THE EFFECTS OF CILIARY NEUROTROPHIC FACTOR ON ADIPOSE TISSUE APOPTOSIS

by

EMILY DUFF

(Under the Direction of Dr. Clifton A. Baile)

ABSTRACT

The administration of Ciliary Neurotrophic Factor (CNTF), a neurocytokine recently implicated for its role in energy homeostasis, results in a reduction of food intake and body weight and induces adipose tissue apoptosis *in vivo.* The first two objectives of this study, determining the effects of CNTF on food intake and body weight as well as adipocyte apoptosis, were tested by injecting rats intracerebroventricular (icv) with CNTF or vehicle for 4 days before sacrificing for data collection and analysis. The high dose of CNTF (3.2 nM) caused a reduction in food intake and body weight, and induced adipose tissue apoptosis in specific fat pads. The third objective was to determine whether CNTF acts directly on 3T3-L1-derived adipocytes to induce apoptosis. CNTF did not cause apoptosis *in vitro*. To confirm that CNTF was acting directly on the adipocytes, Western Blot analysis was used to measure the expression of the activated phosphorylated STAT 3 *in vitro.*

INDEX WORDS: Adipose Tissue, Apoptosis, Ciliary Neurotrophic Factor, CNTF, Leptin, Obesity

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CHAPTER 1

INTRODUCTION

Over the past two decades, the occurrence of obesity among US adults has increased by 74% and about two thirds of US adults are currently either overweight or obese (Mokdad et al. 2003). In addition, the incidence of obesity and overweight seen in US children is also continuing to rise (Ogden et al. 2002). Evidence indicates that overweight and obesity are strongly associated with major health risks like cardiovascular disease, diabetes and cancer (2000). These obesity-related, adverse health consequences have led to an increased number of preventable deaths (Fontaine et al. 2003). Therefore, the prevention and treatment of obesity is imperative to curtail the rising incidence of morbidity and mortality. Overweight and obesity is a complex disorder with multiple causes including both genetic and environmental factors. There have been great advances in recent years toward determining the hypothalamic pathways responsible for the modulation of energy balance. Since the cloning of the leptin gene and characterization of the leptin protein in 1994 (Zhang et al. 1994), a growing number of molecules implicated in energy homeostasis have been discovered, creating limitless possibilities for how bodyweight regulation may occur. Ciliary Neurotrophic Factor (CNTF) is an example of one of these factors that has recently been considered for the role it plays in obesity (Duff et al. 2003). CNTF is a neurocytokine that is released from glial cells in the central and peripheral nervous system. Initial interest in CNTF focused on its role as a trophic factor in nervous system development and maintenance as well as the support of the survival of a variety of neuronal cell types *in vitro* and *in vivo* (Adler et al. 1979; Barbin et al. 1984; Ip et al. 1991; Ip et al. 1992; Clatterbuck et al. 1993; Hagg et al. 1993; Richardson 1994). After a serendipitous finding during a clinical trial in which CNTF was being used to treat the neurodegenerative disease

Amyotrophic Lateral Sclerosis (ALS), researchers discovered that CNTF actually causes weight loss and reduced food intake in subjects (1996). Since this discovery, CNTF has been shown to reduce food intake and body weight in rodent models characterized by leptin resistance (Gloaguen et al. 1997; Kalra 2001; Lambert et al. 2001). CNTF has repeatedly been compared to leptin for its similar mode of action which is often credited to the location of their receptors (CNTFR α and Ob-Rb, respectively) in the arcuate (ARC) nucleus of the hypothalamus. A recent study showed that the central administration of leptin causes the deletion of adipocytes by apoptosis in rat adipose tissue (Qian et al. 1998). The work described here is based on the hypothesis that CNTF, a leptin-like cytokine, would also induce adipose tissue apoptosis when administered intracerebroventricular (icv) in rats. After the finding that the CNTF receptor, CNTFRα, is expressed not only in the arcuate nucleus of the hypothalamus but also by the adipocyte, this hypothesis was extended to include CNTF's direct mode of action on adipocytes to induce apoptosis.

CHAPTER 2

OVERWEIGHT AND OBESITY

Health Implications

The prevalence of overweight and obesity has increased at alarming rates in recent years and is a serious health issue worldwide. From 1995 to 2000, the obese population grew from 200 million to 300 million people worldwide (WHO 2004). In 2000, obesity incidence in the U.S. had increased approximately 61% since 1991 (CDC 2004). The rate of adolescent obesity has increased significantly as well (Troiano et al. 1998). In 1999-2000, an estimated 15% of U.S. children and adolescents aged 6-19 years were overweight (CDC 2004). Overweight is defined as increased body weight in relation to height, when compared to a standard of acceptable or desirable weight (NRC 1989; Stunkard et al. 1993). Obesity is defined as an excessively high amount of body fat or adipose tissue in relation to lean body mass (NRC 1989; Stunkard et al. 1993). Body Mass Index (BMI) is a mathematical formula frequently used to determine whether someone is at risk for overweight or obesity. BMI expresses the relationship (or ratio) of weight-to-height (NRC 1989; Kraemer et al. 1990; Stunkard et al. 1993) in a formula in which a person's body weight in kilograms is divided by the square of his or her height in meters.

$$BMI = \frac{weight_{kg}}{height_{m^2}}$$

Individuals with a BMI of 25 to 29.9 are considered overweight, while individuals with a BMI of 30 or more are considered obese (CDC 2004). Another means to determine whether one is overweight or obese is measuring the waist circumference or waist-to-hip ratio (WHR). This value is mathematically calculated as the waist circumference divided by the hip circumference.

A WHR of 1.0 or higher is considered "at risk" for undesirable health consequences associated with overweight and obesity (CDC 2004).

Research studies have reported that obesity increases the risk of developing a multitude of health conditions including type II diabetes, hypertension, coronary heart disease, ischemic stroke, colon cancer, post menopausal breast cancer, endometrial cancer, gall bladder-disease, osteoarthritis, and obstructive sleep apnea. A more comprehensive list is depicted in Figure 2.1.

- High blood pressure, hypertension
- High blood cholesterol, dyslipidemia
- Type II (non-insulin dependent) diabetes
- Insulin resistance, glucose intolerance
- Hyperinsulinemia
- Coronary heart disease
- Angina pectoris
- Congestive heart failure
- Stroke
- Gallstones
- Cholesystitis and cholelithiasis
- Gout
- Osteoarthritis
- Obstructive sleep apnea and respiratory problems
- Some types of cancer (such as endometrial, breast, prostate, and colon)
- Complications of pregnancy
- Poor female reproductive health (such as menstrual irregularities, infertility, irregular ovulation)
- Bladder control problems (such as stress incontinence)
- Uric acid nephrolithiasis
- Psychological disorders (such as depression, eating disorders, distorted body image, and low self esteem)

Figure 2.1: Physical ailments associated with overweight and obesity (NRC 1989; Stunkard et al. 1993).

In 1999-2000, according to the National Health and Nutrition Examination Survey (NHANES 1999-2000), an estimated 30% of U.S. adults aged 20 years and older (~59 million people) were obese defined as having a body mass index (BMI) of 30 or more. In the same report, an

estimated 64% of U.S. adults aged 20 or older were either overweight or obese, defined as having a body mass index (BMI) of 25 or more (CDC 2004). In addition to being at risk for illnesses such as type II diabetes and hypertension, obese individuals also endure a social stigma (Friedman 2000). This social situation leads to increased psychological disorders including depression and eating disorders. During certain time periods, in many cultures, obesity has been considered to be a sign of affluence and prestige, particularly among cultures were food is less available. In the late twentieth-century, and now modern times, intense pressure to be thin is felt by the majority of individuals, lean and obese (Kuczmarski et al. 1994).

Obesity is a very costly disease, having an enormous economic impact on the U.S. Health care system. Direct and indirect costs associated with obesity must be considered when describing the bearing that this disease has on the economy .(Wolf 1998; Wolf et al. 1998). Direct medical costs include preventative, diagnostic, and treatment services related to obesity. Indirect costs relate to morbidity and mortality costs such as income lost from decreased productivity, restricted activity, absenteeism, and bed days (CDC 2004). In 1998, medical expenses attributed to both overweight and obesity accounted for 9.1% of total U.S. medical expenditures and may have reached as high as $78.5 billion ($92.6 billion in 2002 dollars). Approximately half of these costs were paid by Medicaid and Medicare (Finkelstein et al. 2003).

While the consequences of obesity are clearly evident, the causes are much more complex. There are numerous factors involved in the development of overweight and obesity and it is difficult to attribute the cause to just one factor. Genes, metabolism, behavior, environment, culture, and socioeconomic status are all contributing factors to the causation of obesity. Although obesity has strong genetic determinants, the genetic composition of the population does not change rapidly. Therefore, the large increase in obesity likely reflects major changes in

non-genetic factors (Hill et al. 1998). Behavior and environment are main contributors causing people to be overweight and obese, thus providing the greatest opportunities for prevention and treatment (CDC 2004). Overweight and obesity result from an energy imbalance over an extended period of time. In the United States, a changing environment has included more accessible food that tends to be high in fat, sugar, and calories as well as larger portion sizes. Along with a decrease in physical activity, these factors contribute to an overall increase in calorie intake and decrease in energy expenditure, thus resulting in positive energy balance and weight gain.

