional recovery from cancer.

Chapter 2: Background

Cancer Treatment Fatigue is a Problem

Fatigue associated with cancer treatment interferes with physical and social function, employment, and quality of life (Curt et al., 2000; Grunfeld & Cooper, 2010; Lee et al., 2011; Munir et al., 2009; Pryce et al., 2007; Spelten et al., 2003). Most people treated with cytotoxic chemotherapy experience fatigue (Ahles et al., 2002; Berger et al., 2012; Bower, 2008; Geinitz et al., 2004; Knobel et al., 2001; Minton & Stone, 2008; Ruffer et al., 2003; Schwartz et al., 2000). Fatigue and the associated decline in physical activity is associated with increased fat mass and decreased lean and bone mass (Berger & Higginbotham, 2000; Hadji et al., 2009; Rooney & Wald, 2007). These detrimental changes in body composition decrease physical function (Ganz et al., 2004; Given et al., 2001) and increase risk of bone fracture (Chen et al., 2005; Kanis et al., 1999), cardiovascular disease (Calvo-Romero et al., 2001), and cancer recurrence (Demark-Wahnefried et al., 2012). As the life expectancy of individuals treated for cancer increases (CDC, 2011), the impact of fatigue from cancer treatment also increases. Given the profound effects of fatigue on physical function, quality of life, and long-term health in cancer survivors, the prevention and/or treatment of fatigue is an essential component of functional recovery from cancer.

Definition of Cancer Treatment Related Fatigue

Cancer treatment related fatigue (CTRF) is defined as “a distressing persistent, subjective sense of physical, emotional and/or cognitive tiredness or exhaustion related to cancer or cancer treatment that is not proportional to recent activity and

interferes with usual functioning" (Piper & Cella, 2010). An accepted definition of CTRF assists with communication and clinical assessment of fatigue. However, this definition reveals some of the challenges in measuring fatigue with the intent to understand predictive, associated, or causal factors. Whether or not something is perceived as "distressing" depends upon other factors such as individual expectations. In addition, the level of distress might change based upon reframing of the situation by the individual (Bussell & Naus, 2010).

Reliable and valid measurement of a subjective symptom presents challenges in experimental design since it is difficult to know if one person's experience of fatigue is qualitatively or quantitatively similar from one day to the next, or to another's person's experience, or adequately captured by the instrument used for measurement (Zwarts et al., 2008). It is plausible that fatigue is an outcome from multiple causal mechanisms, influenced by a variety of factors. This complexity complicates unraveling the predictive, associated, or causal factors of CTRF.

Descriptions of CTRF

The sensations and experiences of tiredness or exhaustion associated with cancer and cancer treatment is addressed in qualitative research on cancer related fatigue. In a synthesis of 26 qualitative research studies on cancer-related fatigue, Scott and colleagues (2011) found cancer-related fatigue was often described as abnormal, overwhelming, unpredictable, longer lasting, and more problematic than tiredness prior to cancer and its treatment. Physical fatigue (described by decreased strength, weakness and/or increased need for rest), cognitive fatigue (described as decreased concentration or attention, or weariness), emotional fatigue (described as

decreased motivation or interest and increased fluctuations in emotions), and social impacts of fatigue were commonly reported and similarly described across studies (Scott et al., 2011). The authors concluded that the word “tiredness” does not adequately capture the multidimensional nature of fatigue experienced by those with cancer-related fatigue. The multidimensional nature of fatigue suggests multiple etiological mechanisms and the influence of other symptoms on the perception and intensity of fatigue (Prue et al., 2006). Taken together, fatigue is a complex symptom that is challenging to accurately define and measure for the purpose of identifying causal factors.

Fatigue is a Sickness Behavior

Infection and injury cause physiological and behavioral changes including weakness, fatigue, anorexia, cachexia, loss of motivated behavior, and cognitive impairment (Dantzer, 2001a; Hart, 1988; Kent et al., 1992). These “sickness behaviors” conserve energy and mobilize resources during the acute phase response to aid recovery from infection (Hart, 1988; Kelley et al., 2003). Increased need for rest and decreased exploratory behavior may also decrease risk of predation or injury at a time of physiological impairment (Dantzer & Kelley, 2007) or decrease further risk of exposure to infectious agents (Quan, 2008). Though sickness behavior is considered to benefit recovery from acute infection, prolonged activation of the innate immune response can interfere with recovery and quality of life (Dantzer & Kelley, 2007). Fatigue that persists weeks, months, or years after completion of chemotherapy treatment interferes with functional recovery and is unlikely to have benefits.

Animal Models Elucidate Mechanisms of Sickness Behavior

Activation of the hypothalamic-pituitary-adrenal (HPA) axis has been well studied in animal models. In 1981, Besedovsky and colleagues challenged lymphocytes with an immune stimulant. When they injected the resulting culture supernatant in rats it caused an increase in plasma corticosterone. The causal factor from the culture supernatant was purified and named interleukin-1 (IL-1) (Besedovsky et al., 1981). It was later found that intraperitoneal (ip), intravenous (iv), and intracerebroventricular (icv) administration of IL-1 provoked a dose-dependent increase in adrenocorticotropin hormone (ACTH) and corticosterone secretion in rat, indicating activation of the HPA axis (Besedovsky et al., 1986; Sapolsky et al., 1987).

Lipopolysaccharide (LPS) is an active fragment of endotoxin from gram-negative bacteria that induces the host response to infection. LPS is commonly used in experimental models to elucidate causal mechanisms of sickness behavior. Extensive research indicates that peripheral LPS treatment induces both peripheral and central IL-1 expression and inflammation (Dantzer, 2001b; Espinosa-Oliva et al., 2011; Gayle et al., 1998; Godbout et al., 2005; Skelly et al., 2013). The mechanisms by which peripheral inflammation induce central inflammation are becoming better understood (Capuron & Miller, 2011; D'Mello et al., 2013). Peripheral LPS also induces many sickness behaviors including fatigue behavior (Gaykema & Goehler, 2011; Grossberg et al., 2011; Skinner et al., 2009). Studies on LPS-induced sickness behaviors and on underlying mechanisms of those behaviors inform elucidation of causal mechanisms of cytotoxic chemotherapy induced fatigue.

Animal Models to Measure Fatigue

Fatigue is a complex symptom and measurement of fatigue is complex (Rodriguez, 2000). Objective activity is measured to quantify the human impact of CTRF and may contribute to understanding of etiologic mechanisms. Animal models measuring objective fatigue control some complexity and support elucidation of etiologic mechanisms (Ray et al., 2008; Ray et al., 2011). The animal models of objective fatigue often measure voluntary physical activity.

Animal models of voluntary activity include locomotor activity such as wheel running, home cage ambulatory activity, and exploratory activity in a new environment. Healthy rodents are active during the dark phase. Illness, bacterial endotoxin (Grossberg et al., 2011; Kozak et al., 1994; Skelly et al., 2013), bacterial antigens (Ottenweller et al., 1998), synthetic double stranded RNA in a viral infection model (Katafuchi et al., 2003), inflammatory cytokines (Park et al., 2011), and cytotoxic chemotherapy (Mahoney et al., 2013; Ray et al., 2011; Wood et al., 2006b) suppress activity. Clinically relevant doses of cytotoxic chemotherapy increase inflammatory markers and decrease activity in a rodent model (Mahoney et al., 2013; Wood et al., 2006b), similar to effects in humans (Pusztai et al., 2004; X.S. Wang et al., 2010).

Fatigue is also measured by food-related exploratory activity (Grossberg et al., 2011), immobility in the forced swim test (Swain & Maric, 1997; J. Wang et al., 2010), treadmill running (Balthazar et al., 2009) and time spent on an electrified grid after falling off a treadmill (Butterworth et al., 2009). Some of these models may involve behavioral components that are in addition to fatigue.

Exploratory activity and activity in a novel environment include a motivational component (Tou & Wade, 2002). It is possible to measure motivation using a push lever (Sagara et al., 2010), although reward and fatigue can confound interpretation. The forced swim test is also used to measure depressive-like behavior (Brocardo et al., 2008). The animal must keep their head above water to prevent drowning. The test is usually conducted for 10 to 15 minutes and immobility—defined as the absence of all movements other than those to keep the head above water—is measured. The forced swim test and the electrified grid with the treadmill are likely to differ in results than tests with routine voluntary activity because they are stressful to the animal; all resources are likely to be mobilized for survival.

Similar to food and novel environment exploratory activity, voluntary wheel running may also involve a motivational and reward component. Physical activity in people, the outcome being modeled, likely includes motivation and reward. It may not be possible to separate fatigue behavior from motivation and reward. In addition, inclusion of motivation and reward may strengthen an animal model of activity and fatigue since it may better replicate human fatigue behavior.

Decreased activity is a sign associated with symptoms of fatigue and also of depression, withdrawal, and weakness in people and in rodents. Diet, age, gender, weight, and genetics in people or strain of rodent may also influence activity (Tou & Wade, 2002). Given the complexity of fatigue and the limitations of brain tissue collection in people to unravel neural signaling, a rodent model of fatigue is critical for discovery of the cellular and neural mechanisms underlying CTRF (Dantzer et al., 2012; Jager et al., 2008; Ray et al., 2011; Wood & Weymann, 2013).

Molecular Mechanism of Inflammation

The innate immune system responds to danger signals from infection such as bacterial components and injury indicating cell death (Matzinger, 2002). Peripheral infection or injury triggers an innate inflammatory response characterized by the production of the pro-inflammatory cytokine IL-1β, a causal mechanism of sickness behavior (Kelley et al., 2003; Kent et al., 1992). Cytokines are low molecular weight proteins involved in inflammation. Cytokines promote local inflammation by increasing vascular permeability and cellular adhesion. They also induce production and release of acute phase proteins from the liver, resulting in systemic inflammation. The inflammatory response is considered a first and important step in healing. In contrast, it can become excessive and result in injury or death.

Because of the potentially deleterious effects of inflammation, the cell tightly regulates IL-1β synthesis, processing, and secretion. Secretion of active IL-1β requires two distinct signaling pathways. The first signal pathway induces the 33 kilo Dalton (kDa) protein pro-IL-1β. This involves recognition of the infection or injury danger signal by pattern-recognition Toll-like receptors (TLR) such as TLR-4 on circulating monocytes or tissue macrophages. In the classical M1 activation response, activation of TLR-4 on macrophages results in the synthesis of proIL-1β via activation of nuclear factor kappa B (NFκB) and the stress-activated protein kinases, JNK and p38 (Bankers-Fulbright et al., 1996; Gordon & Martinez, 2010; Moon et al., 2011).

The second signal pathway induces the processing of pro-IL-1β by caspase-1 to the active 17 kDa IL-1β. Caspase-1 is activated by formation of the multiprotein

inflammasome complex in the cell cytoplasm. The inflammasome complex is comprised of the following three essential components: (1) a NOD-like receptor (NLR) such as NLRP3, (2) the adaptor protein containing a caspase-recruitment domain (CARD), abbreviated as ASC (apoptosis-associated speck-like protein containing a CARD), and (3) IL-1β-converting enzyme (ICE) which is also known as caspase-1 (Mariathasan & Monack, 2007; Martinon et al., 2002). Shown in Figure 2.1, the active secretion of mature IL-1β from macrophages occurs in the presence of danger signals (Mariathasan, 2007), including cytotoxic chemotherapy (Sauter et al., 2011; Wong et al., 2012).

Upon binding to its receptor IL-1 receptor type I (IL-1RI) on cell membranes, IL-1β causes receptor activation and stimulates multiple inflammatory responses. The activated IL-1RI binds to the adaptor protein Myeloid Differentiation primary response gene 88 (MyD88) that activates interleukin-1 receptor-associated kinases (IRAK), leading to the phosphorylation and ultimate degradation of the inhibitor of NFκB (I-κB). Degradation of I-κB frees the transcription factor NFκB to translocate to the nucleus and induce transcription of mRNA encoding a variety of inflammatory signals including TNFα, IL-6, and IL-8.

Another inflammatory signaling pathway activated by binding of IL-1β to the IL-1RI is the cJun NH2-terminal Kinase pathway (JNK), a subgroup of the Mitogen-Activated Protein (MAP) kinases involved in inflammatory signaling. Activation of MAP kinase leads to phosphorylation and activation of Signal Transducer and Activator of Transcription 1 (STAT1), which results in expression of inducible nitric oxide synthase (Dunne & O'Neill, 2003). Through these various signal pathways,

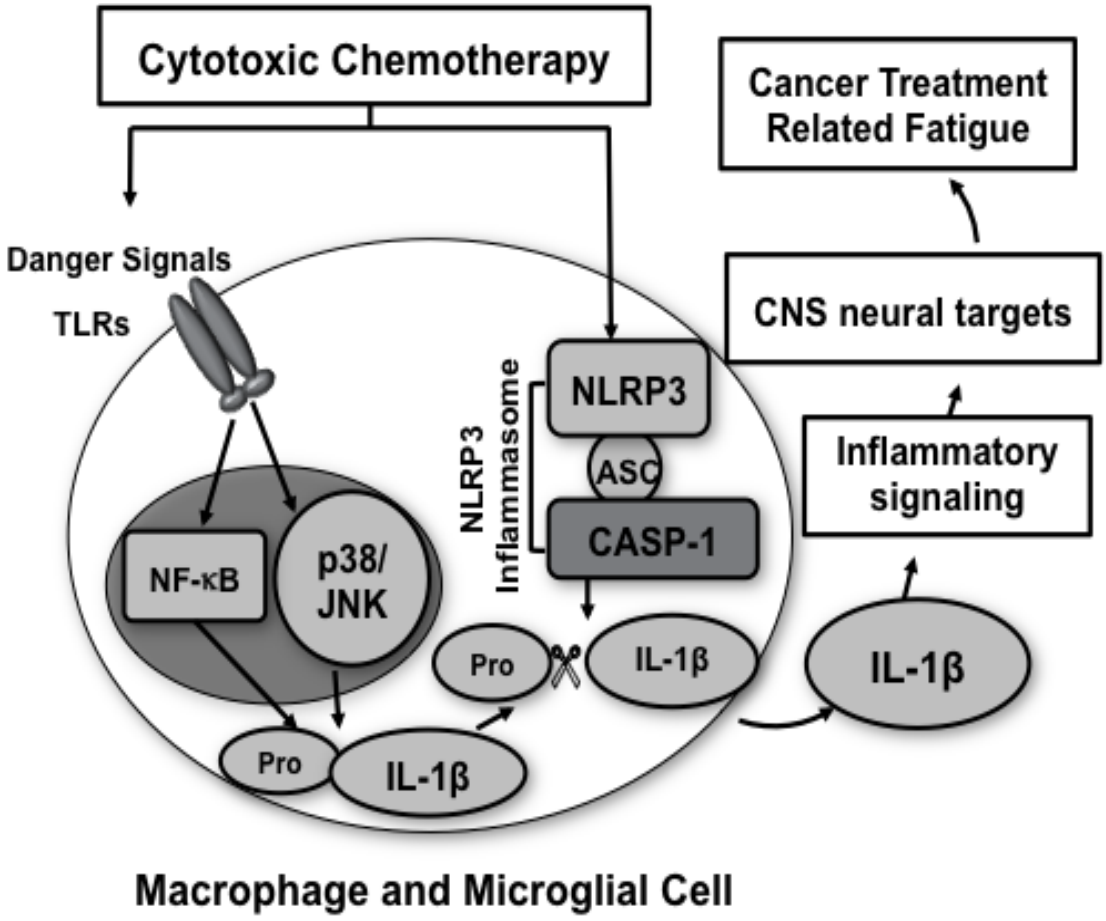

Figure 2.1. Proposed mechanism by which cytotoxic chemotherapy induces IL-1β release and inflammation. Cytotoxic chemotherapy causes cell injury, resulting in release of cellular components that act as danger signals. Danger signals bind Toll-like receptors (TLRs) on macrophages and microglia. Activation of TLRs results in increased expression of pro-IL-1β. Cytotoxic chemotherapy also induces formation of the NLRP3 inflammasome, resulting in cleavage and release of mature IL-1β. IL-1β induces expression of other cytokines causing inflammation. Peripheral inflammation is communicated to the brain, leading to altered neural signaling and fatigue behaviors. Adapted from Wood, L. and Weymann, K. (2013).

IL-1β induces the synthesis of other cytokines such as IL-6 or TNF-α that potentiate inflammation (Basu et al., 2004; Dinarello, 2009). IL-1 β also induces the synthesis of cytokines such as IL-10 and the IL-1 receptor antagonist (IL-1ra) that suppress inflammation. IL-1β (Quan et al., 1998), IL-6 (Vallieres & Rivest, 1997), and TNFα (Nadeau & Rivest, 1999b) are not detected at baseline in wild type (WT) rodents in the brain circumventricular organs including the hypothalamus. These inflammatory cytokines are induced in the brain in response to peripheral inflammation. Inflammation in the brain alters neurotransmitter signaling and induces behavioral responses (Figure 2.2).

Peripheral Inflammation Induces CNS Inflammation and Sickness

A large body of literature demonstrates that peripheral inflammation induces an inflammatory response in the brain at neuroanatomical sites relevant to sickness behaviors (Konsman et al., 2002; Saper et al., 2012; Skelly et al., 2013). Administration of peripheral LPS induces the expression of IL-1β (Quan et al., 1998; Skelly et al., 2013), IL-6 (Skelly et al., 2013; Vallieres & Rivest, 1997), and TNF-α (Nadeau & Rivest, 1999a; Skelly et al., 2013) in brain circumventricular regions that have an attenuated blood brain barrier (Johnson & Gross, 1993). Peripheral inflammation induces central inflammation through humoral, neural, and cellular transmission mechanisms, as shown in Figure 2.2 (Capuron & Miller, 2011; Dantzer et al., 2012). It is possible that these processes occur simultaneously and that the relative importance of these different mechanisms differ for specific sickness responses (Konsman et al., 2002).

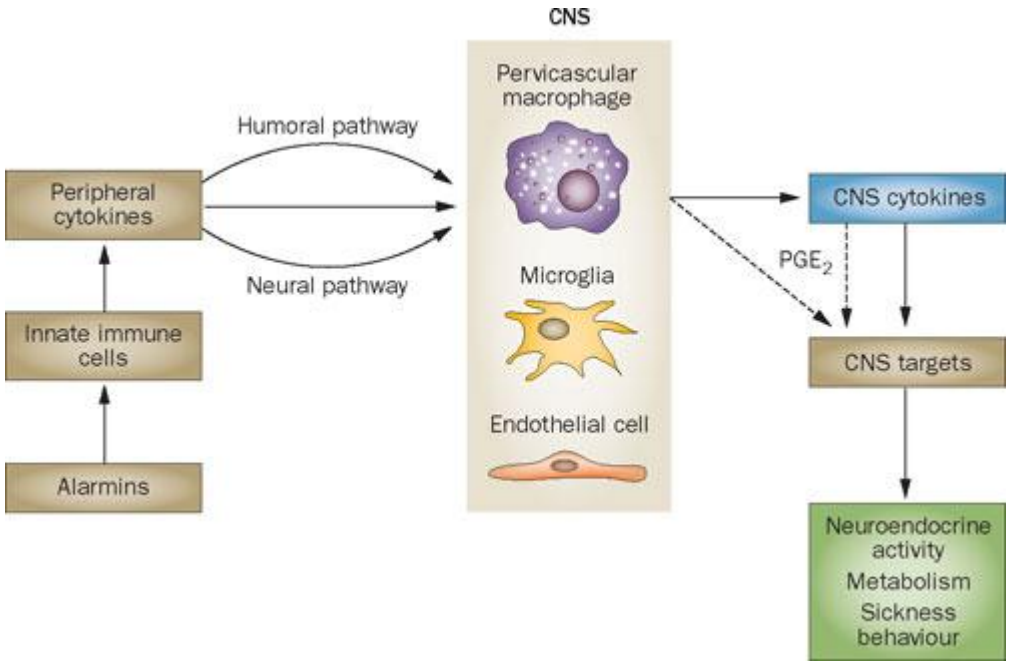

Figure 2.2. Activation of innate immune cells by danger signals (alarmins) leads to production and release of proinflammatory cytokines. This peripheral immune message is relayed to the CNS via humoral and neural communication pathways. This results in local production of proinflammatory cytokines in the CNS, perivascular macrophages, microglia and endothelial cells. The cytokines or the prostaglandins produced ultimately affect neuronal functions, altering metabolism and neuroendocrine activity and resulting in symptoms of sickness. CNS, central nervous system; PGE_2, prostaglandin E_2. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Clinical Oncology (reference citation Dantzer Doi: 10.1038/nrclinonc.2012.88), copyright 2012.