This growing obesity epidemic has gained notable attention in recent years and great measures are being taken to help solve this problem. The Surgeon General recently called for a broad approach to avoid and reduce obesity. He challenged families, schools, work sites, health care providers, communities, and the media to work together to prevent and reduce obesity through three measures: Communication, Action, Research and Evaluation (CDC 2004). In 2000, CDC's Division of Nutrition and Physical Activity funded several state health departments to help them develop and carry out targeted nutrition and physical activity interventions in an effort to prevent chronic diseases, especially obesity (CDC 2004). The CDC recommends reducing calories and increasing physical activity as the safest and most effective way to lose weight (CDC 2004). Unfortunately, this strategy is ineffective for the majority of the obese and overweight population, hence the rising obesity epidemic. Therefore, a better understanding of the physiology behind the cause of obesity is greatly needed. Researchers have made major advances in the past decade in identifying the components of the homeostatic system that regulates body weight, including several genes responsible for animal and human obesity (Friedman 2000).

Physiology and Associated Factors

There are two main views on the causes of obesity. One view suggests that obesity is the result of a fundamental lack of discipline on the part of affected individuals. This view is obviously the target of the diet industry which nets approximately $50 billion a year. An alternative view suggests that body weight (or body fat) is physiologically controlled and that deviations in weight in either direction elicit a potent counter-response that resists change (Kennedy 1953; Friedman 2000). In support of the latter theory, although most people have short-term mismatches in energy balance, most individuals match cumulative energy intake to energy expenditure with great precision when measured over a period that spans many meals (Edholm 1977). This physiological occurrence is an active regulatory process, known as energy homeostasis, that promotes stability in the amount of body energy stored as fat (Schwartz et al. 2000). This accurate regulation of body weight and fat mass emphasizes the precision with which the brain responds to nutritional signals and its capacity to compensate for the disturbances that follow events such as feeding, exercise or alterations in ambient temperature (Williams et al. 2000). Recent advances in the knowledge of molecular mechanisms regulating body fat has resulted in the identification of signaling molecules that affect food intake and that are critical for normal energy homeostasis. A key element of this system is the hormone leptin which is produced by fat tissue and signals to regulatory centers in the brain, specifically the hypothalamus (Friedman et al. 1998). It was the cloning of the *ob gene* and characterization of leptin that supported the idea that body fat content is under homeostatic control (Zhang et al. 1994). During the past few decades, knowledge of the hypothalamus and its role in satiety and hunger has expanded dramatically. The hypothalamus harbors various neurons that have been suggested to be involved in the regulation of feeding and energy balance. It is the specific

neurotransmitters with receptors located on these neurons that have been of particular interest in the area of feeding behavior and energy expenditure (Figure 2.2).

Stimulate feeding	Inhibit feeding
Neuropeptide Y, via Y5 receptor	Glucagon-like peptide-1 (GLP-1), via GLP-1 receptor
Melanin-concentrating hormone, via SLC-1	α-Melanocyte-stimulating hormone, via MC4-R
Orexin-A, via orexin-1 or -2 receptors	Cholecystokinin (CCK) via CCK_A and CCK_B receptors
Galanin, via galanin-1 receptor	Cocaine- and amphetamine-regulated transcript, via an unknown receptor
Agouti gene-related protein, via melanocortin-4 receptor (MC4-R)	Corticotrophin-releasing factor (CRF), via CRF receptors 1 and 2
Glutamate, via N-methyl-D-aspartate receptor	
γ-Aminobutyric acid (GABA), via $GABA_A$ receptor	
Dynorphin, via κ opioid receptor	

SLC-1, somatostatin-like cloned receptor-1.

Figure 2.2: An incomplete list of some hypothalamic neurotransmitters and their receptors implicated in the control of feeding (Williams et al. 2000).

Several key molecules in this hypothalamic network are neuropeptide Y (NPY) and agouti-related protein (AGRP), which stimulate food intake, and α-melanocyte-stimulating hormone (α-MSH) and cocaine-and amphetamine-regulated transcript (CART), which decrease food intake (Stunkard et al. 1993; Erickson et al. 1996; Fan et al. 1997; Friedman 2000). Another set of molecules that are affected by these neuropeptides result in the regulation of energy expenditure. These effectors include uncoupling proteins and peroxisome proliferators-activated receptor-γ (PPAR-γ) co-activator-1 (PGC-1), a key regulator of the genes controlling thermogenesis (Puigserver et al. 1998; Friedman 2000). All of these previously mentioned signaling molecules have been suggested to be involved in the pathway in which leptin modulates food intake and energy expenditure. The regulation of neurons in the arcuate nucleus (ARC) of the

hypothalamus by leptin results in reduced secretion of NPY, reduced expression of AGRP, and increased expression of POMC and α-MSH, and the peptide product of CART (Kristensen et al. 1998; Cone 1999; Wilson et al. 1999; Friedman 2000). Because of its coordinated effects, leptin has promised great potential for a leptin-like product or drug that can activate this extensive signaling pathway involved in energy homeostasis. Leptin has been shown to be clinically effective in individuals who lack leptin (Farooqi et al. 1999) and to eliminate almost completely body fat in transgenic rodent models that overexpress leptin (Ogawa et al. 1999). The finding that many cases of human obesity, similar to diet induced obesity (DIO), are characterized by leptin resistance has hindered progress in the development of leptin as an anti-obesity therapeutic (Kalra et al. 1999). It has been suggested that leptin sensitivity is due to structural aberrations in its receptor, Ob-Rb, or defective transport of leptin across the blood-brain barrier. Downstream signal defects are also a possible cause of leptin resistance (Kalra et al. 1999). This hurdle in leptin research resulted in greater focus on other molecules involved in leptin's signaling pathway. One such molecule is Ciliary Neurotrophic Factor (CNTF) which was found to have a receptor, CNTFRα, with homology to leptin's receptor, Ob-Rb. CNTF is a neurocytokine expressed in glial cells and reduces food intake and body weight in rodents. It became apparent that CNTF and leptin share similarities in molecular events in intracellular signal transduction (Tartaglia 1997), specifically via activation of the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Bjorbaek et al. 1999). CNTF also proved to normalize obesity characteristics in rodent models that are leptin resistant (Gloaguen et al. 1997; Lambert et al. 2001). This finding makes CNTF an attractive candidate for a potential anti-obesity drug. Although it is not listed in the figure below (Figure 2.3), CNTF would be listed with the peptides including POMC and CART as an anorexigenic, or food intake suppressing, molecule.

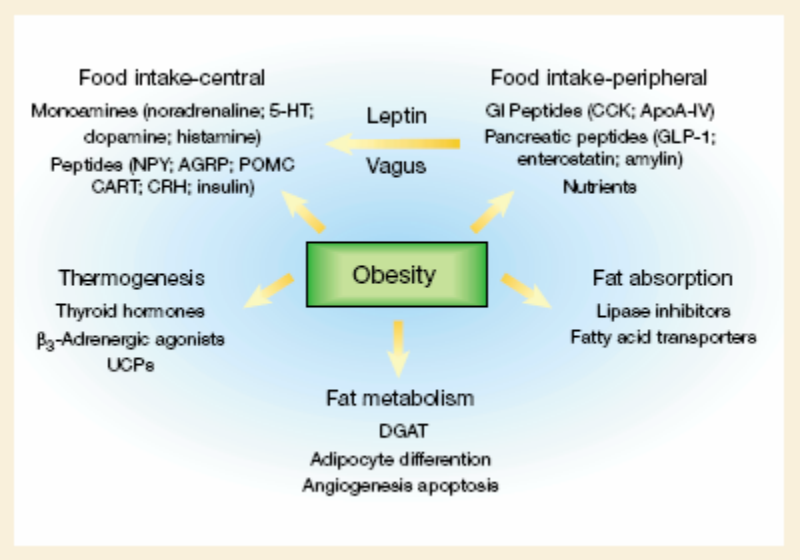


Figure 2.3: Diagram of strategies for molecules targeted against obesity (Bray et al. 2000).

CHAPTER 3

CILIARY NEUROTROPHIC FACTOR (CNTF)

Neurotrophic factors are defined as target-derived, anti-apoptotic molecules that maintain embryonic or adult neuronal cells (Snider et al. 1989). Traditionally, neurotrophic factors have been described as molecules that act exclusively on neuronal cells or those that were discovered as anti-apoptotic molecules for neuronal cells (Sariola 2001). However, research has proven that this definition is arbitrary, because several 'typical' neurotrophic factors affect proliferative and differentiative processes and mechanisms within the nervous system as well as those independent of the nervous system (Lindsay et al. 1989; Cattaneo et al. 1990). Emerging evidence continues to show that loss of appetite and decreased body weight characteristic of infection, injury, and inflammation may be attributed to the central action of several cytokines, including Ciliary Neurtotrophic Factor (CNTF) (Ip et al. 1992; Richardson 1994; Plata-Salaman 1998). CNTF is a neurotrophic factor originally researched for the role it plays in the survival and protection of chick ciliary ganglion (Adler et al. 1979; Lin et al. 1989) *in vitro* as well as other neuroprotective functions. CNTF is a 22-kDa protein that promotes differentiation and survival of a range of cell types in the mammalian nervous system (Richardson 1994). CNTF supports a wide variety of central and peripheral neurons, including sympathetic, embryonic motor, hippocampal, ciliary ganglionic neurons and glial and skeletal muscle cells (Neet et al. 2001). CNTF also contributes several non-neuronal effects such as maintenance of undifferentiated embryonic stem cells (Koshimizu et al. 1996), initiation of an acute-phase response in liver cells (Baumann et al. 1993), and a myotrophic effect on denervated skeletal muscles of mice (Helgren et al. 1994).