Humoral transmission. The blood-brain barrier (BBB) exists throughout the central nervous system (CNS) and provides separation of circulating blood and brain extracellular fluid called cerebrospinal fluid (CSF). The BBB is attenuated or lacking in specific areas including the choroid plexus and a collection of structures along the third and fourth brain ventricles termed circumventricular organs (CVOs). The CVOs include the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and median eminence at the 3rd ventricle in the hypothalamus, and area postrema (AP) in the brainstem (Johnson & Gross, 1993). These areas lack tight junctions between endothelial cells. They also have fenestrated capillaries and specialized ependymal cells with multiple processes that contact CSF and extend into the CVO body. In addition, the SFO, OVLT, and AP have neuronal terminals (Johnson & Gross, 1993). As a result of these properties, cells in the CVOs are in contact with both circulating blood and CSF. This unique characteristic implicates these regions in a number of homeostatic processes, including body fluid homeostasis (Johnson & Gross, 1993) and communication of peripheral inflammation to the brain (Banks & Erickson, 2010; Licinio & Wong, 1997; Tracey, 2002).

Circulating inflammatory mediators induce inflammation and alter neural signaling in the brain. Circulating inflammatory signals induce brain inflammation by the following mechanisms: (1) activation of brain blood vessels in response to circulating cytokines or chemokines and induction of inflammatory mediators (Banks & Erickson, 2010; Dantzer et al., 2008; Quan et al., 2003), (2) direct activation in brain parenchyma after entry at brain CVOs or following transport across the BBB as

evidenced by production of inflammatory cytokines by brain microglial cells (Licinio & Wong, 1997), and (3) detection and propagation of the inflammatory signal at the CVOs and choroid plexus to affect other brain target areas in a paracrine manner (Konsman et al., 2002; Quan, 2008).

Peripheral inflammation induces inflammatory signaling in brain blood vessels (Licinio & Wong, 1997). Briefly, IL-1 binds to the vascular wall at the third ventricle (Hashimoto et al., 1991). Genes involved in inflammatory signaling, including those for IL-1β, caspase-1, and IL-1RI, are constitutively expressed in blood vessels (Wong et al., 1995; Wong & Licinio, 1994). Peripheral inflammation induces IL-1β and nitric oxide expression in blood vessels (Wong et al., 1996). Nitric oxide mediates the induction of cyclooxygenase-2, which is pro-inflammatory, by IL-1 (Wong et al., 1995). Other effects of peripheral LPS, IL-1, and TNFα in blood vessels include activation of cell adhesion molecules, vascular cell adhesion molecules, E-selectin, and complement regulatory proteins (D'Mello et al., 2013; Skelly et al., 2013). Constitutive expression of IL-1ra mRNA in blood vessels suggests a role as an endogenous vascular protectant agent (Licinio & Wong, 1997). Brain blood vessels are important in the communication of peripheral inflammation to the brain.

In addition to brain blood vessels, the CVOs are involved in communication of peripheral inflammation to the brain (Quan, 2008). Three lines of evidence discussed by Quan (2008) are as follows. First, cells in the CVOs express cytokine receptors (Quan et al., 1998; Vallieres & Rivest, 1999), Toll-like receptors (Laflamme & Rivest, 2001; Rivest, 2003) and CD14 (Lacroix et al., 1998) that detect bacterial components. Second, peripheral inflammation induces expression of inflammatory

cytokines by cells such as phagocytic cells inside the CVOs that can affect other brain areas in a paracrine manner (Nadeau & Rivest, 1999a; Quan et al., 1998). Third, cytokine activation in the CVOs is associated with downstream inflammatory signal activation (Harre et al., 2003; Quan et al., 1997). Bidirectional neuronal projections between the CVOs and the hypothalamus, hippocampus, and amygdala could allow for propagation of the inflammatory signal to these other brain regions. This signaling could occur in the absence of inflammatory cytokine receptors in those other brain regions (Dantzer et al., 2008).

Questions remain about the mechanisms by which inflammation affects neural signaling. The receptor IL-1RI, seemingly required on cells in order to respond to IL-1β, is detected in only limited areas of the brain. It is present in BBB-related areas in the brainstem such as the choroid plexus, vascular endothelium, and area postrema. IL-1R1 is also present on neural cell populations including the basolateral nucleus of the amygdala and the arcuate nucleus of the hypothalamus (Ericsson et al., 1994). Propiomelanocortin (POMC) neurons in the arcuate nucleus, involved in feeding behavior, express IL-1R1 (Scarlett et al., 2007). In contrast, other brain neuron populations that show neuron activation in response to IL-1β lack IL-1RI. IL-1RI was not found on orexin neurons in the hypothalamus even though peripheral and central inflammation suppressed orexin cFos expression and neural signaling. In spite of their differences in IL-1RI expression, altered signaling of both POMC and orexin neurons was found to be associated with sickness behaviors (Grossberg et al., 2010; Grossberg et al., 2011). An explanation of these observations is that IL-1 or TNFα induce secondary signaling pathways that mediate the effects associated with

peripheral inflammation (D'Mello et al., 2013; Ericsson et al., 1994; Licinio & Wong, 1997; Skelly et al., 2013). IL-1 may act on brainstem neurons that then signal to other brain neural populations.

Neural transmission. Cutting the vagus afferent nerve removes neural transmission from most of the body to the brain. With vagotomy, a confounding factor is that humoral transmission is intact. However, these signaling responses might be separable in time. The early phase of fever appears to occur prior to the release of cytokines (Blatteis, 2007), and the fever response to low dose LPS is blocked by vagotomy (Romanovsky et al., 1997). This supports neural transmission as a component of fever response. Vagotomy fails to block suppression of feeding from peripheral LPS or IL-1β, suggesting a mechanism other than the vagus nerve for communication to the brain (Schwartz et al., 1997). In addition to neural vagal signaling, localized peripheral inflammation can be communicated directly to the brain through other neural routes (Belevych et al., 2010; Zhang et al., 2008). It is likely that neural transmission works in tandem with humoral or blood-brain barrier-dependent signaling to communicate peripheral immune status to the brain (Quan, 2008).

Cellular transmission. Infiltration of activated monocytes into the brain can result from peripheral inflammation. Monocyte chemoattractant protein-1 (MCP-1) is one mediator of this central inflammatory response (D'Mello et al., 2009; D'Mello et al., 2013; Thompson et al., 2008). MCP-1, also known as CCL2, is a chemokine that regulates migration and infiltration of monocytes/macrophages. Following liver inflammation, D’Mello et al. (2009) reported a 2-fold increase in cerebral MCP-1

levels that depended upon peripheral TNFα signaling and upregulation of the endothelial cell adhesion molecule P-selectin. MCP-1 increases BBB permeability (Stamatovic et al., 2005), which results in increased infiltration of immune cells across the BBB. Liver inflammation is associated with an eight-fold increase of recruited monocytes in the brain, with most of the recruited monocytes expressing the receptor for MCP-1 (D'Mello et al., 2009; Kerfoot et al., 2006). Mice lacking MCP-1 expression show less leukocyte infiltration into brain parenchyma (Thompson et al., 2008). In addition, inhibition of monocyte recruitment into the brain with anti-P selectin and anti-α_4 integrin antibodies is associated with a decrease in sickness behavior as measured by increased activity (D'Mello et al., 2009). Peripheral inflammation induces pro-inflammatory responses in monocytes; chemokines such as MCP-1 increase the movement of these activated cells into the brain, resulting in brain inflammation and sickness behavior.

Administered Cytokines Cause Sickness Behaviors

Direct evidence that sickness behaviors are induced by inflammatory cytokines comes from development of these behaviors after administration of cytokines. Interferon (IFN)α, IFNβ, and IFNγ are cytokines with strong antiviral, immunomodulatory, and anti-proliferative effects. They are used as treatment for multiple sclerosis, melanoma skin cancer, and chronic viral infections such as hepatitis C. Treatment with these cytokines frequently causes fatigue and depression in individuals with the above disorders who previously lacked those symptoms (Capuron et al., 2002; Simone et al., 2006; Wichers et al., 2005). In addition, healthy disease-free males given one dose of IFNβ experienced increased

body temperature and fatigue at 24 hours after administration (Exton et al., 2002; Reichenberg et al., 2001). Interferons administered for treatment provide an opportunity to examine the undesired symptoms induced by these cytokines. A comparison of cytokine induced depression and idiopathic major depression revealed that in addition to considerable overlap of symptoms, IFNα-induced depression was associated with slowed motor response and weight loss (Capuron et al., 2009). These sickness behaviors were associated with serum changes in inflammatory mediators. In a different study, IFN-induced behavioral symptoms were associated with changes in IL-6, and IL-1ra at 180 minutes after administration of IFNβ (Ohno et al., 1998). IFN-induced behavioral changes were also associated with increased serum levels of IL-6, TNFα, and the soluble IL-2 receptor, but not with changes in cortisol levels (Wichers et al., 2007). IFNα may induce depressive symptoms through induction of neurotoxic kynurenine metabolites rather than through tryptophan depletion (Wichers et al., 2005). Taken together, administration of the IFN cytokines induces sickness behaviors that are associated with changes in inflammatory cytokines.

Symptoms experienced by patients with cancer or Hepatitis C infection and treated with cytokines can be described as neurovegetative or psychological (Capuron et al., 2002; Capuron & Miller, 2004). All individuals treated with cytokines developed the neurovegetative symptoms of fatigue, loss of appetite, and sleep disorders. These symptoms appeared within two weeks of cytokine administration and they were resistant to commonly used anti-depressants. Approximately 30-50% of individuals experienced the psychological symptoms of depressed mood, anxiety,

and cognitive impairment. These symptoms appeared later than the neurovegetative symptoms and they were alleviated by anti-depressants (Capuron et al., 2002). Evidence that administered cytokines induce sickness behaviors suggests mechanisms exist within the brain to promote behavioral response. The neurovegetative symptoms of fatigue, loss of appetite, and sleep disorders, and the psychological symptoms likely have different brain signaling pathways that mediate these different symptoms.

Administration of IL-1β in animal models induces sickness behaviors similar to those induced by IFN administration in people. Animals treated with IL-1β have fever (Anforth et al., 1998; Exton et al., 2002), HPA activation (Besedovsky et al., 1981; Sapolsky et al., 1987), fatigue (Bluthe et al., 2000; Exton et al., 2002; Skelly et al., 2013), decreased food intake (DeBoer et al., 2009; Plata-Salaman et al., 1996; Scarlett et al., 2007) and behavioral depression (Anforth et al., 1998). The animal models allow for teasing apart the causal mechanisms of these different symptoms. Since the animal model appears to reflect human clinical observations, research findings from the animal models are likely to be relevant in understanding underlying mechanisms of sickness behaviors in people.

Distinct populations of neurons that are sensitive to inflammation may signal different behavioral responses (Damm et al., 2013; Dantzer et al., 2012; Saper et al., 2012). For example, anorexia following an inflammatory challenge is dependent on direct activation of proopiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus (Grossberg et al., 2010). Decreased locomotor activity is associated with inflammation-suppressed signaling of orexin neurons in the

perifornical lateral hypothalamic area (LHA) (Grossberg et al., 2011). Understanding the specific impact of inflammation on brain neurotransmitter signaling will shed light on neural mechanisms and treatment or prevention of these behavioral responses.

Inflammatory Cytokines Affect Brain Neurotransmitter Signaling

In contrast to the effects of IL-1β on neural signaling in the brain resulting in sickness behaviors, IL-1β signaling is also involved in normal neuron function related to learning and memory. Expression of IL-1β by glial cells in the brain is increased during long-term potentiation of synaptic transmission. Long-term potentiation improves the ability of two neurons to communicate across a synapse. IL-1β binds to the IL-1RI receptor that is abundant in the hippocampus (Besedovsky & del Rey, 1996; Friedman, 2001). The hippocampus is a brain region important in learning and memory. IL-1 receptor antagonist (IL-1ra) blocks IL-1β from binding its receptor, IL-1R1. IL-1ra blocks potentiation. This evidence suggests a normal signaling role of IL-1β in the brain (Schneider et al., 1998). Analogous to the benefit of the inflammatory response in healing, low levels of cytokines may increase synaptic plasticity, learning, and memory. The high levels of cytokines associated with injury and infection may alter this neural signaling, resulting in decreased synaptic plasticity, learning, and memory (Besedovsky & del Rey, 2011; del Rey et al., 2013).

Localized injury or infection in the periphery activates the paraventricular nucleus of the hypothalamus (Belevych et al., 2010). Localized inflammation in the periphery with local expression of IL-1, IL-6 and COX-2—but without detectable levels of IL-1β in the blood or the brain—resulted in fever and in decreased locomotor activity. Furthermore, the resulting febrile and locomotor responses were blunted in animals

deficient in functional TLR4, IL-1RI, IL-6, or COX-2 (Zhang et al., 2008). These results indicate that detectable levels of systemic IL-1β are not required for signaling of localized inflammation by direct neural routes. These results also reflect the difficulty in capturing a transient change in IL-1β levels in discrete brain regions, even when inflammatory signaling and sickness behaviors are present.

Peripheral inflammation alters hypothalamic neural signaling of the HPA axis, of POMC neurons involved in eating behavior, and of orexin signaling involved in fatigue behavior. These effects are thought to promote recovery from infection in the short term. Peripheral inflammation also triggers the cholinergic anti-inflammatory system. Activation of the vagal afferent nerve by IL-1 results in the release of acetylcholine by the vagus efferent nerve. This is the vagal inflammatory reflex (Tracey, 2002). Acetylcholine then binds to the α7 receptor on macrophages and inhibits cytokine production, thus decreasing inflammation (Tracey, 2007; Wang et al., 2003). Stimulation of the HPA axis and the cholinergic anti-inflammatory system are homeostatic mechanisms to limit the inflammatory response. These different lines of evidence indicate that inflammatory cytokines act in the brain to alter neural signaling.

Chemotherapeutic Drugs Induce Inflammation

Cellular and preclinical evidence. A number of cytotoxic chemotherapeutic drugs with different mechanisms of action induce inflammatory cytokines in cellular and preclinical models. Taxanes are mitotic inhibitors that induce tubulin polymerization, causing non-functional microtubules and resulting in antitumor activity. Taxanes induce mRNA expression of TNFα, IL-1α, and IL-1β in mouse

macrophages. Taxanes act through a TLR4/MyD88-dependent pathway for induction of inflammatory cytokines (Byrd-Leifer et al., 2001). Induction of TNFα by taxanes is inhibited by IL-10 (Bogdan & Ding, 1992) and by dexamethasone, both which have anti-inflammatory action (Ding et al., 1990).

Cisplatin, a treatment for solid tumors, binds DNA and results in crosslink adducts. This binding interferes with DNA repair and culminates in apoptosis (Jordan & Carmo-Fonseca, 2000). Treatment of bone marrow-derived macrophages with cisplatin or LPS increased tumoricidal activity and production of TNF and IL-1 (Singh et al., 1991; Suresh & Sodhi, 1991). Cisplatin causes dose-dependent activation of p38 MAPK in proximal renal tubule cells and increased serum levels of TNFα. A hydroxyl radical scavenger prevents the activation of p38 MAPK and TNFα, indicating that hydroxyl radicals have a role in cisplatin-induced activation of p38 MAPK and TNFα. This signaling may mediate cisplatin-induced acute renal injury (Ramesh & Reeves, 2005). This evidence demonstrates that cisplatin induces inflammation that might be directly involved in cell injury.

Adriamycin and daunorubicin are anthracycline anti-tumor antibiotics that inhibit topoisomerase II during transcription, inhibiting DNA replication (Fornari et al., 1994). Anthracyclines are associated with cardiac toxicity leading to increased risk of heart failure (Pai & Nahata, 2000). In mice, adriamycin induces serum IL-1β and IL-1ra. This increase is associated with IL-1RI expression in cardiac tissue (Zhu et al., 2011). Pretreatment of mice with IL-1ra prior to adriamycin administration protects mice from cardiac damage and loss of cardiac function (Zhu et al., 2010). The inflammatory responses to adriamycin and daunorubicin may be mediated by

release of IL-1β through activation of the NLRP3 inflammasome (Sauter et al., 2011). Some of the adverse inflammatory effects of anthracyclines would therefore be reduced by suppression of inflammatory signaling (Sauter et al., 2011; Zhu et al., 2010).

Etoposide, another cytotoxic chemotherapeutic agent, also inhibits toposiomerase similar to the action of anthracyclines. The structure of etoposide differs from anthracyclines. Furthermore, etoposide does not have the cardiac toxicity of the anthracyclines. Etoposide induces an increase in serum levels of IL-6 in healthy mice and induces sickness behaviors of decreased food intake and decreased activity (Wood et al., 2006b). In mouse macrophages, induction of p38 MAPK activity was associated with an increase in IL-6 gene expression and IL-6 protein release into the culture media (Wood et al., 2006b). These mechanistically and structurally distinct cytotoxic chemotherapeutic agents share a common ability to induce an inflammatory response which may be involved in the development of undesired toxicity and undesired cancer treatment related symptoms.

Clinical evidence. A number of clinical studies on cancer report increased levels of IL-6 or TNFα in blood. These inflammatory cytokines are positively associated with advanced disease and worse outcomes in prostate cancer (George et al., 2005), metastatic breast cancer (Ahmed et al., 2006), colorectal cancer (Knupfer & Preiss, 2010), pediatric neuroblastoma (Egler et al., 2008), multiple myeloma (Wierzbowska et al., 1999) and advanced hepatocellular carcinoma (Zhu et al., 2009). These studies are in individuals with cancer, indicating that cancer and treatment increases levels of peripheral cytokines. The cellular and preclinical

evidence discussed above demonstrates that cytotoxic chemotherapy, in the absence of cancer, induces inflammatory cytokines. It is plausible that administration of cytotoxic chemotherapy with the intent of killing cancer cells results in higher levels of inflammatory cytokines because of increased levels of cell death. There are studies aimed to distinguish the inflammatory effects of cytotoxic chemotherapeutic drugs in clinical cancer treatment.

In research examining the effects of cytotoxic chemotherapeutic drugs, raltitrexed treatment for colorectal carcinoma promoted elevated levels of serum IL-6, TNFα, and C-reactive protein whereas 5-fluorouracil-based regimens did not cause this increase (Osterlund et al., 2002). X.S. Wang and colleagues (2010) reported that concurrent chemoradiation therapy for non-small cell lung cancer caused a significant increase in IL-6, IL-10, and soluble TNF-receptor1. The increase in soluble TNF-receptor1 was associated with all 15 symptoms examined. The increase in IL-6 was significantly and positively associated with the five most severe symptoms--pain, fatigue, disturbed sleep, lack of appetite, and sore throat.

Flavopiridol, a flavonoid polyphenolic compound, has antitumor activity thought to be mediated by inhibition of cyclin-dependent kinases. In a phase I clinical study on flavopiridol, most patients developed a proinflammatory syndrome characterized by anorexia, fever, malaise, and pain. There was a transient increase in acute phase inflammatory proteins during this period (Senderowicz et al., 1998). Since acute phase proteins are known to be stimulated by IL-1 and IL-6, serial plasma samples were tested for IL-6. There was a dose-dependent increase in IL-6 at all time points across the 72 hour infusion of drug, and this increase in IL-6 was correlated with

plasma C-reactive protein levels (Messmann et al., 2003). Although timing of evaluation of markers of peripheral inflammation varied in these studies, there is substantial clinical evidence that a variety of cytotoxic chemotherapeutic drugs induce peripheral inflammation.

Cytotoxic chemotherapy induces central inflammation. The effects of cytotoxic chemotherapy on brain inflammation and neurons are not well known (Dantzer et al., 2012; Seigers & Fardell, 2011; Wood & Weymann, 2013). Evidence that chemotherapy induces central inflammation and alters neuron function comes primarily from research on chemotherapy-induced cognitive impairment. Most of this evidence is based on cognitive testing outcomes in both preclinical and human studies.

Many cytotoxic chemotherapeutic drugs induce cognitive deficits in preclinical models (Seigers & Fardell, 2011). Cytotoxic chemotherapeutic drugs decrease neurogenesis and increase cell death in the hippocampus, a brain region involved in cognitive function. Chemotherapy also affects oligodendrocyte precursors *in vivo*, indicating a direct effect on white matter glia (Dietrich et al., 2006).This effect on white matter may explain the reduced speed of information processing seen in some patients after chemotherapeutic treatment (Han et al., 2008). Methotrexate resulted in loss of white matter (Gregorios et al., 1989) and decreased the thickness of the corpus callosum. These changes were associated with memory deficits (Seigers et al., 2009). Oxidative stress, altered signaling of the HPA axis, and reduced cerebral blood flow caused by chemotherapy may also be causal mechanisms of chemotherapeutic-induced cognitive impairment (Seigers & Fardell, 2011). Although

there is some disagreement among studies, there is substantial preclinical and clinical evidence that mechanistically distinct classes of chemotherapeutic agents induce both a peripheral and central inflammatory response that is associated with a number of symptoms of cancer treatment.

Cytotoxic Chemotherapy Induces Sickness Behaviors

Sickness behaviors such as fatigue, pain, and anorexia are well documented in individuals with cancer (Bower, 2008; Chang et al., 2000; Cleeland, 2000). These behaviors often increase during chemotherapy treatment and slowly subside after completion of treatment. In some individuals, sickness behaviors, especially fatigue, persist following treatment (Curt et al., 2000; de Jong et al., 2004; Hofman et al., 2007; Minton & Stone, 2008). There is increasing evidence that these sickness behaviors are associated with cancer or cancer treatment-induced inflammation (Bower & Lamkin, 2013; Cleeland et al., 2003; Dantzer et al., 2012; Liu et al., 2012; Ryan et al., 2007; Saligan & Kim, 2012; Wood & Weymann, 2013).

In clinical studies it is difficult to separate the effects of cancer from the effects of treatment on symptoms. The association of cytotoxic chemotherapeutic drugs with sickness behavior is examined in preclinical studies since the effects of treatment can be separated from the effects of cancer. The chemotherapeutic drug etoposide caused a significant decrease in voluntary wheel running in mice for 24 hours after administration. Although activity levels in the drug-treated animals moved towards baseline between the five administrations of drug, recovery was reduced with each drug administration. The effects of administered etoposide on reduced activity and weight loss were significantly associated with an increase in serum IL-6 (Wood et al.,

2006b).

In a comparison of paclitaxel and nab-paclitaxel, Ray and colleagues (2011) reported that both drugs resulted in reduced voluntary wheel running in mice. Activity was decreased during the first week following drug administration; wheel running activity had not returned to baseline 4 weeks after multiple drug doses. Locomotor activity, measured as movement on the cage floor, was significantly decreased for one week following administration of either drug. Locomotor activity returned to baseline 3 weeks after multiple doses of drug.

Paclitaxel, but not nab-paclitaxel administration was associated with impairment on the wire-hanging test during the first week after drug administration, indicating perhaps greater motor deficits with paclitaxel. Time spent sleeping did not differ between the two drug treatments. The authors conclude that measurement of activity and strength can potentially distinguish toxicity of different drug formulations. In contrast to the findings of Wood et al., (2006b), they found no difference in serum levels of a number of inflammatory cytokines either two hours after the fifth daily administration of the drug or one week following the last drug administration. But, inflammatory cytokine levels were not tested within the first 24 hours after administration of the first dose of drug, when inflammatory cytokines were previously detected in mice (Wood et al., 2006b). In addition, paclitaxel was associated with less severe symptoms of fatigue and muscle aches as compared to CAF (Pusztai et al., 2004), which might be reflected in differences in circulating cytokines.

The cytotoxic chemotherapeutic drug 5-fluorouracil decreases wheel running in a dose-dependent manner in mice. In addition, the chemokine MCP-1 knock-out

mouse returns to baseline activity for distance, time, and speed of running a few days earlier than wild-type, indicating that reduced inflammation in the MCP-1 knock-out leads to an earlier recovery of running activity after 5-fluourouracil treatment (Mahoney et al., 2013).

A clinical study reported musculoskeletal symptoms and cytokines following administration of the chemotherapeutic drugs paclitaxel or a combination cocktail of cyclophosphamide, adriamycin, and 5-FU (CAF) (Pusztai et al., 2004). After one cycle of chemotherapy, all drug-treated participants had a transient increase in muscle aches, fatigue, and nausea that peaked between day 3 and 5 after treatment. Participants given CAF had more severe symptoms than those receiving paclitaxel. Participants receiving paclitaxel every 3 weeks had increased serum IL-6 and IL-8 at the third day after treatment. Levels of IL-8 in this group were positively correlated with flu-like symptoms. Participants receiving paclitaxel weekly had elevated IL-10 on the third day after treatment compared to baseline, and this increase was positively correlated with joint pain. The 20 individuals who received a cocktail of three chemotherapeutic agents had increased flu-like symptoms but decreased levels of IL-8 on the third day after treatment. Although individuals in this study did have cancer, 75% of individuals had stage I or stage II cancer. No participants had stage IV cancer. The onset of symptoms with drug treatment and the resolution of symptoms indicate an association of symptoms with the chemotherapeutic treatment.

Women undergoing chemotherapy treatment for early-stage breast cancer reported higher levels of all symptoms compared to those who had surgery or

radiation therapy (Bower et al., 2011). Symptoms were positively associated with increased serum soluble tumor necrosis factor receptor II (sTNF-RII), perhaps reflective of increased cytokine signaling. In a different study, fatigue in breast cancer survivors was positively associated with serum C-reactive protein, but not with IL-6 or sTNF-RI (Orre et al., 2011). The many different inflammatory mediators, receptors for those mediators, and homeostatic suppressive mechanisms involved in innate inflammatory responses adds complexity in understanding inflammatory pathways induced by different cytotoxic chemotherapeutic agents at different times after treatment.

With the pre-clinical and clinical evidence taken together, chemotherapy causes sickness behaviors in people and in rodents similar to those caused by infection or administration of lipopolysaccharide. There are conflicting results in the few reports where inflammatory markers are examined along with behavioral symptoms following chemotherapy administration. This might result from a transient increase in inflammatory markers as they induce a signaling pathway, or localized effects in brain regions without systemic elevation (Amantea et al., 2010; Clausen et al., 2008). This presents challenges in understanding the inflammatory signaling associated with administered chemotherapeutic agents. The mechanisms by which cytotoxic chemotherapy-induced inflammation affects neural signaling and thus behavior are not well understood (Bower & Lamkin, 2013; Dantzer et al., 2012; Wood & Weymann, 2013).

Evidence Inflammation Underlies CTRF

A variety of mechanisms underlying cancer treatment related fatigue have been

hypothesized. These mechanisms include cytokine dysregulation, vagal nerve activation, hypothalamic-pituitary-adrenal (HPA) axis dysfunction, serotonin dysregulation, and circadian rhythm disruption (Bower & Lamkin, 2013; Dantzer et al., 2012; Ryan et al., 2007). Fatigue is likely to be multi-factorial in origin (Stone & Minton, 2008). Cytokine dysregulation and inflammation can be an underpinning pathology that has downstream effects, but alone it does not describe a mechanism. Cytokine dysregulation and inflammation may underpin many proposed mechanisms of CTRF (Bower & Lamkin, 2013; Cleeland et al., 2003). Inflammation alters specific neuron populations sensitive to inflammation, leading to different symptoms (Saper et al., 2012). Those differences are beginning to get parsed out to understand mechanisms of LPS-induced fatigue (Gaykema & Goehler, 2011; Grossberg et al., 2011). This knowledge will shed light on mechanisms of CTRF. The cytokine dysregulation hypothesis and inflammatory responses underpinning these proposed mechanisms of CTRF are described below.

Cytokine dysregulation hypothesis. Administration of LPS induces fatigue and inflammatory cytokines. In addition, administration of cytokines induces fatigue. These observations led to the examination of cytokine dysregulation in cancer-related fatigue (Bower et al., 2011; Osterlund et al., 2002; Pusztai et al., 2004; X. S. Wang et al., 2010; Wood et al., 2006b). The premise of this hypothesis is that cancer and/or cancer treatment induce an inflammatory state mediated by cytokine dysregulation. In a review of this topic, Schubert and colleagues (2007) analyzed 18 studies with a total enrollment of 1037 participants. They found significant positive correlations between fatigue in cancer patients and IL-6, IL-1ra, and neopterin, a

marker of cellular immune activation synthesized by macrophages. Fatigue did not correlate significantly with IL-1β or TNFα. The authors concluded that despite the diversity in research methodology including the type of cancer, assessment of fatigue, and frequent use of single-time blood sampling, significant correlations were found between cancer-related fatigue and inflammatory markers.

Vagal afferent nerve activation hypothesis. The vagal afferent nerve hypothesis proposes that cancer or cancer treatment induce release of agents that activate vagal afferent nerves. This activation suppresses somatic muscle activity and communicates the peripheral inflammatory state to the brain (Ryan et al., 2007). The communication of peripheral inflammation to the brain is one mechanism to induce a central inflammatory response. In a different mechanism, activation of vagal afferent nerves from the lungs and heart were found to decrease walking activity in a proposed viscerosomatic reflex. The viscerosomatic reflex may signal with heart or lung congestion with the benefit of limiting motor activity (Pickar et al., 1993). Reduced motor activity could be perceived as fatigue in people and could limit motor activity in both people and animal models.

Inflammation influences HPA axis signaling. There is evidence that HPA axis signaling is disrupted with cancer-related fatigue and with chronic fatigue syndrome. In chronic fatigue syndrome there is attenuated diurnal variation in cortisol levels, increased negative feedback to the HPA axis, a blunted HPA axis response, and decreased levels of cortisol (Papadopoulos & Cleare, 2011). These effects on the HPA axis in chronic fatigue syndrome are associated with worse symptoms and worse outcomes. Since low cortisol levels are associated with low physical activity,

depression, and early life stress, more work is needed to unravel causation and relevance of observed changes in HPA axis in chronic fatigue syndrome (Papadopoulos & Cleare, 2011).

Similar to findings with chronic fatigue syndrome, women who had fatigue years after surviving breast cancer had lower morning cortisol levels than breast cancer survivors who did not have fatigue (Bower et al., 2002). Breast cancer survivors with fatigue also had a blunted salivary cortisol response to a stressor compared with women without fatigue, implicating hyposecretion of cortisol with fatigue (Bower et al., 2005). Since cortisol has anti-inflammatory actions, reduced cortisol levels could be associated with elevated inflammation.

Acute and chronic inflammation can affect HPA axis signaling. Acute stressors (Imaki et al., 1991) and acute inflammation tend to increase hypothalamic CRH expression (Besedovsky & del Rey, 1996) whereas chronic inflammation tends to suppress the HPA axis (Shanks et al., 1998). This suggests possible involvement of altered HPA axis signaling in both acute fatigue and persistent fatigue after cytotoxic chemotherapy treatment.

HPA axis signaling might be associated with fatigue through an entirely different mechanism. Recently, IL-1β signaling in the central nervous system was found to induce muscle atrophy via activation of the HPA axis (Braun et al., 2011). Adrenalectomy abrogated this response, indicating the requirement of glucocorticoid signaling. Muscle wasting signaled through IL-1β activation of the HPA axis could also be causative to fatigue.

Inflammation and serotonin dysregulation. Serotonin is a neurotransmitter synthesized from tryptophan in presynaptic terminals and released into the neural synapse. Serotonin is involved in many different signaling processes such as regulation of mood, appetite, memory, and exercise, depending on the location of the receptor where it binds. Changes in behaviors such as mood, appetite, and exercise could underlie fatigue or the perception of CTRF. Thus, dysregulation of serotonin could have a causal role in CTRF.

Central administration of tryptophan results in shorter episodes of physical activity. The duration of running prior to exhaustion was decreased in rats given icv tryptophan (Soares et al., 2007). This fatigue was associated with elevated serotonin in the preoptic area and hypothalamus and decreased levels in the hippocampus (Leite et al., 2010; Soares et al., 2007). The ratio between serotonin and dopamine within the hypothalamus also may contribute to central fatigue (Leite et al., 2010). Administration of a selective serotonin re-uptake inhibitor, which increases synaptic serotonin levels, reduces the physical ability of healthy participants (Wilson & Maughan, 1992). Central administration of IL-1β increases tryptophan in all brain regions (Kabiersch et al., 1988). Thus the central inflammatory response induced by cancer and cancer treatment may increase brain levels of tryptophan and thus of serotonin, leading to fatigue.

In contrast, low levels of tryptophan may be associated with fatigue. Inflammatory cytokines and LPS activate a tryptophan-degrading enzyme, indoleamine 2,3-dioxygenase (IDO) in both the hypothalamus and hippocampus (Andre et al., 2008). Activation of IDO results in decreased levels of synaptic serotonin and increased

levels of kynurenine, a neurotoxic metabolite (Muller & Schwarz, 2007). Interferon-γ (IFNγ) and TNFα upregulate IDO and induce depressive-like behavior in mice (O'Connor et al., 2009). A decreased level of tryptophan along with increased kynurenine was associated with decreased quality of life in people with colorectal cancer (Huang et al., 2002). In cancer patients treated with IFNα, higher depression was associated with lower levels of circulating tryptophan (Capuron et al., 2003). The symptoms of depression can overlap with fatigue behavior, suggesting a possible underlying role of increased IDO activity in fatigue.

Inflammatory cytokines also stimulate the serotonin transporters, resulting in decreased synaptic serotonin levels (Zhu et al., 2006). Gentle exercise may act to decrease fatigue by stimulating synthesis of tryptophan and ultimately resulting in increased serotonin. Since prolonged strenuous physical activity is associated with elevated brain serotonin and fatigue, it is possible that the association between fatigue and serotonin levels is U-shaped, with too little serotonin and too much serotonin associated with fatigue (Jager et al., 2008).

Inflammation disrupts circadian rhythm. Another proposed mechanism of CTRF is the disruption of circadian rhythm. Circadian rhythm of the light-dark cycle is regulated by the suprachiasmatic nucleus (SCN). Normally, light signals from the retina during the light part of the day and melatonin secretion from the pineal gland during the dark cycle signal to the SCN. The SCN has outputs that are relayed through the subparaventricular zone to the dorsal medial hypothalamus. The dorsal medial hypothalamus neurons drive circadian behaviors of sleep, activity, feeding, and corticosteroid secretion. As shown in Figure 2.3, inputs at the level of the

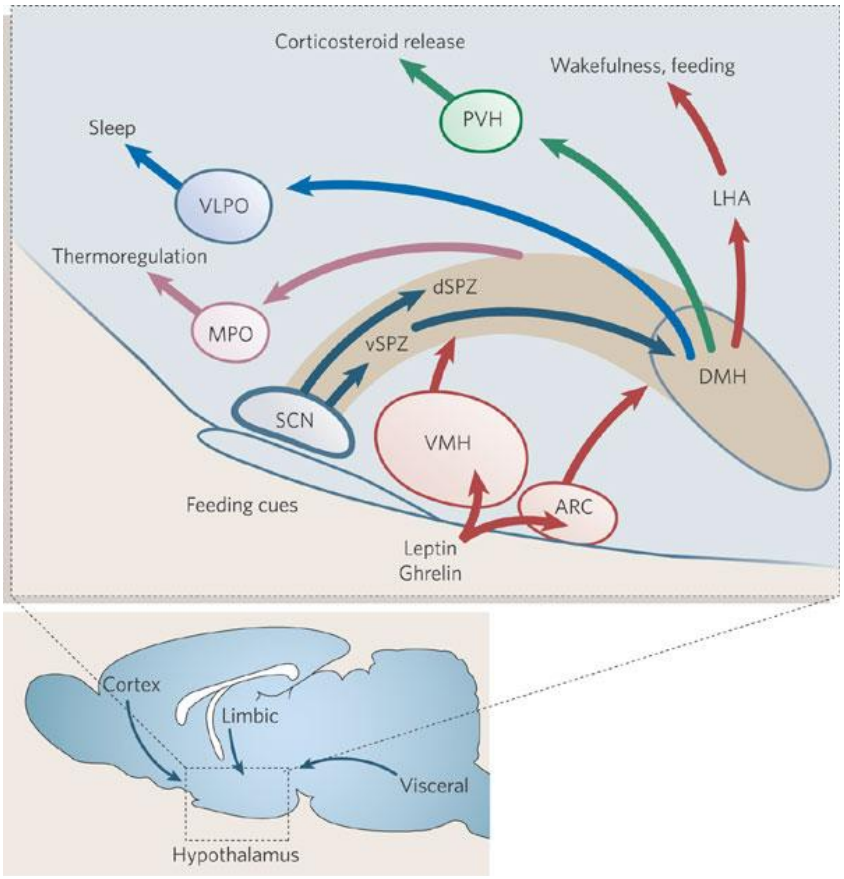

Figure 2.3. The suprachiasmatic nucleus (SCN) is a biological clock. It projects to the ventral (vSPZ)(wake-sleep cycles) and dorsal (dSPZ) subparaventricular zone (body temperature rhythms), and the dorsomedial nucleus of the hypothalamus (DMH)(sleep, activity, feeding and corticosteroid secretion cycles). Outputs from the SPZ are integrated in the DMH with other inputs from arcuate (ARC) nucleus in the hypothalamus. The DMN projects to the VLPO for sleep cycles, to the paraventricular nucleus (PVH) corticotropin-releasing hormone (CRH) neurons for corticosteroid cycles, and to the lateral hypothalamic (LHA) orexin and melanin-concentrating hormone neurons for wakefulness and feeding cycles. Integration in the SPZ and DMH allow circadian rhythms to respond to environmental stimuli, such as food availability, and visceral sensory inputs, cognitive influences from the prefrontal cortex, and emotional inputs from the limbic system. Reprinted by permission from Macmillan Publishers Ltd: Nature. Saper, C.B., Scammell, T.E. & Lu, J. Hypothalamic regulation of sleep and circadian rhythms. *437*, 1257-1263 , 2005.

subparaventricular zone and the dorsal medial hypothalamus allow for integration of internal and external signals such as energy status, visceral sensory inputs, cognitive influences, and emotional inputs from the limbic system that can alter the circadian cycle (Saper et al., 2005).

Peripheral cells, such as blood and bone marrow, also contain a circadian clock with clock genes, similar to but not self-sustained as in the SCN neurons. The peripheral clock genes are likely to be synchronized by the central SCN clock, but humoral signals might allow for uncoupling of the peripheral clocks (Balsalobre, 2002). Endotoxin administered to healthy human participants caused profound suppression of peripheral clock gene expression for 17 hours following administration (Haimovich et al., 2010). Expression of the clock genes were also disrupted in individuals with chronic myeloid leukemia (Yang et al., 2011).

Inflammation disrupts circadian signaling in most experimental models. The reverse is also true. Disruption of circadian rhythm by jet lag or by shift work can disrupt and increase inflammatory responses even in the absence of sleep loss or stress (Castanon-Cervantes et al., 2010).

Inflammation and secondary causes of CTRF. Anemia, depression, and impaired sleep may be altered by inflammation and can result in CTRF (Jager et al., 2008; Ryan et al., 2007). Anemia can result from bone marrow suppression caused by cytotoxic chemotherapy. In addition, inflammatory cytokines are implicated in anemia. Inflammatory cytokines induce the production of hepcidin in liver cells. Hepcidin is a regulator of iron homeostasis and can result in decreased iron availability, resulting in iron deficiency anemia. Inflammatory cytokines can induce

apoptosis of erythroid precursor cells and decrease erythropoiesis, resulting in anemia (Birgegard et al., 2005). In a preclinical study, the cytotoxic chemotherapy drug 5-FU resulted in decreased hematocrit after physical activity levels were returning to baseline and not during the time frame of peak fatigue (Mahoney et al., 2013). Anemia contributes to CTRF, but is not the only mechanism of CTRF.

Inflammation is implicated in symptoms of depression (Miller et al., 2009; Raison et al., 2009). Individuals with depression often report fatigue, but most individuals with CTRF do not report depression (Bower et al., 2011). In addition, paroxetine, a selective serotonin reuptake inhibitor, had no effect on fatigue in patients receiving chemotherapy even though it did lessen the symptom of depression (Morrow et al., 2003). If inflammatory cytokines alter serotonin and other neurotransmitter levels in the brain, then depression could occur (Dunn, 2006). Inflammation disrupts the HPA axis, which is associated with depression (Maes et al., 1998). Bower et al. (2011) reported that although fatigue, sleep disturbance, and depression were elevated among individuals recently treated for breast cancer, only fatigue was positively associated with a serum inflammatory marker. The authors concluded that these three common symptoms might not share an underlying mechanism of inflammation. However, a cross-sectional study might limit detection of changes in inflammatory signaling (Bower et al., 2011). In addition, changes in inflammatory or neural signaling in specific brain regions associated with long-term behavioral symptoms might not be detectable in serum.