Subsequent studies showed that while CNTF possesses the ability to protect and support the nervous system and various non-neuronal cell types, it also plays a role in the physiological pathway involved in energy metabolism (1996; Gloaguen et al. 1997; Lambert et al. 2001).

CNTF Structure, Receptor, and Signaling

There are three major families of neurotrophic growth factors: the neurotrophins of the nerve growth factor (NGF) family, the glial cell-derived neurotrophic factor (GDNF) family, and ciliary neurotrophic factor (CNTF). While the neurotrophins and GDNF families are characterized by their cysteine knot structure, CNTF differs structurally from the other two families and is characterized by its four-helix bundle (Butte 2001). MAD phasing was used to determine that the crystal structure of CNTF was dimeric (McDonald et al. 1995). The basic structure of CNTF is described as four helices, named A-D, with two long cross-over loops (AB and CD) and one short loop (BC) (Figure 3.1) (Kallen et al. 1999; Butte 2001).

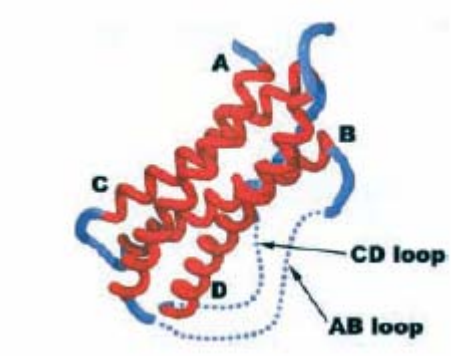


Figure 3.1: Structure of CNTF (Butte 2001).

Although CNTF acts on many of the same neuron types as NGF and GDNF, the cloning, purification, and sequencing of CNTF (Lin et al. 1989; Stockli et al. 1989) led to the discovery that CNTF is unrelated to these neurotrophins and is a member of the gp130 neuropoietic

cytokine family including interleukin-6, interleukin-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), leptin, and cardiotrophin-1 (CT-1) (Boulton et al. 1994; Kishimoto et al. 1994; Pennica et al. 1995).

CNTF's actions are regulated, in part, by a CNTF-specific receptor (CNTFRα) that is most closely related to, and shares subunits with the receptor complexes for IL-6 and leukemia inhibitory factor (LIF) (Sleeman et al. 2000). This structural similarity to IL-6 suggests that the CNTF receptor, like the IL-6 receptor, requires a second, signal-transducing component (Davis et al. 1991). The structure of CNTF's receptor contrasts all known receptors in that it is anchored to cell membranes by a glycosyl-phosphatidylinositol linkage. The isolation of CNTFRα provides a solid means by which to identify cells that are potentially responsive to CNTF. CNTFRα was originally described as being distributed primarily within neural tissues (Davis et al. 1991) but has since been described to be found in skeletal muscle, adrenal gland, sciatic nerve, skin, kidney, liver, and testes (Davis et al. 1991; Ip et al. 1992; Sleeman et al. 2000).

Table 3.1: List of reported distribution of CNTFRα and method of assessment (Sleeman et al. 2000)

Distribution of CNTFRα in Postnatal Rodent

STRUCTURE	TECHNIQUE	Reference
Hypothalamus Nuclei		
•Arcuate	ISH	Gloaguen et al 1997
•Paraventricular	ISH	Gloaguen et al 1997; Lee et al 1997
	IHC	Kordower et al 1997; MacLennan et al 1996
•Supraoptic	ISH	Lee et al 1997
•Lat. Hypothal. Area	IHC	Kordower et al 1997
•Tuberomammilary	ISH	Lee et al 1997
Autonomic Nuclei/Ganglia		
•Intermediolateral N. (Pregang. Symp.)	IHC	Kordower et al 1997; MacLennan et al 1996
•SCG	ISH	Ip et al 1993
	NRT	Kotzbauer et al 1994
	1°	Kotzbauer et al 1994; Wong et al 1995
•Adrenal	NRT	Davis et al 1991
•Ciliary G.	ISH	Ip et al 1993
•DRG	ISH	Ip et al 1993
	IHC	MacLennan et al 1996
•Nodose G.	NRT	Thaler et al 1994
Peripheral Organs		
•Skeletal Mus.	NRT	Davis et al 1991; Ip et al 1993
•Skin	NRT	Ip et al 1993; MacLennan et al 1994
•Liver	NRT	Ip et al 1993
	WB	Kirsh & Hofmann 1994
	1°	Nesbit et al 1996; Schooltink et al 1992
•Kidney	NRT	Ip et al 1993
•Testes	ISH	Ip et al 1993
•Lung	NRT	MacLennan et al 1994
•Bone Marrow	1°	Bellido et al 1996

Peripheral targets including ciliary, dorsal root, and sympathetic ganglia also express the CNTFα receptor. In the CNS, lower motor neurons, whose survival is supported by CNTF (Arakawa et al. 1990; Sendtner et al. 1990), showed strong expression of CNTFRα. Expression has also been found in other regions of the CNS such as the neocortex, the hippocampus, the thalamus, the subependymal zone of the lateral ventricle, and several brainstem nuclei (Lo 1993). The most recent finding reported that CNTFRα is expressed in adipose tissue (Zvonic et al. 2003). The CNTFRα receptor has the ability to be released which would provide a mechanism for regulating the cellular response to CNTF (Davis et al. 1991). Once cleaved from the cell surface, the

soluble CNTFRα has been detected in the serum and the cerebrospinal fluid and has been shown to initiate signaling in cells not responsive to CNTF alone (Davis et al. 1991; Helgren et al. 1994). Other soluble receptor forms that have been found in body fluids include IL-6Rα, gp130 and the LIFRα (Zhang et al. 1998).

CNTFRα is only one component of CNTF's complicated receptor complex. It has been suggested that the CNTF molecule contains three binding sites for each of its three receptors and it is thought to bind sequentially to these receptors resulting in activation of specific signals involved in neuronal survival (Butte 2001). CNTF signaling is initiated when CNTF binds CNTFRα, either in its soluble or membrane bound form (Stahl et al. 1994) in a 1:1 stoichiometry. This initial binding to CNTFRα is not involved in signal transduction (Davis et al. 1993; Davis et al. 1993). After binding, a CNTF-CNTFRα complex forms and two of these heterodimers join together and recruit a 130 kDa transmembrane glycoprotein, the β1 signal transducing receptor, gp130. This step is followed by the recruitment of the β2 signal transducing receptor component, the LIFR protein. A resultant receptor complex forms as a hexamer of CNTF, CNTFRα, gp130, and LIFR in a 2:2:1:1 ratio, respectively (Ward et al. 1994; De Serio et al. 1995; Butte 2001). Another possible binding model that has been suggested is tetrameric and has a 1:1:1:1 CNTF:CNTFRα:gp130:LIFR stoichiometry (Figure 3.2) (Kallen et al. 1999).

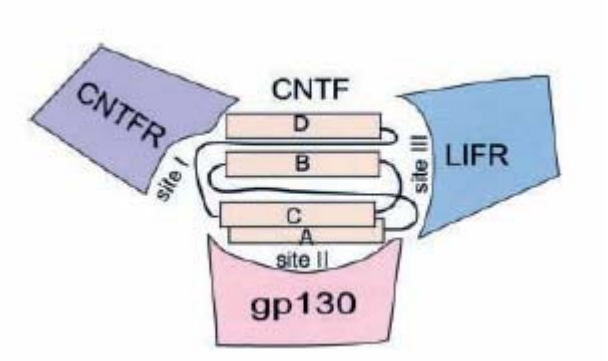


Figure 3.2: CNTF receptor complex. Scheme showing one possible binding model of CNTF with its receptors CNTFα, gp 130, and LIFR in a 1:1:1:1 stoichiometry (Butte 2001).

The formation of the CNTF heterodimer complex results in tyrosine phosphorylation of STAT3 at residue Y705 which triggers intracellular signaling cascades involving the induction of the JAK/STAT and the ras-mitogen activated protein kinase (MAPK) pathways (Taga et al. 1997). The activated form of STAT-3 then dimerizes with STAT-3 or other STAT proteins and translocates to the nucleus where it binds specific DNA sequences present in promotors of cytokine-responsive genes. The events following the phosphorylation of STATs has been associated with enhanced transcriptional activity of responsive genes such as acute phase plasma proteins (Stahl et al. 1994). The crucial step in deciphering cytokine mediated signaling emerged with the discovery of the JAK/STAT pathway in the early 1990s (Touw et al. 2000). Another major difference that the CNTF receptor family has from the NGF and GDNF families is that it is coupled to and signals through the JAK/STAT pathway, rather than via the direct tyrosine kinase activity of NGF and GDNF receptors (Neet et al. 2001).