Inflammation Suppresses Hypothalamic Orexin Neuron Activity

Orexin neuron signaling promotes wakefulness, arousal, and establishment of

motivated behavior (Anaclet et al., 2009; Carter et al., 2013; Harris & Aston-Jones, 2006; Kantor et al., 2013; Saper et al., 2005). Orexin signaling is suppressed with inflammation (Gaykema & Goehler, 2009; Gerashchenko & Shiromani, 2004; Grossberg et al., 2011). Decreased orexin signaling is associated with LPS-induced fatigue behavior (Gaykema & Goehler, 2009; Grossberg et al., 2011) and traumatic brain injury-induced decreased wakefulness and activity in rodents (Willie et al., 2012). No reports in the literature examining orexin signaling as a possible mediating factor of CTRF were found. Background information about orexin signaling and evidence associating orexin signaling with fatigue in neurological disorders is presented.

The Orexin Neuropeptides and Receptors

Orexin neurons secrete two neuropeptides, orexin-A and orexin-B, also known as hypocretin-1 and hypocretin-2, which were independently discovered in 1998 (de Lecea et al., 1998; Sakurai et al., 1998). The neuropeptides are cleaved from a common precursor, preproorexin (also called preprohypocretin), coded by the HCRT gene on mouse chromosome 11 and human chromosome 17 (de Lecea et al., 1998; Sakurai et al., 1998). The orexin-secreting neurons are located in the perifornical area of the lateral hypothalamus. Orexin neurons project throughout brain, including to structures involved in arousal such as the locus coeruleus, the raphe nuclei, the tuberomammillary nucleus, the pontine reticular formation and the laterodorsal tegmental nucleus (Carter et al., 2012; Date et al., 1999; Peyron et al., 1998).

Orexin signaling occurs through two G-protein coupled receptors, orexin-receptor 1 (OXR1) and OXR2. OXR1 and OXR2 mRNA expression have partially overlapping

and complementary distribution, suggesting different physiologic roles in signaling. OXR1 mRNA was observed in the prefrontal and infralimbic cortex, hippocampus, paraventricular thalamic nucleus, ventromedial hypothalamic nucleus, dorsal raphe nucleus, and locus coeruleus. OXR2 has complementary distribution including the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, raphe nuclei, and many hypothalamic nuclei including the tuberomammillary nucleus, dorsomedial nucleus, paraventricular nucleus, and ventral premammillary nucleus (Marcus et al., 2001). Orexin-A has a 2 to 3 order of magnitude stronger affinity for OXR1 as compared with orexin-B (Sakurai et al., 1998). The affinities for OXR2 are similar between the two neuropeptides (Sakurai et al., 1998).

The hypocretin name was coined to reflect the hypothalamic origin of a protein with homology to the gut secretins (de Lecea et al., 1998). The other group who independently discovered these same proteins found that preproorexin mRNA was up-regulated with fasting and that administration of the protein stimulated food intake. They named the proteins “orexin”, from the Greek word for appetite, orexis (Sakurai et al., 1998). However, additional research by the same group found that administration of orexin did not alter daily food consumption and long-term administration did not alter body weight. This suggested a role for orexin signaling beyond regulation of food intake (Yamanaka et al., 1999).

The Ascending Arousal System Promotes Wakefulness

Being awake and responsive to internal and external cues depends on coordinated signaling of many brain areas including the thalamus, hypothalamus, and basal forebrain to integrate and relay information from the brainstem to the

cortex (Miller & O'Callaghan, 2006). The awake state and the asleep state can be simplistically considered as mutually exclusive. In addition, there is an abrupt transition between these states. This abrupt transition is the result of a mutual inhibition between the neurons/neurotransmitters that promote wakefulness and the neurons/neurotransmitters that promote sleep (Saper et al., 2005).

Neurons in the ascending arousal system have increased firing during wakefulness and during enhanced arousal with alertness. The ascending arousal system is comprised of distinct neuron populations and their neurotransmitters, located from the pons to the midbrain reticular formation, shown in Figure 2.4 (Saper et al., 2005). One branch contains the neurotransmitter acetylcholine (ACh), released from both the pedunculopontine nuclei (PPT) and the laterodorsal tegmental nuclei (LDT) in the upper pons. This pathway activates the thalamic relay neurons. The thalamic relay neurons in turn transmit information to the cerebral cortex. The PPT and LDT neurons fire most rapidly during wakefulness and rapid eye movement (REM) sleep, both which are characterized by cortical activation. Consistent with this, the drug donepezil enhances wakefulness by preventing ACh metabolism, thus increasing available ACh.

The other branch of the ascending arousal system includes the noradrenergic locus coeruleus (LC) (Aston-Jones & Bloom, 1981), the serotoninergic raphe nuclei (Guzman-Marin et al., 2000; McGinty & Harper, 1976) the dopaminergic ventral tegmental area nuclei (VTA) (Dahan et al., 2007), and the histaminergic tuberommammillary neurons (TMN) in the posterior hypothalamus (Parmentier et al., 2002). These monoaminergic neurons fire fastest during wakefulness and send

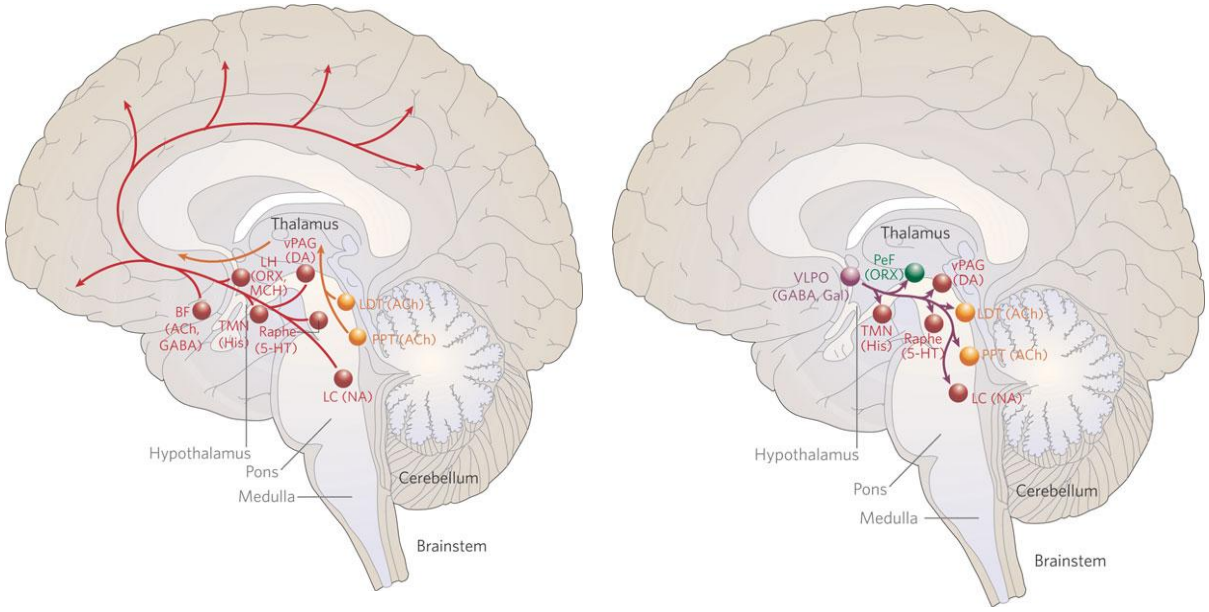

Figure 2.4. The ascending arousal system and reciprocal VLPO inhibitory projections. The ascending arousal system includes the cholinergic (Ach) pedunculopontine (PPT) and laterodorsal tegmental nuclei (LDT) that project to the thalamus and the cortex (yellow pathway-on left). The monoaminergic arousal pathway includes noradrenergic projections from the locus coeruleus (LC), serotonergic (5-HT) projections from the dorsal and median raphe nuclei, histaminergic (His) projections from the tuberomammillary nucleus (TMN), and dopaminergic (DA) from the ventral tegmental area (vPAG). This pathway has input from the lateral hypothalamus (LH) containing orexin (ORX) or melanin-concentrating hormone (MCH), and from basal forebrain (BF) neurons containing γ-aminobutyric acid (GABA) or ACh. The sleep-promoting VLPO has reciprocal inhibitory projections to each of the nuclei in the ascending arousal system (on right). Reprinted by permission from Macmillan Publishers Ltd: Nature. Saper, C.B, Scammell, T.E. & Lu, J. Hypothalamic regulation of sleep and circadian rhythms. *437*, 1257-1263 , 2005

widespread ascending projections to the cerebral cortex. This pathway also has input of melanin-concentrating hormone and orexin from distinct populations of lateral hypothalamic nuclei, and of gamma-aminobutyric acid (GABA) and ACh from populations of basal forebrain neurons. In addition to projections to the cerebral cortex, the monoaminergic nuclei (LC, TMN, and Raphe) project to and inhibit the ventrolateral preoptic nucleus (VLPO), which is primarily active during sleep. The orexin system promotes arousal and wakefulness throughout the active period by activating the ascending arousal system. Both are inhibited by signaling from the VLPO with increased sleep pressure (Adamantidis et al., 2010), shown in the right panel of Figure 2.4 and in Figure 2.5.

The VLPO neurons suppress activity of both the ascending arousal system and orexin signaling (Saper et al., 2005). The VLPO neurons, which express the inhibitory neurotransmitter GABA, project to the neuron populations in the hypothalamus and brainstem involved in wakefulness. The monoaminergic neurons in the locus coeruleus, tuberomammillary nucleus, and Raphe nuclei inhibit signaling of the VLPO neurons. This mutual inhibition between the wake-promoting neurons and the sleep-promoting VLPO neurons comprise the flip-flop switch model, or the sleep-wake switch shown in Figure 2.5 (Saper et al., 2001).

Orexin Signals Wakefulness and Motivated Behavior

Neuronal inputs and projections from orexin-secreting neurons implicate orexin as an intermediate step signaling arousal in response to emotion, metabolic needs, reward-oriented stimuli, and diurnal rhythms as shown in Figure 2.6. One component of arousal is being awake. Orexin neurons show tonic discharge specific to

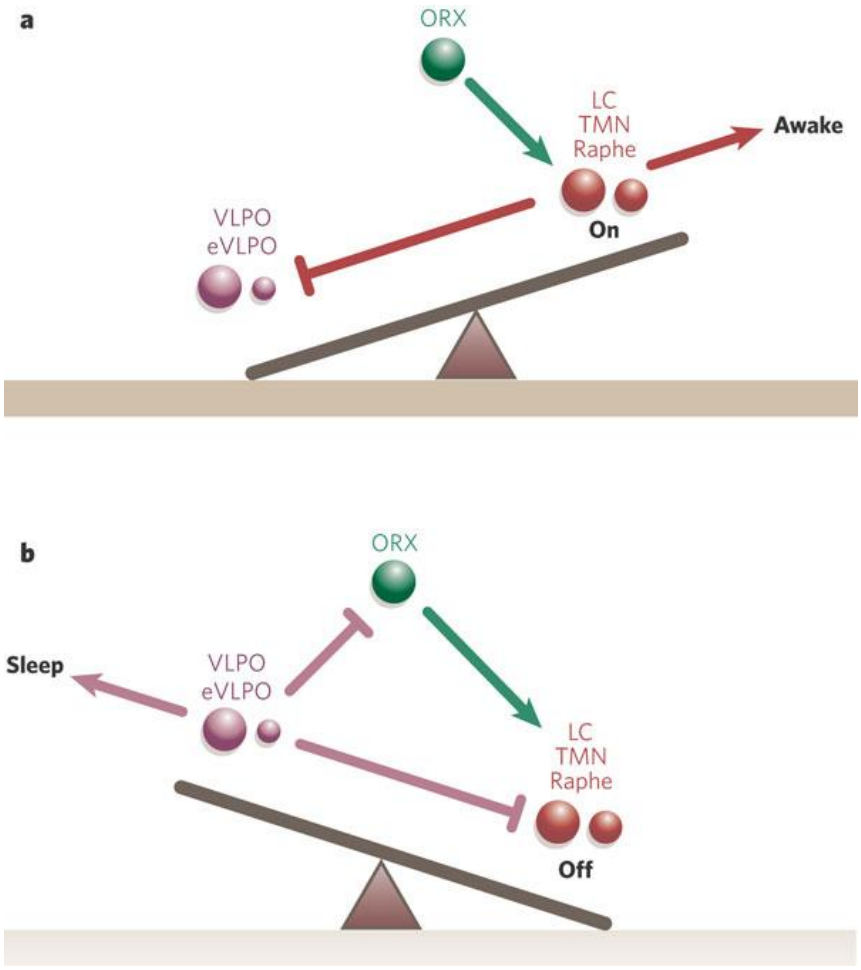

Figure 2.5. Orexin neurons stabilize the sleep/wake flip-flop switch. In wakefulness (a), monoaminergic nuclei (LC, TMN, Raphe) inhibit the ventrolateral preoptic nucleus (VLPO). This prevents inhibition of orexin (ORX) neurons (green) from the VLPO, and ORX reinforce monoaminergic tone. In sleep (b), VLPO inhibits both ORX and monoaminergic nuclei. Direct mutual inhibition between VLPO and monoaminergic neurons result in sharp transitions in state, but has relative instability. Stability is provided by ORX signaling. Reprinted by permission from Macmillan Publishers Ltd: Nature. Saper, C.B., Scammell, T.E. & Lu, J. Hypothalamic regulation of sleep and circadian rhythms. *437*, 1257-1263, 2005.

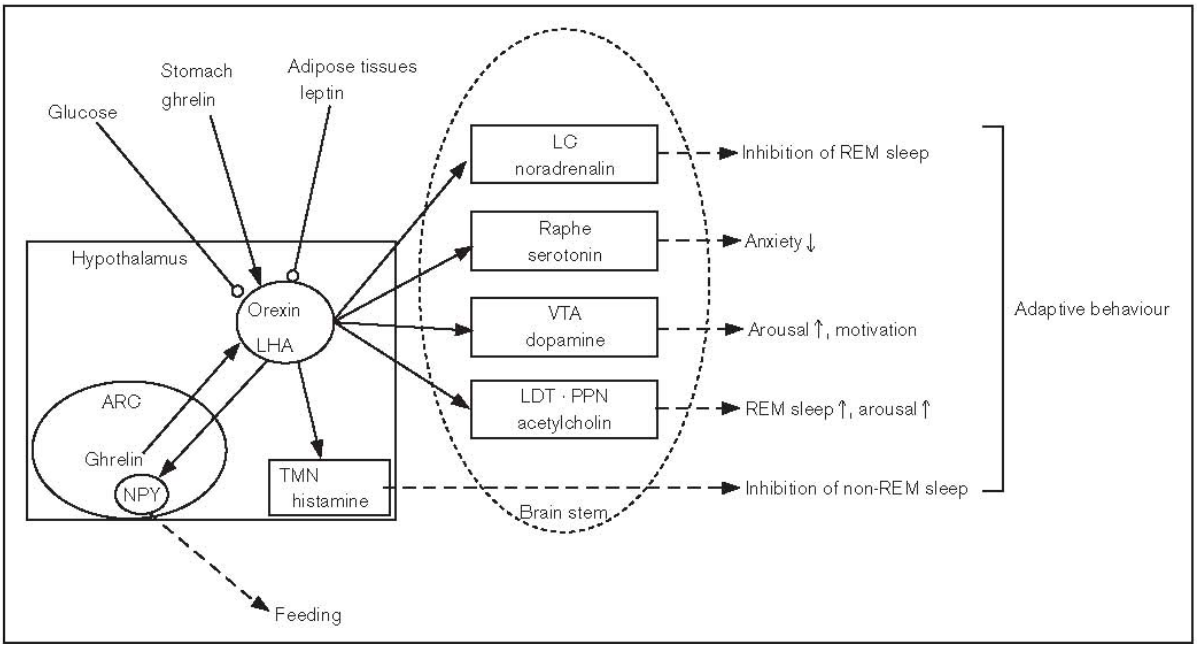

Figure 2.6. Peripheral metabolic signals, leptin, ghrelin, glucose, and circadian rhythms influence orexin neuron function. Orexin neurons in the lateral hypothalamic area (LHA) stimulate hypothalamic nuclei in the arcuate nucleus (ARC) involved in feeding behavior. Orexins increase cortical arousal and promote wakefulness through the tuberomammillary nucleus (TMN), locus coeruleus (LC), Raphe, ventral tegmental area (VTA), and the laterodorsal tegmental (LDT)/pedunculopontine nucleus (PPN). Orexin stimulation of these nuclei modulates reward systems, motor activity, and emotional arousal. From Sakurai, T. (2003). Orexin: A link between energy homeostasis and adaptive behavior. *Current Opinion in Clinical Nutrition and Metabolic Care*, 6, 353-360. Used with permission.

wakefulness (Mileykovskiy et al., 2005; Takahashi et al., 2008). This discharge with active waking is associated with increased muscle tone and movement (Lee et al., 2005). Orexin neurons cease firing during sleep (Lee et al., 2005). Fear triggers an awake state. Consistent with this, orexin neurons receive input from the limbic system which regulates emotionally-stimulated arousal (Sakurai, 2007; Sakurai et al., 2005).

Food seeking is a motivated behavior. Foraging is energy-intensive. It is regulated

along with periods of rest by circadian rhythms and in response to physiologic needs such as energy status. Orexin activity is inhibited by glucose and stimulated by ghrelin (Yamanaka et al., 2003). Glucose signals a high energy state, and as a result suppresses a behavioral response of food-seeking activity. Ghrelin is a neuropeptide associated with hunger. Stimulation of orexin signaling by ghrelin increases food-seeking behavior (Mieda et al., 2004; Yamanaka et al., 2003). Mice lacking orexin neurons did not exhibit increased wakefulness in response to fasting. These findings indicate a role of orexin signaling in integrating internal cues of energy balance and arousal (Mieda et al., 2004; Yamanaka et al., 2003).

Orexin signaling is associated with other motivated behaviors in rodents including sexual, drug-seeking, exploratory, grooming, and eating behaviors (Aston-Jones et al., 2010; Gaykema & Goehler, 2009; Harris & Aston-Jones, 2006; Mileykovskiy et al., 2005). Motivated behavior may signal through stimulation of ventral tegmental area (VTA) dopaminergic neurons (Harris et al., 2005). Orexin signaling may influence dopaminergic activity by modulating responses to medial prefrontal cortex inputs based on diurnal stimuli (Moorman & Aston-Jones, 2010). Orexin neurons have maximal discharge during exploratory behavior and increased discharge with grooming and eating behaviors (Mileykovskiy et al., 2005).

Inflammatory challenge suppressed orexin signaling during the usually active dark period. This suppression was associated with the sickness behaviors of decreased locomotor and exploratory behavior in rodents (Gaykema & Goehler, 2009; Grossberg et al., 2011). Sickness behavior is a component of a motivational system as it is a reorganization of priorities to promote recovery from infection or

injury (Dantzer & Kelley, 2007).

Orexin Signaling Follows a Diurnal Rhythm

Orexin signaling is affected by circadian rhythms driven by the hypothalamic suprachiasmatic nucleus. There is a strong diurnal rhythm in cisternal CSF levels of orexin with highest levels at the end of the wake-active period and the lowest levels at the end of the sleep period in young rats (Fujiki et al., 2001; Yoshida et al., 2001). Zeitzer and colleagues (2003) used the squirrel monkey as a primate model since it has a wake pattern similar to humans. In this primate, orexin levels peaked in the latter third of the daytime wake period. This finding is consistent with that in rats since highest levels in both are towards the end of the wake period. When the wake period was extended by 4 hours in the squirrel monkey, orexin levels remained elevated, suggesting a circadian-independent mechanism influencing orexin levels.

Young, middle aged, and old F344 rats had the same diurnal pattern of CSF orexin levels although the orexin concentration was approximately 10% less in old rats compared to younger rats. The amplitude of the orexin rhythm did not differ among the age categories (Desarnaud et al., 2004). Although CSF orexin levels were decreased in the old rats, this decrease was not from differences in preproorexin gene expression.

In a mouse model of Huntington Disease (HD), the SCN circadian rhythm was normal in the HD-mice, but orexin neuron activity did not follow a circadian profile. Since neuronal activity was measured at only two time points it was not possible to determine if the circadian variation of orexin activity was blunted or phase-shifted. The observation of increased orexin neuron activity during the daytime hours (the

sleep period for mice) indicates an intervention in Huntington Disease targeted to reduce orexin signaling during the period of rest rather than stimulating orexin signaling during the period of wakefulness (Williams et al., 2011).

Absence of Orexin Signaling in Narcolepsy

The absence of orexin signaling underlies narcolepsy-cataplexy (Chemelli et al., 1999; Lin et al., 1999; Nakamura et al., 2011). Narcolepsy-cataplexy is a sleep disorder characterized by excessive and unpredictable daytime sleepiness, disrupted nocturnal sleep, and cataplexy, the sudden occurrence of muscle atonia. Narcoleptic patients with cataplexy have non-detectable or low levels of orexin-A in the CSF and they lack evidence of preproorexin messenger RNA in the hypothalamus (Nakamura et al., 2011; Peyron et al., 2000). In humans, the subcategories of narcolepsy either with or without cataplexy were associated with CSF orexin deficiency, with lowest levels associated with cataplexy (Nakamura et al., 2011). Evidence from canine and human narcolepsy research indicates that total daily sleep and REM sleep time does not differ between those with and without narcolepsy (Broughton & Aguirre, 1987), but that sleep and wake times are fragmented with narcolepsy.

Stimulant Drugs to Treat Narcolepsy and CTRF

Stimulant drugs to treat narcolepsy shed light on possible interventions to treat CTRF. Methylphenidate, which increases dopamine signaling, was used in the past to promote arousal in people with narcolepsy. The effect of methylphenidate on cancer-related fatigue was examined in four small clinical studies. Only one of the four studies had a significant effect of treatment on fatigue (Bruera et al., 2013;

Lower et al., 2009; Minton et al., 2011; Moraska et al., 2010). Although the benefit of methylphenidate to treat CTRF is questionable, there is debate that adequate dosing and identification of the appropriate population to treat are important to consider before this potential treatment is ruled out (Stone, 2013).

Modafinil, a drug that increases histamine in the brain via orexin neuron signaling (Ishizuka et al., 2010), has replaced the use of methyphenidate in narcolepsy treatment. Modafinil is used to treat severe daytime sleepiness in narcolepsy. It was found to lessen severe but not moderate fatigue in individuals with cancer in a small clinical trial (Jean-Pierre et al., 2010). Modafinil decreased excessive daytime sleepiness but not reports of fatigue following traumatic brain injury (Kaiser et al., 2010). Modafinil also improved cognitive performance after chemotherapy (Kohli et al., 2009) and in individuals with brain tumor (Gehring et al., 2011). Histamine signaling is downstream from orexin signaling and these two neuron populations have different functions in promoting arousal (Anaclet et al., 2009). As a result, an orexin agonist may be more suitable than histamine to increase functional arousal (Anaclet et al., 2009; Ishizuka et al., 2010).

An oral agonist of the orexin receptor has shown success in clinical trials to treat narcolepsy and is reported to be close to market release after bioavailability of the drug formulation is improved (NIH RePORT #1U01NS080400-01). If cytotoxic chemotherapy disrupts orexin neuron function, then the oral orexin agonist is a rational intervention to target chemotherapy-induced fatigue.

Orexin Signaling in Neurological Disorders and Fatigue

Following the discovery of the association of disrupted orexin signaling in

narcolepsy-cataplexy disorder, other neurological disorders characterized by disrupted sleep and excessive daytime sleepiness were examined for orexin neuron dysfunction.

In a comprehensive search of the literature, thirty-three studies in humans were identified where either orexin neurons or cerebrospinal fluid levels of orexin-A was examined. Seven of these studies used postmortem tissue. There were three additional studies that examined orexin neurons in animals only. Case studies and studies on narcolepsy were not included in this summary. Table 2.1 summarizes the results of orexin neuron counts, orexin-A CSF levels, and an association with sleep or fatigue levels in the disorders examined. Examining the results as a whole, it appears that brain injury from trauma, a bleed, or a tumor consistently results in decreased orexin-A levels in CSF.

CSF Orexin Levels Reduced with Brain Injury

CSF levels of orexin-A were examined after traumatic brain injury (TBI) in three studies that enrolled at total of 115 living participants. All three studies reported reduced levels of orexin-A in CSF (Baumann et al., 2005; Baumann et al., 2007; Ripley et al., 2001). In the one longitudinal study, Baumann (2007) reported that 25 of 27 participants had low CSF orexin-A the first few days after TBI, and 4 of 21 participants had low CSF orexin-A at 6 months after TBI. The time of CSF collection was not provided even though there might be a diurnal rhythm of orexin-A levels in humans. Seventy-two percent of the participants had new-onset sleep-wake disturbances at 6 months after TBI, and 25% reported excessive daytime sleepiness

Table 2.1.

Summary of differences in orexin cerebrospinal fluid levels and neuron counts between listed disorders and control groups in each study, and whether or not an association was found between indicators or reduced orexin activity and fatigue.

	Number of studies identified					
	CSF orexin-A		Number of orexin neurons		Less orexin associated with fatigue	
Disorder	No difference found	Lower in disorder	No difference found	Lower in disorder	No	Yes
Parkinson Disease	5	3	0	2	4	0
Multiple Sclerosis	5	0	NT	NT	1	0
Alzheimer Disease	3	1	1	1	0	2
Huntington Disease	2	1[a]	NT	1[a]	NT	NT
Guillain-Barre	1	3	NT	NT	1	0
Fibromyalgia	1	0	NT	NT	NT	NT
Traumatic Brain Injury	0	3 + 1[a]	1[a]	1	0	1 + 1[a]
Cerebral hemorrhage	0	2	NT	NT	NT	NT
Brain Tumor	0	1	NT	NT	NT	NT

CSF=cerebrospinal fluid. NT=not tested. 1[a] refers to a study in an animal model in addition to the clinical studies listed. More detailed information on the studies listed is provided in the text.

at 6 months after TBI. Orexin neuron counts were determined post-mortem in 4 individuals, 7 to 42 days after TBI. Those with TBI had a 27% reduction in orexin neurons. It was not reported if this decrease was specific for orexin neurons or if there were similar decreases across many brain regions. Findings from an experimental model of TBI in mouse were consistent with the results in people. Experimental TBI in mouse resulted in decreased levels of orexin-A, blunted diurnal

amplitudes in the hypothalamus, and decreased wakefulness and motor activity (Willie et al., 2012).

Ripley et al. (2001) examined CSF orexin-A levels in healthy people and in those with a variety of neurological disorders (shown in Table 2.2). Healthy control participants had an average orexin-A level of 318 pg/mL (range 224-653). Orexin-A levels in CSF were lowest in those with narcolepsy. Patients with narcolepsy had an average level of CSF orexin-A of less than 100 pg/mL (range <100 to 649). The 1 participant with subarachnoid hemorrhage had a level of 127 pg/mL. The 6 participants with TBI had an average level of 147 pg/mL (range 129-442). The 14 participants with a CNS cancer had an average level of 208 pg/mL (range 136-370). The group of 11 participants with Guillain-Barre´ had reduced CSF orexin-A, with an average of 230 pg/mL (range <100 to 720). These results are in contrast to other studies where CSF levels of orexin-A were low in some, but not in all individuals with Guillain-Barre´ (Kanbayashi et al., 2002; Nishino et al., 2003). A different group reported 9 of 11 participants with Guillain-Barre´ had normal levels of CSF orexin-A (Baumann & Bassetti, 2004).

CSF Orexin Levels Normal in Many Neurologic Disorders

Results are also mixed concerning CSF orexin-A levels in individuals with Parkinson Disease (PD) and Alzheimer Disease (AD). Of the 8 studies enrolling individuals with PD and examining orexin-A levels in CSF, 5 of the studies showed no difference in CSF orexin-A levels between individuals with and without PD (Compta et al., 2009; Drouot et al., 2011; Overeem et al., 2002; Ripley et al., 2001; Yasui et al., 2006). Three studies reported low levels of CSF orexin-A associated

Table 2.2.

Summary of cerebrospinal fluid levels of orexin-A with different disorders

Group	Number	CSF level orexin-A (pg/ml)	
		Median	Range
Healthy controls	48	318	224-653
Narcolepsy	42	< 100 (37 undetected)	<100 to 649
Subarachnoid hemorrhage	1	127	
Traumatic Brain Injury	6	147	129-442
CNS Cancer	14	208	136-370
Guillain-Barre´	11	230	<100 to 720
Acute lymphocytic leukemia (no CNS invasion)	30	278	153-420
Multiple Sclerosis	10	288	220-493
Alzheimer Disease	24	298	221-428
Cerebral infarct	5	342	132-605

Note. Adapted from CSF Hypocretin/orexin Levels in Narcolepsy and Other Neurological Conditions, by Ripley, B., Overeem, S., Fujiki, N., Nevsimalova, S., Uchino, M., Yesavage, J., Di Monte, D., Dohi, K., Melberg, A., Lammers, G.J., Nishida, Y., Roelandse, F.W., Hungs, M., Mignot, E., and Nishino, S., 2001. CSF hypocretin/orexin levels in narcolepsy and other neurological conditions. Neurology 57, 2253-2258. Lower limit of detection 100 pg/mL.

with PD (Asai et al., 2009; Drouot et al., 2003; Fronczek et al., 2007).

One study with 19 individuals with advanced PD reported CSF orexin-A levels negatively correlated with disease severity. These levels were not correlated with sleepiness reported on the Epworth Sleepiness Scale (ESS), nor with age, disease duration, or levadopa dose (Drouot et al., 2003). Another study of 25 individuals

reported lower levels of orexin-A in CSF with longer disease duration (Asai et al., 2009). Two studies using postmortem samples of individuals with and without PD found decreased number of orexin neurons in the PD group (Fronczek et al., 2007) and greater loss of orexin neurons with increased disease progression (Thannickal et al., 2007). In addition to decreased neurons, Fronczek et al. (2007) also found 40% lower orexin-A levels in prefrontal cortex CSF of those with PD and 25% reduced orexin-A levels from brain ventricle samples of those with PD.

In AD, three studies reported normal CSF orexin-A levels in the combined total of 47 participants with AD (Baumann et al., 2004; Friedman et al., 2007; Ripley et al., 2001). Decreased levels of orexin-A among those with AD and a 40% decrease in orexin neurons among those with AD were found in a post-mortem study. Information on daytime sleep collected prior to death showed that 2 participants with excessive daytime sleep had the lowest orexin-A levels (Fronczek et al., 2011).

The 5 studies on multiple sclerosis (MS) reported normal levels of CSF orexin-A (Constantinescu et al., 2011; Kanbayashi et al., 2002; Knudsen et al., 2008; Papuc et al., 2010; Ripley et al., 2001). The 1 study on Fibromyalgia (Taiwo et al., 2007), and the 2 studies on Huntington Disease in living humans also found normal CSF levels of orexin-A (Baumann et al., 2006; Meier et al., 2005).

It is not known if orexin-A CSF levels in humans have diurnal variance as seen in rodents. This lack of knowledge is in part due to limitations in obtaining CSF in awake and healthy individuals at multiple time points throughout the day. In the large study by Ripley and colleagues (2001), CSF was collected between 0700 and 1700 for all participants other than 9 healthy controls who were tapped at the following

times: 4 tapped between 1800-1900, 4 tapped between 2200-2300, and 1 tapped at 0200. The authors reported no significant difference in CSF orexin-A level based on the time of collection. However, with controls ranging from 224 to 653 pg/mL, a significant difference in time of collection in the sample of 9 controls taken at a single point in time might be difficult to detect. It remains to be shown if CSF levels of orexin-A have a diurnal fluctuation in healthy people.

If the diurnal variance in humans is similar to that in rodents, then one methodological concern of these studies is the time of cerebrospinal fluid sample collection. Many of these studies stated that CSF was collected between 0900 and noon, a time when orexin-A levels might be at their lowest in humans (Salomon et al., 2003; Yoshida et al., 2001; Zeitzer et al., 2003). Other studies stated that CSF collection was 0900-1700. Some studies do not provide a time of collection. In a primate model with a wake pattern similar to humans, Zeitzer and colleagues (2003) reported that orexin levels peaked in the latter third of the day, towards the end of the wake period, consistent with findings in rodents. The nadir occurred at the beginning of the wake period, as is also observed in rodents (Desarnaud et al., 2004; Grossberg et al., 2011). There was a robust 31% daily oscillation of CSF orexin-A levels in the primate model, consistent with findings in rodents (Zeitzer et al., 2003).

An additional study on CSF levels of orexin-A in people further complicates whether or not CSF levels of orexin-A in people shows a pattern similar to that in rodents or non-human primates. In a clinical study examining healthy controls and people with depression, Salomon and colleagues (2003) reported orexin-A levels in

CSF lowest at 1300 and highest at 0100, 2 hours after lights out. This pattern was similar comparing those supine with an indwelling lumbar catheter and the subset of ambulatory participants who received multiple lumbar punctures for CSF collection. There was a significant 10% daily oscillation of CSF orexin-A levels in control participants and a dampened diurnal, 3% oscillation among depressed participants. The authors concluded that the dampened diurnal variation in orexin-A, seen also in a rodent model of TBI (Willie et al., 2012), might be associated with depression (Salomon et al., 2003). Their findings indicating lowest levels of orexin-A at 1300 is not consistent with results in rodents where CSF levels of orexin-A are lowest at the end of the sleep period and highest at the end of the awake period.

Another consideration in interpreting CSF levels of orexin-A is the relationship between the percent reduction of orexin neurons and orexin-A CSF levels. Rodents with a loss of 73% of orexin neurons showed 50% decrease in CSF orexin-A levels. Rodents with a 14% loss of orexin neurons showed no difference in CSF orexin-A levels (Gerashchenko et al., 2003). As a result, although CSF levels of orexin-A might be the only samples available from human participants, these levels might not accurately reflect disrupted signaling of orexin neurons or loss of orexin neurons sufficient to disrupt signaling but not sufficient to decrease CSF levels. Additional research is needed to fully understand the diurnal pattern of CSF levels of orexin-A in people and to understand the clinical significance of CSF levels of orexin-A.

Inflammation Disrupts Orexin Signaling and is Associated with Fatigue

Hypothalamic orexin neuron function during the dark phase is suppressed by lipopolysaccharide (LPS) administration and is associated with decreased dark

phase locomotor activity in rodents (Grossberg et al., 2011). Orexin signaling was measured by a decreased percentage of orexin-A neurons expressing nuclear cFos and decreased CSF levels of orexin-A at the end of the active period. Furthermore, central replacement of orexin-A prevented the onset of the LPS-induced lethargy. Similarly, LPS-induced inflammation suppresses expression of cFos in hypothalamic orexin neurons and decreases exploratory and other behavioral activities (Gaykema and Goehler, 2009).

The question remains how inflammation, whether by administration of central IL-1β or by peripheral LPS, at a mechanistic level affects orexin neurons. Administration of IL-1ra partially restored locomotor activity in the LPS-treated rodents, indicating a role of IL-1 signaling in the behavioral response to LPS. Blocking prostaglandin signaling in LPS-treated rodents was found to have no effect on the decrease in locomotor activity associated with LPS treatment, suggesting that prostaglandin signaling is not required for the lethargy behavioral response to LPS. In addition, LPS-induced fatigue was not dependent on melanocortin signaling (Grossberg et al., 2011). Neurotensin-expressing GABAergic interneurons, which have an inhibitory effect on orexin neurons when activated by leptin (Leinninger et al., 2011), and are activated during LPS-induced inflammation, may have a role in mediating orexin neuron inhibition following an inflammatory challenge (Grossberg et al., 2011). This mechanism needs to be more fully elucidated.

The evidence that inflammation-inducing treatments disrupt orexin signaling and this disruption is associated with fatigue (Gaykema & Goehler, 2009; Grossberg et al., 2011) suggests that chemotherapy-induced inflammation may disrupt orexin

neuron signaling. Disruption of orexin signaling may be one mechanism underlying chemotherapy treatment related fatigue. The purpose of my dissertation research is to test if orexin signaling has a role in cytotoxic chemotherapy-induced fatigue.

Chapter 3: Methods

The purpose of this research was to determine if cytotoxic chemotherapy induces inflammatory gene expression in the hypothalamus or brainstem and to determine if single or multiple doses of these drugs suppress orexin neuron activity in rodents. If orexin neuron activity was suppressed, then an additional purpose was to test if orexin administration reverses chemotherapy-induced fatigue. This chapter describes the experimental methods used, justification of the methods, and data analysis. These experiments are part of a larger goal to understand the etiologic neural mechanisms of fatigue induced by cytotoxic chemotherapy.

Animals

Mice and rats were used in experiments. Female C57BL/6J mice (strain #000664; 8-12 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME). The C57BL/6J inbred mouse strain has a high baseline level of wheel running activity (Lightfoot et al., 2004) and running activity is sensitive to change in response to administered cytotoxic chemotherapeutic drugs (Mahoney et al., 2013; Ray et al., 2011; Wood et al., 2006b). Male Sprague Dawley rats (250-350 g) were purchased from Charles River Laboratories (Wilmington, MA). Mice and rats show similar responses to both the absence of orexin signaling and the administration of orexin-A (Anaclet et al., 2009; Furutani et al., 2013; Gerashchenko et al., 2001; Gerashchenko et al., 2003). Mice were used in order to obtain voluntary wheel running activity after cytotoxic chemotherapy treatment. Rats were used for measurement of orexin-A in cerebrospinal fluid and to determine the effect of administration of orexin-A on ambulatory activity. Animals were maintained in

pathogen-free rooms on a normal 12hour light/dark period with lights on from 0600 to 1800 with *ad libitum* access to food (rodent diet 5001, Purina Mills) and water. In experiments where data was collected on animals more than 48 hours after receiving cytotoxic chemotherapy, both sham-treated and cytotoxic chemotherapy-treated animals were given water containing 150µg/mL amoxicillin (oral suspension, Sandoz). Animals were weighed daily during experiments. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Oregon Health & Science University Department of Comparative Medicine Institutional Animal Use and Care Committee.

Cytotoxic Chemotherapy Administration

Animals were administered a combination of cyclophosphamide (Cytoxan), doxorubicin (Adriamycin), and 5-fluorouracil (5-FU) (CAF) at concentrations of 167mg/kg, 4mg/kg, and 167mg/kg respectively in mice and 75 mg/kg, 4 mg/kg, and 75 mg/kg in rats. This adjuvant drug cocktail is commonly used for treatment of breast cancer. The body surface area normalization method (Reagan-Shaw et al., 2007) was used to calculate the mouse dose in mg/kg based on the human dose of Cytoxan (500mg/m^2), Adriamycin (50mg/m^2) and 5-FU (500mg/m^2) (Smalley et al., 1983). Mice administered 4 separate doses of CAF at 3-week intervals did not meet any criteria for euthanasia during treatment (unpublished observations). Drug concentrations in rat were based on the following reports: the drug combination given at 65 mg/kg Cytoxan, 6.5 mg/kg Adriamycin, and 65 mg/kg 5-FU at 21 days apart with euthanization required after the second dose when 6.5 mg/kg of

adriamycin was in the cocktail but not with 6.5 mg/kg methotraxate (Little, 2007); Cytoxan at 75 mg/kg (Fardell et al., 2012); and 5-FU at 75 mg/kg in a cocktail given weekly for 4 weeks (Briones & Woods, 2011). In preliminary work we treated rats with 75/4/75 mg/kg of CAF, respectively, for 2 doses given 8 days apart. Weight and activity in treated rats returned to baseline after 8 days. Cyclophosphamide was purchased from Baxter Healthcare Corporation. Doxorubicin-HCL was purchased from Bedford Labs (Bedford, OH, USA), dissolved in sterile, deionized water to attain a stock solution of 2 mg/ml, and stored at 4°C. 5-FU was purchased as a 50mg/ml solution from American Pharmaceutical Partners (Schaumburg, IL, USA).