The commonality that CNTF's cytokine family shares is the usage of the high-affinity signal-transducing subunit, gp130. The similar utilization of this subunit has been suggested to be associated with the redundant functioning and biological activities of this cytokine family (Nakashima et al. 1998). This family has been associated with its pleiotropic effects in immune,

hematopoietic, and neural systems (Sariola 2001). IL-6 requires gp130 and LIFRβ in a heterodimer, and CNTF requires CNTFRα, gp130, and LIFRβ (Gearing et al. 1991; Ip et al. 1992; Davis et al. 1993; Stahl et al. 1993; Simpson et al. 1997).

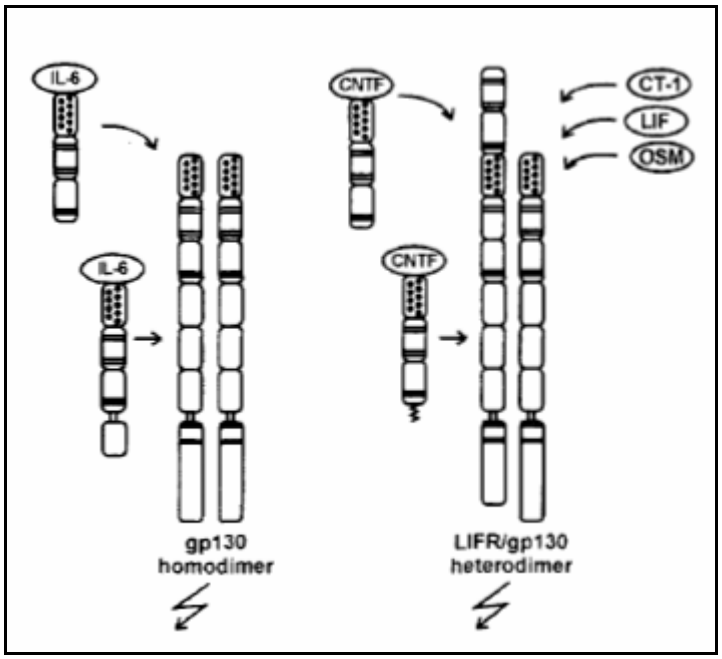


Figure 3.3: CNTF signaling. Dimerization of gp130 induces intracellular signaling. Schematic representation of gp130, the LIFR and the membrane bound receptors for IL-6 and CNTF. The glycosyl-phosphatidylinositol cell membrane anchor of the CNTFR is indicated by a zigzag. Homodimerization of gp130 leading to intracellular signaling is induced by the IL-6/IL-6R complex. Heterodimerization of gp130 with the LIFR leading to intracellular signaling is induced by LIF and OSM, CT-1 and the CNTF/CNTFR complex (Simpson et al. 1997).

It is interesting to note that the *soluble* extracellular domain of CNTFRα, cleaved from its GPI anchor by phospholipase, can itself act with the gp130/LIFRβ complex to initiate signaling upon CNTF binding (Davis et al. 1993; Marz et al. 1999; Neet et al. 2001). Each of these neurotrophic receptors share the common theme that the highest affinity and specificity are formed by the complete complex of two or more membrane-bound polypeptide chains. The protein-protein interactions that generate the structured receptor complex and resultant formation of the binding

site(s) for the neurotrophic ligands are very important when considering the signaling and biological actions of these receptor complexes (Neet et al. 2001).

The structural difference between human and rat CNTF should be observed when considering potential human applications involving CNTF. Human and rat CNTF share 85% sequence identity, support the survival of chicken embryo ciliary ganglia neurons *in vitro*, but display a 4-5 fold difference in specific activity (Panayotatos et al. 1993). This great difference between human and rat CNTF was found to be attributed to a single amino acid. Using arginine residue 63 of rat CNTF as a substitute into the human sequence produced a protein with the properties of rat CNTF. Conversely, substituting the human CNTF glutamine residue 63 into rat CNTF generated a protein possessing properties of human CNTF (Panayotatos et al. 1993). These findings are important in determining the exact structure of CNTF as well as the practical applications in developing potential therapeutic proteins.

Nervous System Protection/Regeneration

Ciliary Neurotrophic Factor (CNTF) is expressed in glial cells within the central and peripheral nervous systems. Specifically, Schwann cells in the peripheral and oligodendrocytes in the central nervous systems express CNTF (Oppenheim et al. 1991; Stockli et al. 1991). Original interest in CNTF stemmed from the finding that it had the ability to support the survival of parasympathetic neurons of the chick ciliary ganglion in vitro (Adler et al. 1979). Over the next two decades, many studies were conducted that identified various roles that CNTF plays in neuronal as well as non-neuronal cell types, proving its versatility. In culture, CNTF promotes the survival of various neuronal populations and acts as a differentiation and trophic factor on glial cells (Barbin et al. 1984; Lillien et al. 1988; Arakawa et al. 1990; Ip et al. 1991; Magal et al. 1991; Louis et al. 1993). Exogenously administered CNTF prevents the death of damaged

neurons in several injury models in vivo (Sendtner et al. 1990; Hagg et al. 1993) and prevents the typical symptoms associated with progressive motor neuron degeneration in mouse models (Sendtner et al. 1992). Interestingly, unlike mice lacking CNTF, mice lacking CNTFRα die perinatally and display severe motor neurons deficits. This finding suggests that CNTFRα may be a receptor for a secondary ligand that plays a critical role in the developing nervous system (DeChiara et al. 1995).

CNTF lacks a classical signal peptide sequence of a secreted protein, suggesting that it cannot be secreted through conventional pathways. Instead, it has been suggested that CNTF conveys its cytoprotective effects only after its release from glial cells via a mechanism induced by injury. Release of CNTF has been detected after nerve and central nervous system injury (Ip et al. 1993) and administering CNTF to nerve stumps supports the survival of denervated motor neurons (Sendtner et al. 1990). It has also been established that stress to the retina upregulates the expression of CNTFRα messenger RNA (mRNA) (Wen et al. 1995). In the CNS, the increased expression of CNTF is limited to astrocytes contiguous to the damage site, supporting the notion that astrocytes are crucial in maintaining or restoring neuronal function associated with brain injury (Rudge et al. 1995). In culture, CNTF also causes the differentiation of the O-2A glial progenitor into a type 2 astrocyte in the developing optic nerve (Hughes et al. 1988; Lillien et al. 1988). CNTF mRNA is typically found at very low levels in the normal rat central nervous system, but after being subjected to intentional lesioning of specific CNS areas, CNTF mRNA is significantly upregulated (Ip et al. 1993). A similar effect, showing an increase in CNTF mRNA, was found with induction of injury in the Schwann cells of the peripheral nervous system (Friedman et al. 1992). Administration of CNTF has been shown to rescue motor, sensory, thalamic, septal, and striatal neurons as well as photoreceptors and oligodendrocytes

(Sleeman et al. 2000). Thus, application of exogenous CNTF has been shown to have profound effects as a neuroprotective factor in response to nervous system damage.

Several studies using animal models have shown that administration of CNTF provides protection following CNS injury, including the ability to support striatal output neurons in a pharmacological model of Huntington's disease (Anderson et al. 1996; Emerich et al. 1996; Emerich et al. 1997; Emerich et al. 1997). CNTF's neuroprotective effect in striatal output neurons has been suggested to occur by direct actions as there is definite expression of the entire CNTFR complex in the striatum (Sleeman et al. 2000). In vivo applications of CNTF has been shown to prevent degeneration of chick spinal motoneurons during development (Oppenheim et al. 1991) and correlatively, motor neurons have been shown to possess the CNTF receptor (Ip et al. 1993). Helgren et al. has shown that CNTF reduces the denervation-induced atrophy of muscle and attenuates the reduced twitch and titanic tensions that result from muscle denervation (Helgren et al. 1994). This finding confirms that in addition to CNTF's known neurotrophic actions, CNTF also exerts myotrophic effects.

In the CNS, centrally administered CNTF prevents axotomy-induced cell death of neurons in the anteroventral and anterodorsal thalamic nuclei of the adult rat (Clatterbuck et al. 1993). Exogenous administration of CNTF has also been shown to prevent axotomy-induced degeneration of substantia nigra dipaminergic neurons in the adult rat (Hagg et al. 1993). This is a significant finding because the degeneration of these specific neurons is a major component in the manifestation of Parkinson disease (Hagg et al. 1993). In addition to Parkinson's, Huntington's, and Alzheimer's diseases, CNTF has also been a major focus in the research associated with amyotrophic lateral sclerosis (ALS), one of the most experimentally tractable of the neurodegenerative diseases. Studies have shown that in the spinal cord of ALS patients there is a marked increase in the hybridization

signal for CNTFRα subunit mRNA overlying motor neurons (Duberley et al. 1995). In one study included a double-blind placebo-controlled clinical trial conducted to evaluate the safety, tolerability, and efficacy of subcutaneous administration of recombinantly produced human CNTF (rHCNTF) in slowing disease progression in patients with ALS. The results showed that there were no statistically significant treatment effects between the rHCNTF and placebo treated patients. There were, however, side effects of rHCNTF treated patients which included anorexia, weight loss, and cough (1996). Similar effects were seen in another placebo-controlled trial treating 570 ALS patients with rhCNTF at 3 doses for 6 months. At all of the tested doses, rhCNTF had no beneficial effect on limb strength, pulmonary function, arm and leg megascores, activities-of-daily-living outcome measure, and survival. The rhCNTF treated patients exhibited dose-dependent adverse effects including cough, asthenia, nausea, anorexia, weight loss, and increased salivation (Miller et al. 1996). After a similar outcome was later reproduced in experiments using rodents (Martin et al. 1996), CNTF quickly became associated with its potential effects on metabolic pathways and recognized as a new candidate for therapeutic applications for obesity. Peripheral administration of CNTF has been shown to produce cachexia, atrophy of skeletal muscles, and anorexia accompanied by weight loss in rats (Shapiro et al. 1993; Henderson et al. 1994; Espat et al. 1996; Martin et al. 1996). Central administration of CNTF has also produced anorexia and body weight loss, and neuropeptide Y (NPY) gene expression was suppressed in the arcuate nucleus (ARC) of the hypothalamus in rats (Kalra et al. 1998; Xu et al. 1998; Lambert et al. 2001). Comparatively, peripheral and central administration of leptin, a secreted protein from adipocytes, causes a reduction in food intake and body weight loss in genetically obese and normal rodents and decreased hypothalamic NPY gene expression (Campfield et al. 1995; Sahu 1998). It has been suggested that CNTF acts via a leptin-like mechanism (Gloaguen et al. 1997).

Leptin-like Characteristics

Leptin is a 146-amino acid cytokine-like peptide released into the blood from fat cells in response to feeding and has been shown to cause a pronounced effect on the suppression of food intake and body weight reduction (Zhang et al. 1994). Its 1994 discovery at Rockefeller University (Zhang et al. 1994) had researchers hopeful that a new anti-obesity agent had been found. Soon after leptin's discovery, a hurdle was encountered with the finding that many cases of obesity are characterized by leptin resistance (El-Haschimi et al. 2000; Lin et al. 2000; Lin et al. 2001; Jacobson 2002; Scarpace et al. 2002). Along with the ongoing investigation to determine leptin's exact mechanism, researchers began looking for other signaling molecules involved with this regulation of energy homeostasis. One such molecule, CNTF, was originally implicated for its role in the nervous system but has recently been shown to have profound effects in modulating body weight and food intake. CNTF has been compared to leptin for its similar effects on food intake, weight loss and energy expenditure. Several recent studies report that CNTF and leptin negatively regulate the food intake-stimulating signals of neuropeptide Y (NPY), agouti-related protein (AgRP) and gamma-aminobutyric acid (GABA) in the arcuate nucleus of the hypothalamus. In addition, they positively regulate cocaine-amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC), which is the source of the food intake-inhibiting peptide, melanocyte-stimulating hormone (α-MSH) (Kalra et al. 1998). CNTF and leptin have been shown to activate a similar pattern of STAT (Signal Transducer and Activator of Transcription) factors in neuronal cells. In addition to the similar effects on food intake and weight loss in rodent models, CNTF has been compared to leptin because of the findings that CNTF's receptors have characteristics similar to those of the leptin receptors, including their distribution within hypothalamic nuclei involved in feeding (Kalra 2001; Lambert et al. 2001). Several studies have shown that the leptin receptor (OB-Rb) is predominantly expressed in brain regions associated with the control of food intake and energy

expenditure, including the arcuate, ventromedial, and paraventricular hypothalamic nuclei. To support that CNTF similarly targets hypothalamic satiety centers, *in situ* hybridization was performed, and the results showed that the arcuate and paraventricular nuclei of the mouse hypothalamus express mRNAs for CNTF receptor subunits (Ip et al. 1996). In addition, both leptin and CNTF induce transcription of several cytokine-inducible inhibitors of signaling, such as suppressor of cytokine signaling 3 (SOCS-3) in the arcuate nucleus of the hypothalamus (Ziotopoulou et al. 2000).

In a 1997 study, Gloaguen *et al.* made a significant finding that CNTF and leptin activate a similar pattern of STAT factors in neuronal cells and that the mRNAs for CNTF and leptin receptors are both located in mouse hypothalamic nuclei associated with energy balance regulation (Gloaguen et al. 1997). CNTF's ability to trigger the activation of receptor-associated kinases of the Jak family, receptor phosphorylation, and recruitment and activation of STAT transcription factors is a result of the formation of its heterodimer receptor complex (Kishimoto et al. 1994; Ip et al. 1996; Murphy et al. 1997; Heinrich et al. 1998; Gadient et al. 1999). Leptin has an analogous affect on the Jak-STAT pathway via the induction of its receptor's, OB-R, homodimerization (Tartaglia 1997). Activation of STAT3 appears to be required the physiological actions of leptin and CNTF (Duan et al. 2001). The Gloaguen et al. study showed that CNTF centrally administered to *ob/ob* mice (lack functional leptin) reduced adiposity, hyperphagia, and hyperinsulinemia which are associated with leptin deficiency. Interestingly, the research also supported CNTF's effectiveness in reducing similar obesity-related phenotypes in *db/db* (lacking leptin receptor) and diet-induced obesity (DIO) mouse models which can both be described as displaying leptin resistance (Gloaguen et al. 1997).

In 2001, a paper by Lambert *et al.* (Lambert et al. 2001) extended these findings that CNTF reduces the obesity phenotype associated with leptin resistance by showing that a derivative of CNTF, $CNTF_{Ax15}$, normalized the obesity related characteristics of *ob/ob, db/db* and DIO mouse models. CNTF administration resulted in decreased food intake, body weight reduction, and the

normalization of plasma insulin and lipid levels (Lambert et al. 2001). This study also addresses a suggestion that CNTF is acting via a non-leptin like pathway more similar to those activated by cachectic cytokines, believed to be structurally related to CNTF. The research disputes this possibility by showing that CNTF activates hypothalamic signaling similar to those activated by leptin and acts very differently than interleukin-1, a prototype cachectic cytokine. This explains why CNTF's weight-reducing effects are free from side-effects typically associated with related cytokines (Lambert et al. 2001). A recent study suggests, however, that chronic CNTF administration in the brain causes a potentially harmful inflammatory action, unlike leptin, and more similar to the effects of Leukemia Inhibitory Factor (LIF), a related pro-inflammatory cytokine (Prima et al. 2004).

Another important characteristic that CNTF and leptin share is their similar interaction with NPYergic signaling in the hypothalamus (Xu et al. 1998). Neuropeptide Y (NPY) was identified in the hypothalamus as a cytokine that transmits stimuli for stimulation of food intake (Kalra et al. 1996; Kalra et al. 1996). NPY-producing neurons are located in the brain stem and in the arcuate nucleus (ARC) of the basal hypothalamus and innervate specific hypothalamic sites including the paraventricular nucleus (PVN) (Chronwall 1989; Everitt et al. 1989). Studies using rodent models have shown that NPY synthesis in the ARC and release in the PVN are augmented during normal feeding at night or after fasting conditions and dietary restrictions (Sahu et al. 1988; Brady et al. 1990; White et al. 1990; Kalra et al. 1991; Dube et al. 1994). Studies in which NPY action was blocked by immunoneutralizion or by administering an NPY Y1 receptor antagonist resulted in the inhibition of feeding (Dube et al. 1994; Kanatani et al. 1996). Based on these findings, researchers began to investigate the actions of CNTF and leptin, two seemingly anorectic cytokines, on their potential modulation of the NPY signaling pathway.

There is evidence that leptin down-regulates the hypothalamic NPY system associated with physiology of the control of food intake (Stephens et al. 1995). Peripheral and central administration of CNTF cause reduction of food intake and body weight (Moldawer et al. 1988; Henderson et al.

1994; Martin et al. 1996; Xu et al. 1998). CNTF peripherally and centrally administered to rats resulted in a decrease in prepro-NPY mRNA levels in the hypothalamus compared to pair-fed control rats that displayed augmented hypothalamic gene expression (Xu et al. 1998). In this same study, pre-treatment of rats with CNTF resulted in a significant decrease in NPY-induced food intake. This study also reported that icv leptin injections decreased body weight, reduced food intake, and suppressed NPY gene expression compared with the control PBS-treated rats (Xu et al. 1998). Leptin also reduced NPY-induced feeding in a time dependent manner (Xu et al. 1998). A related study showed that CNTF and leptin suppressed hypthalamic NPY gene expression and significantly attenuated luteinizing hormone (LH) suppression in response to food deprivation (Kalra et al. 1998). The importance of this finding is that the upregulation of the NPY system may be correlated with the reduction of pituitary gonadotropin secretion and that the NPYergic signaling may be the communication link between the neural mechanism regulating reproduction and energy balance (Kalra et al. 1998).