Animals were administered the drugs in two separate intraperitoneal (ip) injections to prevent precipitation of adriamycin by 5-fluorouracil. Cytoxan and Adriamycin were combined in a volume of 1 mL normal saline (NS) in mice and in 2 mL NS in rats and injected ip. Sixty minutes later, animals were injected ip with 1 mL (mice) or 2 mL (rats) NS containing 5-FU. Sham-treated mice were injected with the same volumes of NS without drug. This injection volume was used to reduce localized tissue inflammation at the injection site, to provide fluid, and to improve absorption of the drug. Animals were returned to their home cages after injection.

Experiment 1: The Effect of CAF on Brain Inflammatory Gene Expression

The purpose of this experiment was to determine if peripherally administered cytotoxic chemotherapeutic drugs induce inflammatory gene expression in the hypothalamus or brainstem. Mice housed 5 to a cage were injected with CAF or NS between 0800 and 1000 (for 3, 6, 24, and 48hour samples) or between 1400 and 1600 (for 16hour samples). At 3, 6, 16, 24, and 48 hours post injection, mice were

deeply sedated with ketamine-xylazine (150 mg/kg-15 mg/kg ip) then perfused with 25 mL ice cold RNase-free 0.01 molar phosphate buffered solution (PBS). Whole brains were removed and hypothalamic blocks and brainstems excised. Hypothalamic blocks containing part of the thalamus and median eminence attached were excised by making coronal cuts at the rostral extent of the optic chiasm caudal to the mammillary body and saggital cuts along the optic tracts. The cortex was removed at the corpus callosum. Brainstems were excised by cutting rostral to the cerebellum and gently removing the cerebellum. Tissue was placed in RNAlater® (Ambion) solution on ice, and stored at 4˚C up to 48 hours prior to ribonucleic acid (RNA) extraction.

Total RNA was extracted from hypothalamic and brainstem tissue using QIAGEN RNeasy kits (QIAGEN, Inc., Valencia, CA) and included digestion of the homogenate with proteinase K (1% by volume) and removal of deoxyribonucleic acid (DNA) from total RNA using ribonuclease-free deoxyribonuclease (QIAGEN). Total RNA was quantified (Nanodrop) and stored at -80 C. Complementary DNA (cDNA) was generated using the TaqMan Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA). Each cDNA synthesis reaction (10 µL) contained 100 ng RNA, 1 µL 10x RT buffer, 2 µL 25mM MgCl2, 1.6 µL 10mM deoxynucleotide triphosphates, 0.6 µL 50µM random hexamers, 0.4 µL RNAse inhibitor, 0.6 µL Multiscribe RTase, and nuclease-free water. Reverse transcriptase reactions were run on an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) programmed for 10 min at 25 C, 60 min at 37 C, and 5 min at 95 C. Samples were diluted with 40 µL nuclease-free water and stored at 4 C until RT-PCR was

performed.

Relative levels of interleukin-1 beta (IL-1β), IL-1 receptor type I (IL-1RI), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and monocyte chemoattractant protein-1 (MCP-1) mRNAs were measured in brain tissue collected from CAF and sham-treated mice 3, 6, 16, 24, and 48 hours after injection (n≥5 per treatment and time). These cytokines and chemokine were tested because they are induced in serum in mice and in murine macrophages after treatment with cytotoxic chemotherapeutics (Elsea et al., 2008; Sauter et al., 2011). Quantitative RT-PCR was performed on an ABI 7300 Real-Time PCR System using prevalidated TaqMan master mix and commercial mouse-specific primer probes (IL-1 β, #Mn01336189_m1; IL-1RI, #Mn00434237_m1; IL-6, #Mn00446190_m1; TNFα, #Mn00443260_g1; MCP-1, #Mn00441242_m1; Applied Biosystems). Each RT-PCR reaction contained 5 µL of 2x Master Mix, 0.5 µL primer probe, and 5 µL of 2 ng/µL of cDNA. Reactions were run in duplicate or triplicate. Raw cycle threshold values from Eukaryotic 18S ribosomal subunit and GAPDH endogenous controls were compared between groups to validate that gene expression of internal controls was not affected by experimental treatment. Gene expression is expressed as fold change relative to sham treated group using the $2^{-\Delta\Delta C}{}_T$ method (Livak & Schmittgen, 2001) (ABI 7300 relative quantity study software version 1.3). Statistical analyses were conducted on the ΔC_T values for each gene since those values have a normal distribution.

Experiment 2: The Effect of CAF on Fatigue and Orexin Neuron Function

The purpose of this experiment was to determine if administration of a single

dose of CAF affects hypothalamic orexin neuron function after drug administration during the period of acute fatigue. Voluntary wheel running was measured in 12 mice housed individually in shoe-box cages with an activity wheel. Wheel turns were collected automatically in 15 minute periods with a magnetic reed switch (MiniMitter) and the Vital View Data Acquisition System (Vital View, Bend, OR). After acclimation for 10 days, baseline wheel running activity was collected for 10 days. Running activity was calculated as the percent change in voluntary wheel running activity from baseline for each mouse.

To indirectly quantify orexin neural activity, cFos expression in orexin neurons was examined and quantified with immunohistochemistry (IHC) as described previously (Grossberg et al., 2011). Briefly, animals were deeply anesthetized using sodium pentobarbital (65 mg/kg) and killed by transcardial perfusion with PBS followed by fixation with 4% paraformaldehyde (PFA) between 1915 and 1945, 24 hours after sham or CAF injection (6 animals per treatment). The 24hour treatment time coincides with acute reduction in locomotor activity after CAF injection. Animals were treated and tissue collected on sequential nights to allow for collection of tissue for all 12 animals between the desired times. Brains were post-fixed in PFA overnight and cryoprotected in 20% sucrose for 24 hours and frozen. Brains were sectioned at 30µm into a 1:4 series on a freezing microtome. Sections were blocked in 5% normal donkey serum then incubated for 72 hrs at 4C in primary antibody containing rabbit anti-cFos antibody (SC-52, Santa Cruz Biotechnology) diluted 1:5000 and goat anti-orexinA antibody (SC-8070, Santa Cruz Biotechnology) diluted 1:1500. Sections were washed then incubated 2 hrs at room temperature in donkey

anti-rabbit Alexa 594 and donkey anti-goat Alexa 488 (both 1:500, Invitrogen). Separate sections were incubated in the absence of primary antisera to ensure specificity of secondary antibodies. Sections were mounted and cover slipped in Aqua Polymount (Polysciences, Warrington, PA). Slides were viewed and photomicrographs obtained under a fluorescent microscope with appropriate filters (model 4000 DM, Leica Microsystems; or model LSM710, Carl Zeiss). Orexin-A positive neurons with and without nuclear localization of cFos immunoreactivity (IR) were counted (group assignment masked) using a dual red/green filter and counts confirmed with images at 488 and 594 taken separately with no significant differences in counts. Orexin neuron subpopulation locations in the dorsal medial hypothalamus (DMH), the perifornical hypothalamic area (PFA), and the lateral hypothalamic area (LHA) were based on mouse brain stereotactic measurements (Paxinos & Franklin, 2001). An external investigator photographed images with group assignment masked.

Justification of voluntary activity as a measure of fatigue. Objective levels of activity, such as actigraphy, are one measure to quantify fatigue in people following cytotoxic chemotherapy treatment. Similarly, volitional activity in animals is measured as cage locomotor activity or voluntary wheel running activity. Healthy rodents are active during their dark phase. This activity is suppressed by administration of bacterial endotoxin (Grossberg et al., 2011; Kozak et al., 1994), bacterial antigens (Ottenweller et al., 1998), synthetic double stranded RNA (poly I:C) in a viral infection model (Katafuchi et al., 2003), and with administration of cytotoxic chemotherapy (Mahoney et al., 2013; Ray et al., 2011; Wood et al., 2006b)

in a dose-dependent manner (Mahoney et al., 2013; Skinner et al., 2009). Furthermore, decreased voluntary wheel running was consistent with and a more sensitive sickness response to peripheral inflammation as compared with decreased food intake, decreased body mass, and fever (Skinner et al., 2009). Ray and colleagues (2011) reported that wheel running and cage locomotor activity were both decreased with similar patterns following cytotoxic chemotherapy administration.

Justification of c- Fos expression as a marker of neural activity. Nuclear expression of the cellular phosphoprotein (cFos) inducible transcription protein is commonly used as an indirect marker of neural activity. The cFos protein is translocated from the cytoplasm to the nucleus approximately 1 hour after stimulus activation, provides a quantifiable cellular resolution of neural activity (Morgan & Curran, 1991; Sagar et al., 1988)..cFos expression in hypothalamic orexin neurons has been shown to be suppressed by inflammatory challenge (Gaykema & Goehler, 2009; Grossberg et al., 2011).

Experiment 3: The Effect of CAF on CSF Levels of Orexin-A

Orexin-A neuropeptide levels were measured in CSF collected from rats at 16 hours after drug or sham treatment (n=9 per group) as an indicator of orexin neuron activity. Rats were used in order to obtain sufficient volume of CSF. Individually housed rats were administered CAF or NS between 1500 and 1600. At 0630-0730 the next day, rats were anesthetized with 2% isofluorane and a needle inserted percutaneously into the cisterna magna to collect 50 uL CSF. Animals were returned to their home cage on a warming mat during recovery. Protease inhibitor (Roche) was added to the CSF samples according to manufacturer's instructions. Samples

were frozen on dry ice and stored at -80C. The amount of orexin-A in each CSF sample was measured using a commercial radioimmunoassay (RIA) kit (Phoenix Pharmaceuticals). RIA was performed in duplicate using 25 uL of CSF according to the manufacturer's instructions. The interassay variability assessed by replicate analysis of a 250 pg/ml standard was 0.43%.

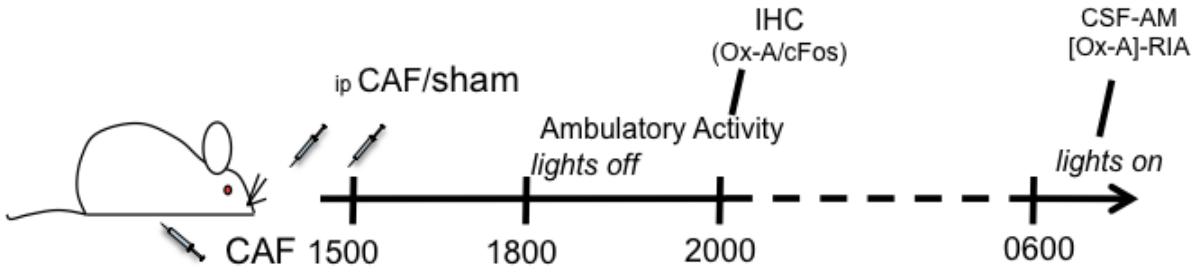

Figure 3.1. Depiction of timing of tissue collection to determine orexin neuron activity. Interperitoneal (ip) injection of cytotoxic chemotherapy (CAF) or sham at 1500 and 1600 on day 1, followed by collection of cerebrospinal fluid (CSF) the following morning in rats. For immunohistochemical (IHC) visualization of cFos in orexin-A+ neurons (Ox-A), mice were treated on day 1 with CAF or sham, and brains were collected approximately 24 hours later between 1915-1945. In experiment 4, bolus Ox-A was given centrally at 1730. Ambulatory activity was evaluated for the first 2 hours of the dark phase and throughout the dark phase.

Experiment 4: The Effect of Exogenous Orexin-A on Ambulatory Activity

The purpose of this experiment was to determine if central replacement of orexin-A neuropeptide restores activity in CAF-treated rats. Voluntary home-cage ambulatory activity and body temperature were measured in rats with telemetric transponders (MiniMitter) surgically implanted in the scapular region as described

previously (Grossberg et al., 2011). A ventricular cannula was surgically implanted into rats (n=20) 3 to 4 days after transponder implantation as described previously (Grossberg et al., 2011). Briefly, a 22 gauge sterile guide cannula with obturator stylet (Plastics One, Roanoke, VA) was implanted into the lateral ventricle and fixed in place with multiple screws and dental cement. The coordinates used were 1.5 mm lateral to midline,1.0 mm posterior to bregma, and 4.1 mm below the skull surface (Paxinos & Watson, 1998). After 3 days of recovery, animals and the lateral ventricle cannula were handled daily for 3 days to minimize stress responses with intracerebroventricular (icv) administration of fluid. Animals were allowed to acclimate for at least 5 days following ventricular cannulation surgery. Placement of cannula in the lateral ventricle was confirmed with return of clear cerebral spinal fluid prior to icv administration of orexin-A/saline. Body temperature and movement on the x-, y-, and z- axes were recorded in 5 minute intervals throughout the experiment with Vital View Data Acquisition System (Vital View, Bend, OR). Baseline ambulatory activity was collected for 3 days. Rats were injected ip with CAF or NS between 1430 and 1630 (CAF n=12; NS n=8).

Orexin-A neuropeptide was administered centrally 24 hours after CAF or sham injections. Rats were given 1 µL of either sterile 0.9% saline (NS) or I µL of saline with 1 µg of orexin-A (Ox-A) (California Peptide Research) into the lateral ventricle cannula between 1720 and 1740 (ip CAF-treated rats n=6 per icv group; ip NS-treated rats n=4 per icv group). The 1 µg dose of orexin-A was selected because it increased early dark phase activity in LPS-treated rats but not in sham-treated rats (Grossberg et al., 2011). Locomotor activity after ip CAF or sham treatment and after

icv administration of NS or Ox-A was compared to baseline for each animal. Inactive bouts were assigned when a 5-minute interval had a recorded movement of 5 counts or less.

Experiment 5: Orexin Neuron Activity after Multiple Doses of CAF

The purpose of this experiment was to determine if administration of multiple cycles of cytotoxic chemotherapeutic drugs is associated with diminished hypothalamic orexin neuron activity. Voluntary wheel running activity (VWRA) was measured in mice as described in Experiment 2 except wheel turns were summed over 60 min intervals. After establishing a 10-day baseline VWRA mice were separated into 2 groups. Mice in group 1 (n= 6) were injected with CAF while mice in group 2 were injected with NS. Mice were injected with an additional 3 doses of CAF or NS at 21-day intervals, mimicking a clinically relevant treatment regimen. All mice were provided with drinking water supplemented with antibiotics (150 µg/mL amoxicillin oral suspension, Sandoz) to prevent infection related to neutropenia, secondary to chemotherapy-induced bone marrow suppression.

Three weeks after the fourth dose of CAF or NS treatment, while CAF-treated animals still had fatigue, mice were terminally sedated between 1915 and 2000, perfused transcardially with 25 mL PBS followed by 25 mL 4% PFA as described in Experiment 2. Whole brains were removed, frozen, and sectioned for IHC as described in Experiment 2. Following IHC staining, sections (masked for treatment) were counted for nuclear visualization of c-Fos protein in neurons immunoreactive for orexin-A.

Statistical Analysis

Data were graphed and analyzed using GraphPad Prism 5. Comparisons between two groups at a single time point were performed using two-tailed Student's t-test. Comparisons between two treatments at multiple time points were performed using two-way ANOVA with post hoc Bonferonni-corrected t-test. Comparisons among 3 or more groups were examined with a one-way ANOVA with post hoc Bonferonni-corrected t-test. Repeated measures ANOVA was used to examine change in repeated measures of activity between treatment groups. Differences between groups were considered significant when $p<0.05$.

Chapter 4: Results

The purpose of this research was to determine if cytotoxic chemotherapy induces inflammatory gene expression in the hypothalamus or brainstem and to determine if single or multiple doses of these drugs suppress orexin neuron activity in rodents. If orexin neuron activity was suppressed, then an additional purpose was to test if orexin administration reverses chemotherapy-induced fatigue. The components of Figure 1.1 examined in this study are the effects of peripherally administered cytotoxic chemotherapy on central inflammation, orexin signaling, and fatigue.

Cytotoxic Chemotherapy Induces Brain Inflammatory Signaling

Since previous evidence suggests behavioral change such as fatigue/lethargy is mediated by neural signaling in the brainstem or hypothalamus, I tested if peripherally administered cytotoxic chemotherapy induces inflammatory gene expression in those brain regions. I examined gene expression of IL-1R1, IL-1β, IL-6, TNFα, and MCP-1 at 3, 6, 16, 24, and 48 hours after administration of NS or CAF. There was no increase in RNA expression of any of these inflammatory genes in either brain region at 3 hours after treatment. IL-1R1 gene expression was significantly increased in both the brainstem ($p<0.01$) and hypothalamus ($p<0.001$) at 6 hours after treatment (Figure 4.1A). IL-6 gene expression was also significantly increased in both the brainstem ($p<0.001$) and hypothalamus ($p<0.01$) at 6 hours after treatment (Figure 4.1B and C). This increase did not approach significance at any other time point tested (Figure 4.1C). TNFα gene expression was significantly induced in the hypothalamus at both 16 ($p<0.05$) and 24 hours ($p<0.01$) after treatment (Figure 4.1D). Although the average expression was elevated, it was not

significantly increased in the brainstem at 24 hours after treatment likely due to variability (Figure 4.1D). MCP-1 expression was significantly increased in both the brainstem ($p<0.05$) and hypothalamus ($p<0.05$) at 24 hours after treatment (Figure 4.1E), but not at other time points tested. Lastly, IL-1β gene expression was significantly increased in the brainstem, but not in the hypothalamus, at 48 hours after treatment ($p<0.05$) (Figure 4.1F). Taken together, these results indicate that cytotoxic chemotherapy induces inflammatory gene expression in both the brainstem and hypothalamus within 48 hours of treatment.

Reduced Activity after CAF is Associated with Decreased Orexin Signaling

CAF administered intraperitoneally to rats 3 hours before the start of the dark phase caused a significant reduction in ambulatory activity during the dark phase (Figure 4.2A-I). CAF-treatment caused a significant reduction in dark phase activity counts ($t(18)=7.42$, $p<0.001$) (Fig 4.2A), total minutes of activity ($t(18)=4.60$, $p<0.001$) (Figure 4.2B), average duration of sustained activity between inactive bouts ($t(18)=4.22$, $p<0.001$) (Figure 4.2C), and average rate of activity throughout the dark phase ($t(18)=7.93$, $p<0.0001$) (Figure 4.2D). CAF-treatment caused a significant increase in average duration of inactive bouts ($t(18)=2.59$, $p<0.05$) (Figure 4.2E) and total minutes of inactivity ($t(18)=4.96$, $p<0.001$) (Figure 4.2F) during the dark phase.