There are several suggested ways that CNTF may act on NPY signaling of the hypothalamus. One is that CNTF restricts the release of NPY at the appetite stimulating targets in the PVN (Kalra et al. 1991; MacLennan et al. 1996). Another likely site of anorectic action of CNTF may be the ARC where NPY-producing neurons are located (Kalra et al. 1999; Pu et al. 2000). CNTF has also been shown to suppress the feeding induced by NPY (Xu et al. 1998). The strong evidence that CNTF reduces body weight and food intake is likely associated with anorexia caused by the attenuation of NPY signaling. A study in which continuous intraventricular infusion of CNTF induced anorexia and body weight loss also showed that concomitant infusion of NPY fully counteracted the CNTF-induced responses (Pu et al. 2000). Again, this supports the idea that the potent anorectic effects of CNTF is likely a result of the attenuation of the food intake stimulating effects of NPY in the PVN and surrounding neural hypothalamic sites (Kalra et al. 1999). In the same study, central CNTF administration resulted in a marked suppression of leptin concentrations suggesting that the anorectic

effects of CNTF are likely exerted independently of leptin through CNTF receptors located in the ARC-PVN, in close proximity to where NPY exerts its orexigenic effects (Pu et al. 2000). In a study comparing the effects of leptin and CNTF on the NPYergic signaling system, CNTF appeared to be slightly more effective than leptin on a molar basis (Xu et al. 1998). The confirmation that both CNTFRα immunoreactivity and CNTFRα mRNA are expressed in various hypothalamic sites, including the PVN, supports the suggestion that CNTF influences the NPY signaling of the hypothalamus (MacLennan et al. 1996; Lee et al. 1997).

Although CNTF shares many similarities with leptin, there is evidence for the differences between the two cytokines. For instance, leptin administered to food deprived rodents reversed the starvation-induced increase in agouti-related protein (AgRP) whereas CNTF administration had no effect on AgRP (Ziotopoulou et al. 2000). Leptin's effect on AgRP might be explained by the fact that its receptor, Ob-Rb, is expressed in corticotrophin-releasing hormone (CRH)-containing neurons. Another disparity is that continuous leptin administration prevents a decrease in POMC mRNA expression in fasted rats but CNTF administration to fasted animals has no effect on POMC expression (Ziotopoulou et al. 2000). One of the most significant findings was CNTF's success in normalizing the obese phenotype in rodent models resistant to leptin. CNTF reduced body weight and food intake under conditions in which exogenous leptin was ineffective, such as in db/db and diet-induced obese mouse models (Gloaguen et al. 1997; Marsh et al. 1999). This finding is significant for the future of obesity therapeutics because many cases of human obesity involve leptin resistance (Sahu 1998). In addition, the anorectic effects of centrally administered CNTF are more profound and longer lasting then other related cytokines (Xu et al. 1998). Thus, CNTF's ability to sustain anorexia and reduce body weight for a considerable period of time makes it an attractive candidate for a potential obesity therapeutic as rebound weight gain is typically a problem associated with weight loss. It should be noted again that recent research on the safety of CNTF administration should be considered with the application of CNTF on obesity therapeutics due to alteration of genes

leading to an inflammatory response in the brain (Prima et al. 2004). Another recent study reported that CNTF and leptin induce distinct patterns of immediate early gene expression in the brain, supporting the hypothesis that CNTF and leptin act via both similar and distinct CNS sites. In addition, the results showed that CNTF administration, but not leptin, induced fever and cyclooxygenase-2 mRNA expression (Kelly et al. 2004).

CNTF and AXOKINE®: Obesity Applications

After the discovery that CNTF induced marked weight loss in patients with amyotrophic lateral sclerosis (ALS), researchers began speculating on its potential as an anti-obesity therapeutic (1996). CNTF has previously been characterized by the biological actions that it shares with other cytokines, which include induction of fever, hepatic acute phase protein responses, anorexia, weight loss, muscle wasting and cachexia (Shapiro et al. 1993; Henderson et al. 1994; Espat et al. 1996; Martin et al. 1996). Unlike the prototypical cachectic cytokines, studies have shown that CNTF can induce weight loss without exhibiting the typical deleterious characteristics of these cytokines (Xu et al. 1998; Lambert et al. 2001). In contrast to a typical cachectic cytokine such as interleukin-1 or forced dieting, CNTF does not activate the pituitary-adrenal stress axis (Lambert et al. 2001). In addition, CNTF-treated animals appear to sustain their weight loss after cessation of treatment longer than animals subjected to forced dieting (Lambert et al. 2001). This lack of rebound weight gain after CNTF treatment suggests its potential success as a weight loss and weight maintenance agent.

In 1993, a genetically engineered variant of CNTF named recombinant human variant (rhv) CNTF, was developed after the finding that human and rat CNTF differ only at one point in their amino acid sequences, specifically, residue 63 (Panayotatos et al. 1993). The advantages of rhv CNTF is its increased potency and improved pharmacological properties (Panayotatos et al. 1993). As previously stated, both leptin and rhvCNTF are capable of normalizing many of the characteristics associated with obesity in ob/ob rodent models (Campfield et al. 1995; Halaas et al.

1995; Pelleymounter et al. 1995; Lambert et al. 2001). The finding that diet induced obesity models (DIO) already have elevated leptin levels and are not responsive to additional exogenous leptin (Lambert et al. 2001) was very disappointing to researchers. A similar effect has been seen in obese humans with elevated serum leptin levels that are unresponsive to high doses of exogenous leptin (Heymsfield et al. 1999). In contrast, rhvCNTF is known to cause weight loss in DIO mice, suggesting that this cytokine may circumvent the leptin resistance associated with the DIO and other leptin resistant mouse models (Kalra 2001; Lambert et al. 2001).

By 1999, Regeneron Pharmaceuticals had developed a re-engineered version of CNTF named AXOKINE® and were conducting the first stages of clinical trials for the drug as a candidate for obesity therapeutics. AXOKINE® is a modified version of human CNTF in which the C terminus has been truncated by 15 residues, the free cysteine has been replaced with an alanine, and the glutamine at position 63 has been switched to an arginine. It is three to five times more potent than the CNTF parent molecule as measured by *in vitro* neuronal survival assays (Peterson et al. 2000).

Systemic administration of AXOKINE® is able to specifically activate STAT-3 and phosphorylated STAT-3 (pSTAT-3) in the median eminence and arcuate nucleus, key hypothalamic areas involved in regulation of feeding (Figure 3.4).

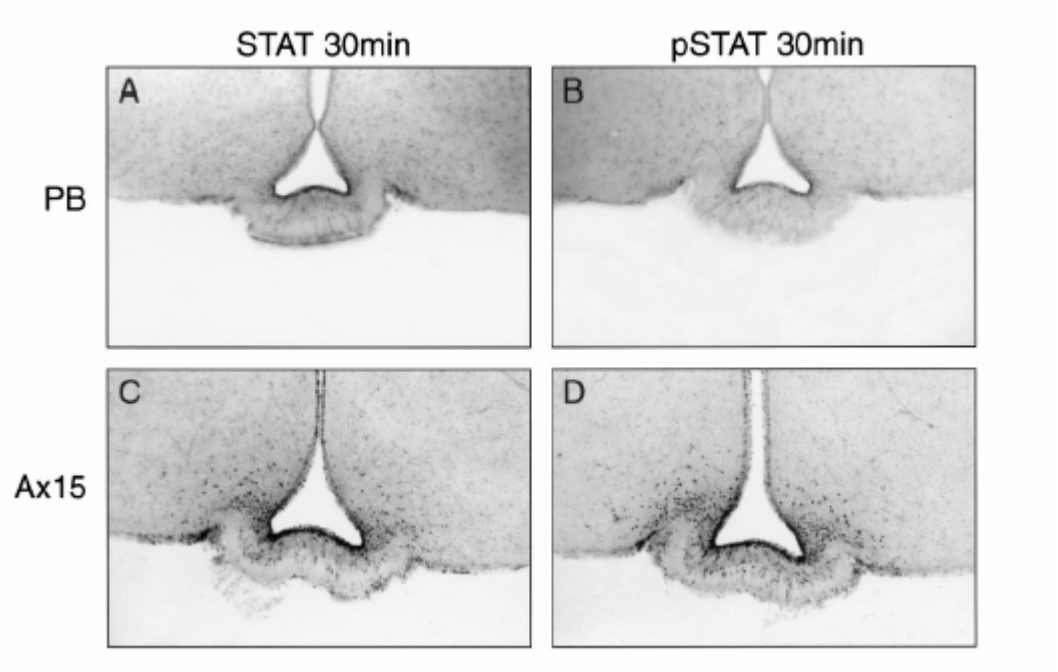


Figure 3.4: **CNTF induction of STAT-3 expression *in vivo.*** Immunohistochemical detection of STAT-3 and pSTAT-3 in the basal hypothalamus of the ob/ob mouse at the level of the arcuate nucleus and median eminence after i.v. injection of phosphate buffer (PB) or AXOKINE® (Sleeman et al. 2000).

AXOKINE® is more effective in reducing body weight when delivered intracerebrovenricularly (icv) than when given systemically (Anderson et al. 1998). CNTF or AXOKINE® is more potent than leptin at reducing weight in diet-induced obesity rodent models and has been shown to reduce body weight and normalize serum insulin levels in leptin receptor-deficient *db/db* mice (Gloaguen et al. 1997; Anderson et al. 1998; Marsh et al. 1999). AXOKINE® also activates signaling in tissues not previously known to express CNTFRα (Peterson et al. 2000)which supports the idea of a soluble form of the CNTFRα receptor (Davis et al. 1993).

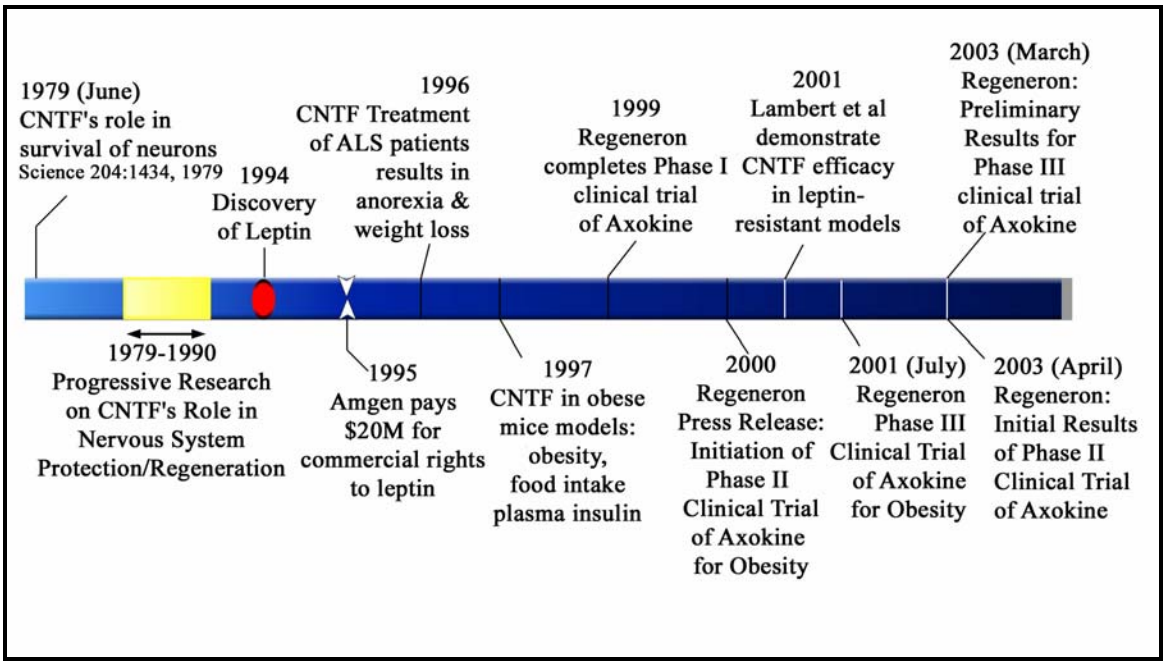


Figure 3.5: **CNTF/AXOKINE Discovery Timeline.** 1979-Present; The progression of obesity research including major landmark discoveries for CNTF and leptin (Duff, 2003, original design).

The progression of CNTF and AXOKINE® is depicted in the above timeline (Figure 3.5). Phase II clinical trials for AXOKINE® were underway by the year 2000, and results were released in an April 2003 press release (unpublished data, 3/31/2003, available at: http://www.regeneron.com/investor/press_detail.asp?v_c_id=169). The trial design was a double-blind, randomized, placebo-controlled study with the objective to assess the short-term safety and efficacy of AXOKINE® treatment compared with placebo with respect to weight loss in overweight and obese individuals diagnosed with Type 2 diabetes. The findings showed that 12 weeks of treatment with AXOKINE® (1.0µg/kg/day) resulted in statistically significant and dose-dependent weight loss. Trends towards normalizing blood glucose and other metabolic parameters characteristic of obesity and Type 2 diabetes were observed during this short-term study (unpublished data, 3/31/2003, available at: http://www.regeneron.com/investor/press_detail.asp?v_c_id=169). On March 31, 2002, U.S. Patent and Trademark Office granted Regeneron U.S. Patent No. 6,472,178 covering an isolated nucleic acid molecule that encodes AXOKINE®, an expression vector containing this molecule, and methods of production of the molecule (unpublished data, 3/31/2003, available at: http://www.regeneron.com/investor/press_detail.asp?v_c_id=153). A Phase III pivotal study for AXOKINE® began in 2001 and the preliminary results were released in March 2003. The Phase III trial was much larger and longer than Phase 2, consisting of approximately 2000 overweight and obese non-diabetic subjects including 1467 AXOKINE®-treated subjects and 501 placebo-treated subjects. During a 12 month time period subjects received either placebo or AXOKINE® (1.0µg/kg/day) as subcutaneous injections. AXOKINE® treatment resulted in a statistically significant reduction in body weight: however, the magnitude of the overall weight loss was small. In addition, AXOKINE® associated weight loss was limited by antibodies that developed

after three months of AXOKINE® treatment. Aside from the antibody development, physicians and researchers agree that AXOKINE® was well tolerated and its efficacy was comparable to currently available drugs. As AXOKINE® Phase III trial has progressed, Regeneron is completing analysis of its ongoing pilot study as well as the short-term studies that have been started throughout the trial (unpublished data, 3/31/2003, available at: http://www.regeneron.com/investor/press_detail.asp?v_c_id=169). In the March 2003 press release, a Regeneron spokesperson stated "Obesity is a complex metabolic disease similar to type 2 diabetes, and like diabetes will probably require combination therapies to achieve optimal efficacy and the dramatic weight losses that people have been hoping for. Its (AXOKINE's®) unique and well-defined mechanism of action makes AXOKINE® a potentially attractive candidate as part of an obesity regimen." (unpublished data, 3/31/2003, available at: http://www.regeneron.com/company/press_detail.asp?v_c_id=169). It is clearly evident that Regeneron is hopeful that AXOKINE®, its most advanced therapeutic candidate, will be successful in Phase III of clinical trials and become marketable as a therapeutic to treat obesity and type II diabetes.

CHAPTER 4

APOPTOSIS: PROGRAMMED CELL DEATH

Apoptosis

The mechanisms that underlie apoptosis or programmed cell death have been intensely studied in recent years for several reasons. Apoptosis is a physiological process in which selected cells are deleted in a rapid, efficient manner through the signal-induced activation of an intrinsic self-destructive cellular process (Qian et al. 2001). A lack of apoptosis may cause cancer or autoimmune diseases, whereas excessive apoptosis may result in neurodegeneration. While there are still many components of this complex process yet to be discovered, great progress has been made in understanding the apoptotic mechanisms (Hengartner 2000). The apoptotic process is characterized by a decrease in mitochondrial membrane potential, activation of caspases, loss of plasma membrane asymmetry, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA (Gullicksen et al. 2004). Distinct DNA fragments of oligonucleosomal size (180-200 bp) is a biochemical hallmark of apoptosis that can be visualized on a gel as a distinct ladder of bands at multiples of approximately 180 base pairs (Bortner et al. 1995; Gullicksen et al. 2004).

Mitochondria are key cellular structures involved in the apoptotic mechanism (Figure 4.1). The apoptotic proteins Bcl-2 and Bax are also crucial components in the regulation of apoptosis. Overexpression of Bax increases the rate of apoptotic death by acting on mitochondria to interrupt the electron transport chain, thus promoting the release of cytochrome *c* into the cytoplasm (Oltvai et al. 1993; Green et al. 1998).

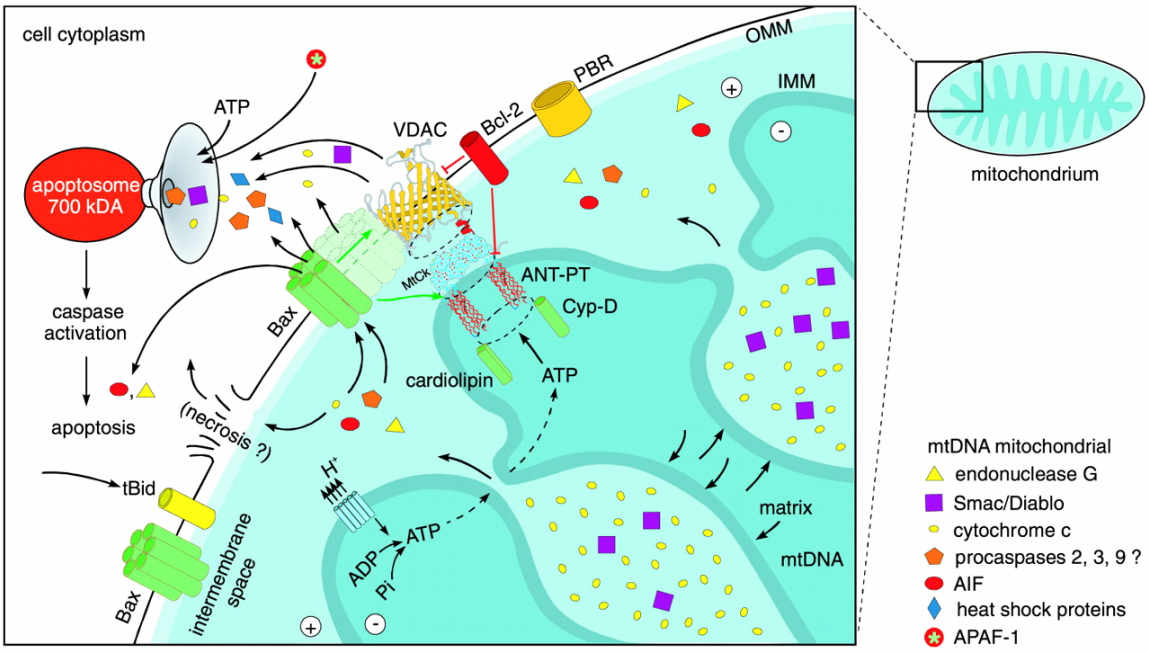


Figure 4.1: Mitochondrial Apoptosis (Mayer et al. 2003)