Though the maximum decrease in activity occurred 6 to 12 hours after treatment (Figure 4.2G), activity was significantly decreased during the first 2 hours of darkness, 4 hours after CAF treatment (Figure 4.2H). During the first 2 hours of

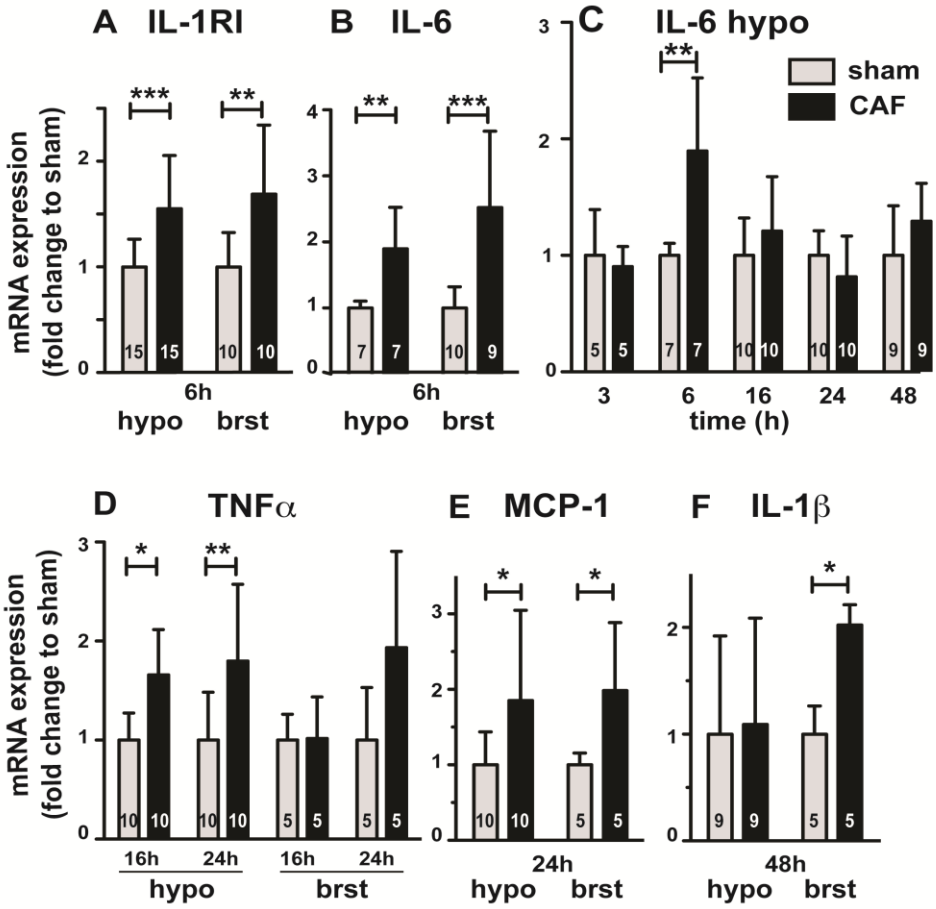

Figure 4.1. Hypothalamic (hypo) and brainstem (brst) mRNA levels of inflammatory genes after ip sham or cyclophosphamide, adriamycin, and fluorouracil (CAF) administration in mice. Significant differences in gene expression are presented as fold-change relative to sham. The mean and standard error are graphed. The number is of animals in each group is given in bars. A, IL-1R1 gene expression is induced in both brain regions 6h after ip CAF treatment. Expression after ip CAF administration is not different from sham at other time points tested. B, IL-6 gene expression is significantly induced 6 h after CAF treatment relative to sham in both brain regions. C, IL-6 gene expression in the hypothalamus after CAF treatment is not significantly different than sham at time points other than 6 h after treatment. D, TNFα gene expression at 16 h and 24h after CAF treatment is significant only in the hypothalamus. E, MCP-1 gene expression is significant in both brain regions at 24 h after treatment. F, IL-1β gene expression is significantly induced in the brain stem at 48 h after treatment. Interleukin 1 receptor type 1 (IL-1R1), Interleukin (IL)-1β (IL-1β), Tumor Necrosis Factor alpha (TNFα), Monocyte chemoattractant protein (MCP-1/CCL-2). *p<0.05, **p<0.01, ***p<0.001.

darkness (1800-2000), activity in CAF-treated animals was 57±3% of baseline activity (n=12) compared to sham (86±9%, n=8) ($t(18)=3.28$, $p<0.01$) (Figure 4.2H). Baseline activity between the groups prior to treatment was not significantly different at 1800-2000 ($p=0.2$) or throughout the dark phase, 1800-0600 ($p=0.1$) (Figure 4.2G). Locomotor activity, both ambulatory and voluntary wheel running, was initiated at the start of darkness in both treatment groups. Total counts of dark phase ambulatory activity in rats and voluntary wheel running activity in mice slowly returned to baseline 8 and 10 nights, respectively, after administration of a single dose of cytotoxic chemotherapy (Figure 4.2I). Irrespective of treatment, both rats and mice had limited activity during the light phase (Figure 4.2C and G). There was no difference in the counts of activity ($p=0.6$) or the average duration of bouts of activity ($p=0.5$) (Figure 4.2C) or inactivity ($p=0.1$) during the light phase between CAF and sham-treated animals.

Since impaired orexin signaling is associated with decreased locomotor activity in rodents, I tested if orexin signaling was decreased during the period of acute fatigue/lethargy following treatment with cytotoxic chemotherapy. CAF-treated rats had a 49% reduction in CSF levels of orexin-A compared to sham-treated rats (Figure 4.3) (mean-CAF=400 ± 27 pg/mL, n=9; mean-sham=813 ± 43 pg/mL, n=9; $t(16)=8.04$, $p<0.001$).

To further examine the effect of chemotherapy on orexin neurons, I examined cFos IR in orexin neurons 24 hours after chemotherapy treatment. Hypothalamic orexin neurons in mice treated with CAF had significantly reduced nuclear cFos IR compared to sham treated controls (n=6 per group; $p<0.05$) (Figure 4.4A).

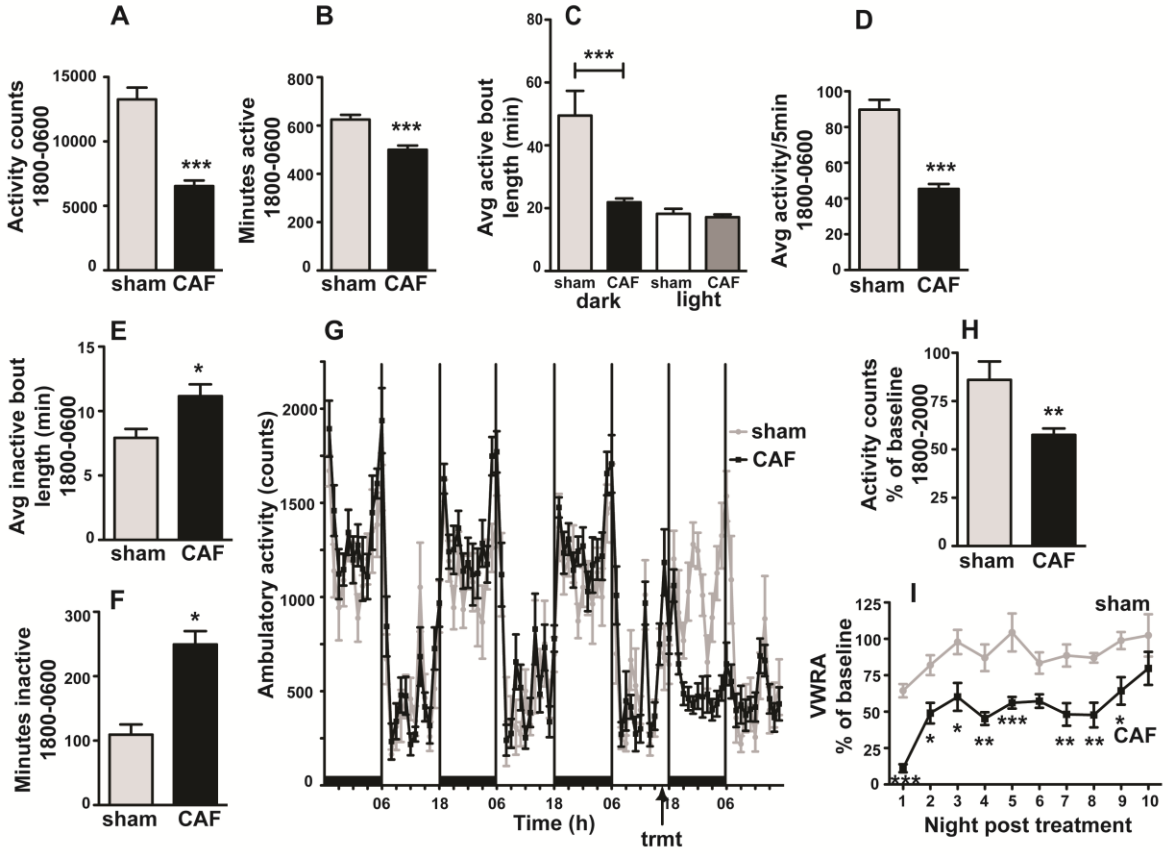

Figure 4.2. Cytotoxic chemotherapy reduces locomotor activity in rats and mice. A, Locomotor activity summed across the dark phase is reduced following CAF treatment in rats. B, Total minutes of activity during the dark phase is reduced following CAF treatment. C, The average duration of bouts of activity are decreased during the dark phase but not during the light phase after CAF treatment. D, The average rate of activity (average of movement during 5 minute bins that had activity) is decreased during the dark phase. E, CAF increases the average duration of inactive periods during the dark phase. F. CAF treatment increases the total minutes of inactivity during the dark phase. G, Baseline activity in the dark and light phase prior to and after CAF or sham treatment. H, CAF treatment decreases ambulatory activity calculated as % of baseline activity in the first 2 hours of the dark phase, 1800-2000. Rats n=8 sham, n=12 CAF. I, Voluntary wheel running activity in mice (n=6 each treatment) slowly returns to baseline at 10 nights after treatment with CAF. *p<0.05, **p<0.01, ***p<0.001.

Colocalization of cFos in orexin neurons in the dorsomedial hypothalamus (DMH), perifornical area (PFA), and the lateral hypothalamic area (LHA) subpopulations (Figure 4.4B) revealed a significant effect of treatment ($F(1,30)=13.32$; $p=0.001$) and anatomic location ($F(2,30)=18.15$; $p< 0.001$). cFos immunoreactivity in orexin neurons was significantly different between treatment groups in the dorsomedial hypothalamus (Bonferroni-corrected t-test $p<0.05$) (Figure 4.4A). There was no difference in cFos immunoreactivity in orexin neurons between treatment groups in the perifornical area and the lateral hypothalamic area. The total number of orexin IR neurons counted was not different between treatment groups ($p=0.9$). The numbers for one set of the four sets taken of each brain are as follows (mean ± SEM): all regions: sham 737 ± 53.5; CAF 743 ± 47.6; DMH: sham 229 ± 27.8; CAF 212 ± 9.2; PFA: sham 224 ± 17.2; CAF 244 ± 23.7; LHA: sham 284 ± 19.5; CAF 286 ± 25.6.

Administration of Orexin Restores Ambulatory Activity after Chemotherapy

To determine if decreased orexin neuron signaling underlies the reduction in ambulatory activity following treatment with cytotoxic chemotherapy, I administered intracerebroventricular (icv) orexin-A neuropeptide or the NS diluent to rats. A 1 μg bolus of orexin-A, given 30 minutes before the start of the dark phase, restored activity in CAF-treated animals. Among the chemotherapy treated rats, animals that received icv orexin-A had more activity ($p<0.05$) (Figure 4.5A) and a higher rate of activity ($p<0.05$) (Figure 4.5C) in the first 2 hours of the dark phase and throughout the 12 hour dark phase ($p< 0.05$) (Figure 4.5B and D) than animals that received icv NS. CAF-treated rats given orexin-A had increased duration of activity at the start of the dark phase as compared to icv NS (Bonferroni $t=2.61$, $p<0.05$) (Figure 4.5E).

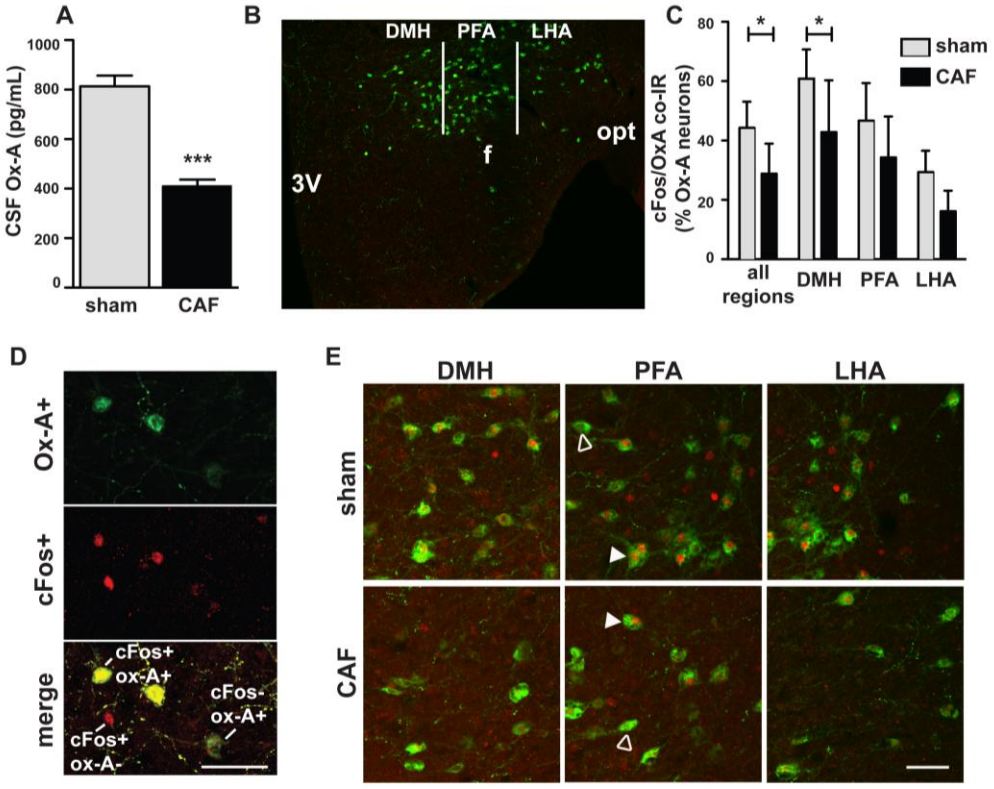

Figure 4.3. CAF treatment decreases dark phase orexin neuron activity. A, Rats treated with CAF at 1600 had decreased orexin-A (Ox-A) in cerebrospinal fluid (CSF) collected the following morning (n=9 each treatment). B, Image of mouse hypothalamic area (near Bregma – 1.7 mm) illustrating orexin neurons in the dorsal medial hypothalamus (DMH) (width of 500 µm lateral from 3rd ventricle-3V), perifornical hypothalamic area (PFA) (width of 500 µm lateral from DMH and includes the fornix (f)), and lateral hypothalamic area (LHA) (400 µm lateral from PFA to optic nerve (opt)) subpopulations counted. C, Percentage of orexin neurons with cFos immunoreactivity (IR) is less in CAF-treated mice, indicating CAF treatment reduces the normal evening rise of cFos in hypothalamic orexin neurons. Percentage of orexin neurons with cFos coexpression given for all orexin neurons in the hypothalamus and for separate anatomic subpopulations (n=6 each treatment). Data graphed is mean ± SEM. D, Photomicrographs of DMH orexin neurons after sham treatment showing orexin-A IR (green)-top panel, cFos IR (red)-middle panel, and the merged image showing 2 of the 3 orexin-A+ neurons co-stain for nuclear cFos (yellow cells), one orexin-A+ neuron is negative for cFos (green cell), and a cell negative for orexin-A that is positive for nuclear cFos (red nucleus). E, Hypothalamic orexin neurons subpopulations costained for cFos. Open arrowhead indicates cells expressing orexin-A but not nuclear cFos; closed arrowheads indicate cFos nuclear expression in orexin-A+ neuron. Scale bars=50 µm. *p<0.05, ***p<0.001.

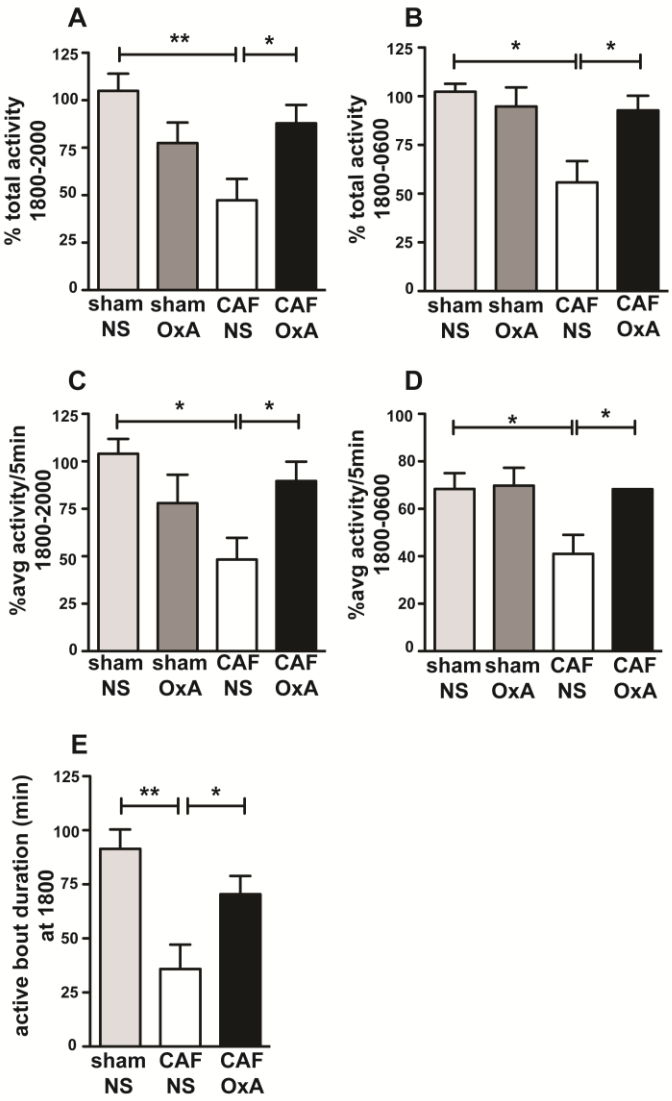

Figure 4.4. Central orexin-A administration restores ambulatory activity counts (A, B) and the average activity rate per 5 minute bin (C, D) during the first 2 hours of the dark phase (A, C) and throughout the dark phase (B, D) as compared to baseline. Activity calculated as percentage of baseline activity for each animal. E, Orexin-A administration increases duration of the first bout of activity at the beginning of dark phase. Legend: sham/NS= ip sham and icv NS (n=4); sham/OxA= ip sham and icv OxA (n=4); CAF/NS= ip CAF and icv NS (n=6); CAF/OxA= ip CAF and icv Orexin-A (n=6). Sham/OxA was not significantly different than sham/NS in any of the measures. CAF/OxA was not significantly different than sham/NS in any of the measures. *p<0.05, **p<0.01.

Dark phase total activity (Figure 4.5B), rate of activity (Figure 4.5D), and duration of activity at the start of the dark phase (Figure 4.5E) in CAF-treated animals given icv orexin-A was not significantly different than activity in sham-treated control animals given icv NS. CAF-treated animals that did not receive icv orexin-A showed the expected reduced activity (57% of baseline) at the beginning of the dark phase ($p<0.01$) (Figure 4.5A) and throughout the dark phase (51% of baseline) ($p<0.05$) (Figure 4.5B). Taken together, orexin replacement in CAF-treated rats restored dark

phase activity—rate, duration, and total amount—similar to that in control animals.

Orexin Neuron Activity Suppressed after Multiple Cycles of Chemotherapy

The effect of four cycles of CAF on hypothalamic orexin neuron cFos IR was examined since prior work in our laboratory showed that persistent fatigue can occur after multiple CAF cycles. CAF-treated mice had significantly reduced cFos IR in orexin-A-expressing neurons compared to sham treated controls (n=4 each treatment, $t(6)=3.59$, $p=0.01$) (Figure 4.6A and B). Across all regions, 22.1% of orexin-A-expressing neurons had cFos IR in CAF-treated mice vs 49.2% in sham. The 3 hypothalamic subpopulations of orexin neurons showed a significant effect of treatment ($F(1,12)=14.99$; 53% of variation, $p<0.01$) and anatomic location ($F(2,12)=53.13$; 20% of variation, $p<0.001$). Post-hoc analysis showed a significant decrease in cFos IR in both the DMH ($t=3.98$, $p< 0.01$) and PFA ($t=4.61$, $p< 0.001$) orexin neurons (Figure 4.6B). No difference in cFos nuclear IR between treatment groups was observed in the LHA. Rostrocaudal location had no observed effect on cFos IR in orexin neurons. The number of orexin-A+ IR neurons counted across the hypothalamus and in each region did not differ with treatment ($p=0.8$; data not shown).

Cytotoxic Chemotherapy Reduces Body Temperature

Though fever is often associated with infection and LPS-induced decreased activity, rats treated with cytotoxic chemotherapy had significantly reduced dark phase body temperature (96.1% of dark phase baseline (range 34.1C to 36.8C); $p<0.001$, n=12) compared to sham-treated controls (99.8 % of dark phase baseline (36.7C to 37.9C), n=8). Body temperature during the light phase was similar in both

groups and did not differ from their light phase baseline temperature. Dark phase body temperature returned to baseline temperature the second night after cytotoxic chemotherapy treatment (data not shown), even though activity during the second night after drug treatment remained approximately 50% decreased from their dark phase baseline. Body temperature on the second night after sham or drug treatment, which had returned to baseline, was not further affected by central administration of the orexin-A neuropeptide or NS (data not shown).

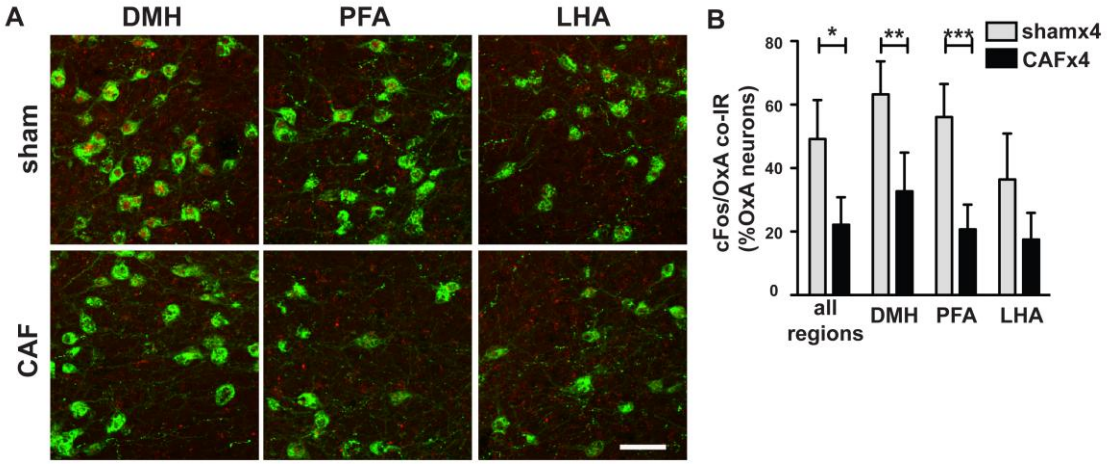

Figure 4.5. The normal evening rise of cFos in orexin neurons in the hypothalamus is reduced in mice after multiple cycles of CAF treatment is given in doses 21 days apart. A, Representative photomicrographs of cFos (shown in red nuclei) in orexin neurons (shown in green) in DMH, PFA, and LHA in sham and CAF-treated mice. B, Percentage of orexin neurons with cFos IR following treatment with ip sham or CAF, given for all orexin neurons in the hypothalamus and for separate anatomic subpopulations. n=4 each treatment. Scale bar=50µm. *p<0.05, **p<0.01, ***p<0.001

Chapter 5: Discussion

Fatigue is a common symptom associated with infection and cancer chemotherapy treatment. Infection induces peripheral inflammation that alters neural signaling in the brain, resulting in sickness behavior that conserves energy and promotes recovery from infection in the short term (Dantzer et al., 2008; Kelley et al., 2003; Konsman et al., 2002). Since cytotoxic chemotherapy is known to induce peripheral inflammation, fatigue associated with cytotoxic chemotherapy may have underlying mechanisms similar to that caused by infection (Bower & Lamkin, 2013; Cleeland et al., 2003; Dantzer et al., 2012; Saligan & Kim, 2012). The neural mechanisms associated with cytotoxic chemotherapy induced fatigue have not been previously reported.

I report here three important results from this research. First, peripherally administered cytotoxic chemotherapy induces inflammatory gene expression within 48 hours after treatment in both the brainstem and the hypothalamus. Second, cytotoxic chemotherapy suppresses hypothalamic orexin neuron function. This is demonstrated with reduced orexin-A neuropeptide concentration in the cerebrospinal fluid after treatment with cytotoxic chemotherapy and failure of normal physiological activation of orexin neurons (as measured by cFos nuclear localization). Third, central replacement of the orexin-A neuropeptide restores ambulatory activity in cytotoxic chemotherapy treated animals. Taken together, these findings indicate a role for orexin neuron activity in cytotoxic chemotherapy-induced fatigue. To my knowledge, this is the first report of altered neural signaling associated with cytotoxic chemotherapy induced fatigue/lethargy.

My findings are consistent with studies demonstrating decreased orexin function in other conditions involving central inflammation. Traumatic brain injury and treatment with the bacterial endotoxin lipopolysaccharide (LPS) both result in decreased orexin neuron activity (Baumann et al., 2005; Gaykema & Goehler, 2009; Gerashchenko & Shiromani, 2004; Grossberg et al., 2011; Willie et al., 2012). In addition, decreased orexin neuron activity is associated with the fatigue that accompanies both of these conditions.

Cytotoxic Chemotherapy Induces Brain Inflammatory Gene Expression

The cytotoxic chemotherapeutic drugs used in this study, cyclophosphamide, adriamycin, and fluorouracil, acutely increase circulating inflammatory cytokine levels in mice (Sauter et al., 2011; Wong et al., 2012; Wood et al., 2006b). Fluorouracil was recently found to increase circulating MCP-1 levels at 5 and 15 days after multiple doses of drug (Mahoney et al., 2013). Yet, it is not known if these cytotoxic chemotherapeutics induce hypothalamic inflammation. LPS induces peripheral inflammation, central inflammation in the hypothalamus and brainstem, and sickness behaviors such as anorexia and fatigue (Capuron & Miller, 2011; Gayle et al., 1998). Chemotherapy induces similar sickness behaviors, suggesting a common mechanism with inflammatory stimuli such as LPS. But, we observed a decrease in core body temperature after CAF injection not followed by a febrile period. This is in contrast to the typical body temperature response after peripheral inflammatory insults. This observation suggests a different hypothalamic response pathway may be active after cytotoxic chemotherapy.

I found that CAF cytotoxic chemotherapy induced a similar pattern of

inflammatory gene expression in the brainstem and hypothalamus. This similarity could result from inflammatory signaling from the periphery communicated to both regions due to the attenuated blood brain barrier in those regions, vagal signaling (Bluthe et al., 1994), or signaling from the brainstem to the hypothalamus (Gaykema & Goehler, 2011).

I was surprised to not detect elevated IL-1β gene expression at or prior to 6 hours after treatment since IL-1β is considered a key initiating cytokine for induction of inflammatory signaling. Adriamycin induces formation of the NLRP3 inflammasome protein complex that cleaves pro-IL-1β to the active IL-1β (Sauter et al., 2011). This release of IL-1β could act to induce IL-1R1 and IL-6 gene expression at 6 hours after treatment without increased IL-1β gene expression. I found IL-1β gene expression was significantly induced in the brainstem at 48 hours after treatment, indicating a possible biphasic inflammatory response.

One limitation of this study is that I did not examine gene expression beyond 48 hours after treatment. As a result, I do not know if the late release of IL-1β induced a second increase of IL-6 or IL-1R1 gene expression. It was not feasible to simultaneously measure the concentration of cytokines in these brain regions, so I do not know if the active form of IL-1β was increased in the hypothalamus or brainstem following treatment. In addition, I tested only the common intraperitoneal route of administration of drug. It is possible that an intravenous route could result in different central effects (Bluthe et al., 1996). The presence of central inflammation following cytotoxic chemotherapy suggests a mechanism by which these drugs can alter neural signaling. However, prostaglandin and central IL-1 signaling were not

required in LPS-induced fatigue/lethargy (Grossberg et al., 2011). It is not known how inflammation alters orexin neuron signaling. This area warrants additional study.

Physical Activity to Measure CAF-Induced Fatigue

The change in ambulatory activity in rodents after treatment with cytotoxic chemotherapy in this study captures the salient descriptions of chemotherapy-induced fatigue in people including decreased total activity, decreased duration of activity, slowed activity, and increased need for rest (Scott et al., 2011). Activity during the dark phase slowly returned to baseline in the subsequent 8-10 nights after treatment in rodents. My results are consistent with other reports of decreased activity with cytotoxic chemotherapy in rodents (Mahoney et al., 2013; Ray et al., 2011; Wood et al., 2006b). I collected ambulatory activity data in 5 minute periods after treatment with cytotoxic chemotherapy. This supports a detailed analysis of activity and inactivity duration and rate not found in previous reports of total dark phase activity after cytotoxic chemotherapy.

Decreased Orexin Signaling as a Component of Fatigue

In nocturnal rodents, orexin neurons signal throughout the dark phase (Estabrooke et al., 2001; Lee et al., 2005), with discharge specific to wakefulness, exploration, and motivated behavior (Mileykovskiy et al., 2005; Takahashi et al., 2008). Orexin neurons project densely to neurons involved in arousal and wakefulness including noradrenergic neurons in the locus coeruleus (Carter et al., 2012) and monoaminergic, cholinergic, and histaminergic neurons in the raphe nuclei, the tuberomammillary nucleus, the pontine reticular formation, and the laterodorsal tegmental nucleus (Date et al., 1999; Peyron et al., 1998). Suppression

of orexin neuron function with traumatic brain injury or administration of LPS is associated with fatigue (Willie et al., 2012; Grossberg et al., 2011). Replacement of orexin restores activity after administration of LPS (Grossberg et al., 2011). In addition, orexin peptide replacement in orexin knockout rodents restores locomotion and results in restored wakefulness (Anaclet et al., 2009; Kantor et al., 2013). I report here that cytotoxic chemotherapy decreases orexin neuron function and induces fatigue during the dark phase; exogenous administration of the orexin neuropeptide reverses this fatigue. My results are consistent with earlier work on effects of orexin on dark phase activity in rodents.

Results from acute and chronic administration of cytotoxic chemotherapy in this study support the functional division of orexin neurons described previously. The dorsomedial hypothalamus and perifornical area orexin subpopulations regulate arousal and stress responses (Harris & Aston-Jones, 2006; Nollet et al., 2011; Yoshida et al., 2006). I found a significant suppression of the dorsomedial hypothalamus orexin neurons after a single dose and multiple doses of cytotoxic chemotherapy, and suppression of the perifornical area orexin neurons after multiple doses of chemotherapy. My findings are similar, but not identical to those of Grossberg et al. (2011) who reported orexin neuron activity in the perifornical area subpopulation is suppressed by LPS-induced inflammation and this suppression is associated with decreased locomotor activity.

It is not surprising that LPS and cytotoxic chemotherapy may have slightly different effects on orexin neurons. LPS causes decreased activity in rodents for less than one day, while cytotoxic chemotherapy reduces activity for many days following

a single treatment. LPS induces hypothalamic IL-1β gene expression within 2 hours after treatment, a result I did not observe with cytotoxic chemotherapy. LPS induces higher IL-6 gene expression after treatment as compared to my results with a single dose of cytotoxic chemotherapy (Grossberg et al., 2011). LPS often induces a febrile response, and I found cytotoxic chemotherapy induces hypothermia. Taken together, this indicates different effects between these agents on neuron populations in the hypothalamus.

Elevated inflammatory gene expression induced by LPS as compared to chemotherapy treatments could result since cytotoxic chemotherapy binds DNA and interferes with RNA and protein synthesis (Longley et al., 2003; Vacchelli et al., 2012). Differences in the rate of induction and severity of hypothalamic inflammation could result in different effects on neurons and subsequent symptoms.

Comparison of the effects of LPS and chemotherapy on orexin neuron subpopulations in different studies is challenging. The orexin subpopulations are approximate and difficult to discern on some hypothalamic sections. Since identification of subpopulations is subjective, it is important to note that I found a significant decrease in orexin neuron activity across all regions following both acute and chronic treatment with cytotoxic chemotherapy.

My visual observation of decreased orexin neuron activity indicated by decreased nuclear localization of cFos was confirmed with finding that CSF concentration of orexin-A was decreased after administration of cytotoxic chemotherapy. Orexin peptides are released from orexin neurons throughout the active phase, which presumably drives a diurnal fluctuation of orexin-A in CSF (Desarnaud et al., 2004). I

measured CSF concentration of orexin-A at the morning peak level and not at the nadir prior to the dark period since it was previously shown that only the morning peak level was altered in rodents given peripheral LPS (Grossberg et al., 2011).

Decreased CSF levels of orexin-A can result from the loss of orexin neurons. CSF levels of orexin-A were decreased 50% following loss of 73% of orexin neurons with saporin neurotoxin targeted lesions (Gerashchenko et al., 2003). In a follow-up study, the same group found a six day central infusion of LPS decreased the number of orexin neurons (Gerashchenko & Shiromani, 2004). Since both peripheral and central LPS are known to induce central inflammation, this indicates that orexin neurons are sensitive to damage from inflammation. The acuity and duration of the inflammation may influence the effect on orexin neurons

In this study I did not find any difference in the number of hypothalamic orexin neurons between treatment with cytotoxic chemotherapy and sham. Similar to my results, a single dose of LPS and experimental traumatic brain injury were also found to decrease CSF levels of orexin-A at the end of the dark phase without any change in the number of hypothalamic orexin neurons (Grossberg et al., 2011; Willie et al., 2012). There are many potential causes of decreased neuropeptide release from neurons other than the permanent loss of neurons. For example, orexin neurons are particularly sensitive to endoplasmic reticulum stress, and this impairment may affect correct folding of orexin-A (Michinaga et al., 2011; Obukuro et al., 2013). Since orexin neuron number is not decreased but their function secreting orexin-A neuropeptide is impaired from chemotherapy, this suggests that recovery of full orexin signaling may be possible at some point following treatment with cytotoxic

chemotherapy.

Morning orexin-A levels in CSF need to be tested beyond 16 hours after treatment with chemotherapy. If CSF levels of orexin-A return to baseline many days after cytotoxic chemotherapy treatment, and if return to baseline is slower after multiple doses, then this supports an intervention to restore orexin neuron function after cytotoxic chemotherapy. It is important to determine if restored CSF levels of orexin-A are associated with restored baseline activity after cytotoxic chemotherapy treatment.

Exogenous Orexin Restores Activity after Cytotoxic Chemotherapy

If chemotherapy induces fatigue by decreasing orexin neuron function, then orexin replacement should reverse this fatigue. I found central replacement of orexin-A restored ambulatory activity movement and the rate of activity in the first two hours of the dark phase when healthy rodents have highest activity. Administration of orexin-A to CAF-treated rats also increased the duration of sustained activity at the start of the dark phase. Similar to my results, restoration of this normal pattern of arousal and sustained wakefulness was recently reported in rodents given orexin-A producing stem cells following orexin neuron ablation (Kantor et al., 2013). In my study, chemotherapy treated animals given central orexin-A showed normal patterns of grooming, had restoration of movement in the cage, restoration of the rate of activity, and increased duration of activity upon wakening, indicators that orexin-A restored functional activity rather than inducing hyperactivity or pathological activity.

Centrally administered orexin-A tends to increase activity for two hours (Kiwaki et

al., 2004; Samson et al., 2010). Though the effect on activity was less than that measured during the first 2 hours after administration, I was surprised to find that exogenous orexin-A stimulated activity in CAF-treated rats across the 12-hour dark phase. One explanation for this longer activity is that orexin-A stimulates orexin-A release (Yamanaka et al., 2010). Physical activity in animals may also stimulate increased CSF levels of orexin-A, resulting in a sustained active period (Wu et al., 2002). The increased dark phase activity measured in CAF-treated rats after administration of orexin-A did not extend into the light phase. It is not surprising that an exogenous bolus of orexin-A did not affect locomotor activity 12 hours after administration. My results indicate that the normal rodent diurnal pattern of light phase inactivity was not affected. Activity beyond 24 hours after administration of central orexin-A was not examined in this study.

Administration of orexin-A to control rats—those not treated with CAF—did not significantly affect dark phase activity, consistent with earlier reports where orexin-A was given at a similar concentration at the start of the dark phase (Anaclet et al., 2009; Grossberg et al., 2011). A dose of orexin-A four times greater than that used in my study was found to increase dark phase activity in control animals for two hours after orexin-A treatment (Kotz et al., 2002). Increased dose of orexin-A increases activity (Kiwaki et al., 2004; Nakamura et al., 2000; Samson et al., 2010) perhaps explaining this difference between my findings and earlier work.

Administration of orexin-A during the light phase has repeatedly been shown to increase activity in control rodents for up to two hours during the normally inactive light phase (Fenzl et al., 2011; Kiwaki et al., 2004; Nakamura et al., 2000; Novak &

Levine, 2009; Samson et al., 2010). Rodents are active during the dark phase. Treatment in this study was aimed at restoring normal dark phase activity, not at producing a pharmacologic enhancement of activity during the light phase when rodents are normally less active. The approach in this study was based on the long-term goal of restoring functional activity in people with chemotherapy-induced fatigue.

There are a few additional areas of study to better understand the role of orexin neuron function in cytotoxic chemotherapy-induced fatigue before targeted interventions with orexin agonists are tested. The rate of return of CSF orexin-A levels to baseline after single and multiple doses of cytotoxic chemotherapy treatment needs to be measured. The effect of exogenous orexin-A on activity after multiple doses of chemotherapy needs to be tested. The effect of exogenous orexin-A in female rats should be examined. The effects of other types of cytotoxic chemotherapeutic agents on orexin neuron function have not yet be tested.

Implications

Understanding the underlying mechanisms of cytotoxic chemotherapy-induced fatigue can support the development of interventions to prevent or treat this fatigue. This study supports earlier research indicating that suppression of the central inflammatory response and restoration of orexin signaling are both potential targets to prevent or treat cytotoxic chemotherapy induced fatigue (Grossberg et al., 2011; Wong et al., 2012; Wood & Weymann, 2013). I found that central replacement of orexin-A neuropeptide restored functional activity during the active phase in rats. Central replacement of orexin-A is not clinically feasible. However, an oral agonist of

the orexin receptor is in clinical trials to treat narcolepsy (NIH RePORT #1U01NS080400-01), a disorder in which orexin signaling is impaired (Chemelli et al., 1999; Lin et al., 1999).

Although the focus of this study was on acute fatigue following cytotoxic chemotherapy, I found orexin signaling was disrupted following multiple doses of chemotherapy, indicating a similar underlying neural mechanism with acute and persistent fatigue. In addition, Grossberg and colleagues (2011) found the number of orexin expressing cells in the hypothalamic PFA was significantly reduced in a sarcoma tumor bearing rodent model. Those findings indicate that chronic inflammation associated with cancer may also suppress orexin neuron function and may underlie fatigue. Fatigue often persists following multiple doses of cytotoxic chemotherapy, interfering with functional recovery from cancer and cancer treatment. It will become clinically feasible to test the effect of the oral orexin agonist on persistent fatigue associated with both cancer and cancer treatment.

My results support a connection of the components of the conceptual model framing this study, that cytotoxic chemotherapy induces central inflammation, impaired orexin signaling, and fatigue behavior (Figure 1.1). I have not demonstrated in this study if the effect of chemotherapy on orexin neurons requires either peripheral or central inflammation. Though the chemotherapy drugs used in this study do not have brain or CNS penetration at therapeutic levels, there is evidence of some penetration (Rinne et al., 2012; Soussain et al., 2009; Tangpong et al., 2007). Fluorouracil is toxic to CNS progenitor cells and to non-dividing oligodendrocytes, resulting in damage to CNS myelin tracts. This damage did not

correlate with chronic inflammation or extensive vascular damage (Han et al., 2008). It is possible that cytotoxic chemotherapy has a direct effect on hypothalamic orexin neurons.

A limitation of this study is that other potential contributors to cancer treatment related fatigue including impaired sleep, anemia, anorexia, cachexia, cognitive deficits, depression, and pain were not examined. There are treatments for anemia, depression, and pain. Orexin replacement is unlikely to address fatigue resulting from other underlying factors. The complexity of fatigue suggests that assessment for different possible contributors to fatigue will need to be individually examined in order to gain benefit from targeted interventions.

There are no broadly successful interventions to treat cytotoxic chemotherapy-induced fatigue. Drugs that stimulate arousal, such as methylphenidate and modafinil, have limited success in the treatment of persistent fatigue with cancer (Bruera et al., 2013; Jean-Pierre et al., 2010; Lower et al., 2009; Minton et al., 2011). An orexin agonist may be more suited to increase functional arousal as compared to altering dopamine or histamine signaling (Anaclet et al., 2009). Physical exercise is a non-pharmacologic intervention that has shown benefit in reducing cancer-related fatigue (Kummer et al., 2013; McNeely & Courneya, 2010; Mitchell et al., 2007). However, adherence to a regular schedule of physical activity is a barrier to this intervention (Wenzel et al., 2013).

An important area of future research is examining the effects of orexin-A replacement after multiple cycles of cytotoxic chemotherapy. Depending upon results of current clinical trials of the orexin agonist to increase wakefulness in

people with narcolepsy, clinical studies with the orexin agonist should be conducted in people with persistent fatigue following cancer therapy. It is possible that fatigue in the first few days following chemotherapy treatment for cancer is protective to decrease the impact of those drugs on brain neurons. This should be tested. Fatigue that persists weeks or months after treatment with cytotoxic chemotherapeutics is unlikely to be beneficial. Targeted interventions are needed for this persistent fatigue. Based on results presented here and previous research on orexin, I propose that treatment with an orexin agonist may promote functional activity, daytime wakefulness, and increase success in maintaining physical activity in people treated with cytotoxic chemotherapy. Treatment for cytotoxic chemotherapy-induced fatigue will improve functional recovery from cancer.

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