Proteolytic molecules called caspases are then activated and act to carry out early events of the apoptotic process. The Bcl-2 protein, located in the mitochondria, endoplasmic reticulum, and nuclear membranes, prevents the release of cytochrome *c* and disrupts caspase activity (Korsmeyer et al. 1993). Thus, Bcl-2 antagonizes the action of Bax and inhibits Bax-induced apoptosis (Rosse et al. 1998; Murphy et al. 2000). The ratio of Bcl-2/Bax is the ultimate determinant of apoptotic susceptibility (Korsmeyer et al. 1993).

Adipose Tissue Apoptosis

Adipose Tissue mass is regulated by both the number and average volume of adipocytes (Kim et al. 2000). The Bax and Bcl-2 proteins involved in the apoptotic mechanism have also been implicated in the role they play in energy homeostasis and metabolism. Attenuation of Bcl-2 activity has been correlated with apoptosis of brown adipocytes while augmentation is associated with inhibition of apoptosis (Navarro et al. 1999). Daily injections of recombinant leptin in rats caused a significant increase in Bcl-2 expression and Bcl-2/Bax ratio in granulose cells (Almog et al. 2001).

Following leptin treatment, there is a prolonged recovery period before body weight and fat stores return to baseline levels (Kaibara et al. 1998). Thus, it has been suggested that this delayed recovery of body weight is a result of loss of adipose tissue, and not just the depletion of lipid stores (Qian et al. 1998; Baile et al. 2000).

Recent studies have demonstrated that intracerebroventricular (icv) injection of leptin in rats causes apoptosis of adipocytes in inguinal fat pads of rats (Qian et al. 1998; Gullicksen et al. 2003). The method used to determine this apoptosis involves DNA laddering characteristic of apoptosis. In the later stage of apoptosis, activated nucleases cause internucleosomal cleavage of DNA into small fragments resulting in an increasing amount of small (<1500bp) soluble DNA that can be measured and related to total DNA (Gullicksen et al. 2003). It has also been shown that leptin directly acts on adipocytes to decrease lipogenesis and increase lipolysis, which results in the mobilization of stored lipid (Fruhbeck et al. 1997; Wang et al. 1999). A delay in body weight recovery after leptin administration has been suggested to be related to a loss of adipocytes (Gullicksen et al. 2003). Exogenous leptin resulted in the reduction of the number of large fat cells in specific fat pads with a concomitant increase in the number of small fat cells (Gullicksen et al. 2003). Although the mechanism for the induction of apoptosis is not yet known, it can be assumed that finding other molecules exhibiting similar apoptotic effects as leptin will be a significant step towards discovering the overall neuronal pathway and mechanism responsible for centrally-induced adipose tissue apoptosis.

Tumor necrosis factor α (TNFα) is a cytokine that has been associated with obesity, Type II diabetes mellitus and cachexia (Hotamisligil et al. 1994; Argiles et al. 1997; Ventre et al. 1997). TNFα significantly affects lipolysis and the cell cycle of adipocytes and has been shown to induce apoptosis in several cell types (Petruschke et al. 1993; Nagata 1997; Porras et al. 1997). Adipocytes of obese animals and humans display significant increases in TNFα expression (Zhang et al. 1996).

TNFα has been shown to induce caspase 3-dependent apoptosis in lipid-filled adipocytes from white fat tissues *in vitro* (Qian et al. 2001). In contrast, insulin has an anti-apoptotic function in adipocytes that involves the antagonism of TNFα (Qian et al. 2001). Conjugated linoleic acid isomer Trans-10, Cis-12 induces body fat loss, adipose tissue apoptosis (Hargrave et al. 2002) and increases the level of TNFα in white adipose tissue of mice (Tsuboyama-Kasaoka et al. 2000).

To date, there have been no studies to measure the lipid mobilization and response time of CNTF. Although several studies have shown a consistent loss in body weight and reduction in food intake in rodents treated with CNTF (Gloaguen et al. 1997; Kalra et al. 1998; Xu et al. 1998; Ziotopoulou et al. 2000; Kalra 2001; Lambert et al. 2001), until recently there has not been a focus on the mechanisms for reducing adipocyte size and the possible complete elimination of adipocytes. It has been shown that after cessation of CNTF treatment, there is not an immediate rebound in weight gain (Gloaguen et al. 1997; Xu et al. 1998; Lambert et al. 2001). This finding along with many similar actions of CNTF to leptin raises the possibility that CNTF treatment induces the deletion of adipocytes as well. A recent study reported that intracerebroventricular (icv) administration of CNTF induces adipose tissue apoptosis in epididymal and retroperitoneal white adipose tissue (Duff et al. 2004).

While there have been several *in vivo* studies involving rodent models to determine the effects of CNTF on the physiology of metabolism and obesity, few *in vitro* experiments have been performed to date. Therefore, central effects have been confirmed but direct and peripheral effects of CNTF need to be explored more. A recent study examined the regulation and activation of STATs and proteins by CNTF in cultured 3T3-L1 adipocytes with the objective of determining whether CNTF could have effects on peripheral tissues such as white adipose tissue (Zvonic et al. 2003). In this experiment, the researchers demonstrated that CNTF administration results in the activation of STAT3 in preadipocytes, cultured 3T3-L1 adipocytes and in rodent adipose tissue. The effects of CNTF on cytosolic and nuclear extracts prepared from fully differentiated 3T3-L1 adipocytes were dose-dependent (Zvonic et al. 2003).

CNTF treatment of differentiated 3T3-L1 adipocytes resulted in a time-dependent activation of STAT 3 (Figure 4.2).

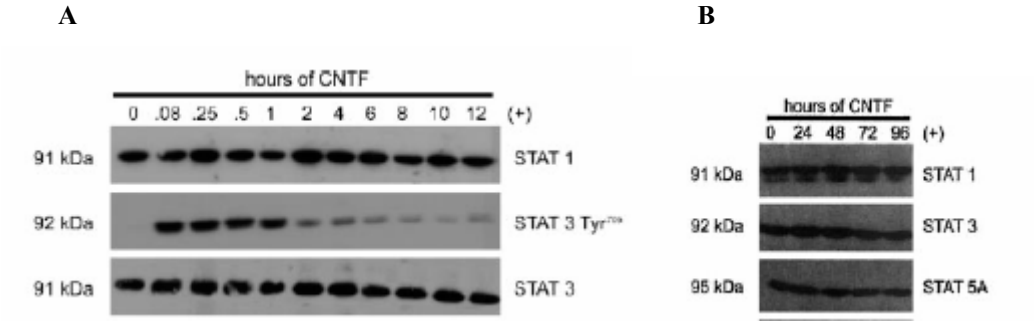


Figure 4.2: **CNTF induction of STAT-3 expression *in vitro.*** The effects of acute (A) and chronic (B) CNTF administration on the expression of STATs (Zvonic et al. 2003).

This study also provided the first evidence that CNTFRα is expressed in adipose tissue (Zvonic et al. 2003). This research supports the notion that the ability of CNTF to result in weight loss may not be solely mediated by the central nervous system. This compilation of evidence along with the fact that the efficacy of CNTF treatment on cultured adipocytes is demonstrated by the activation of STAT 3, provided a sound basis for testing the effects of CNTF on apoptosis of fully differentiated adipocytes.

There have been many other studies to support the findings of the above mentioned papers and this research provides a solid background for which the following two experiments were conducted. There is convincing data showing that CNTF is effective in reducing the obesity related phenotypes in rodents when administered icv. Recent studies have also shown that central administration is not the only means of mediating weight loss and that cultured adipocytes are also responsive to CNTF. The research also shows that CNTF acts via a leptin-like signaling pathway. Therefore, leptin's ability to induce adipose tissue apoptosis presents an interesting question as to whether another related cytokine, such as CNTF, is involved in this apoptotic process as well.

CHAPTER 5

CILIARY NEUROTROPHIC FACTOR INJECTED ICV INDUCES ADIPOSE TISSUE APOPTOSIS IN RATS[1]

[1] Duff, E., C. L. Li, et al. (2004). "Ciliary Neurotrophic Factor Injected ICV Induces Adipose Tissue Apoptosis In Rats." Apoptosis. Accepted by *Apoptosis, 6/01/2004*

ABSTRACT

Recent findings show that ciliary neurotrophic factor (CNTF) and leptin have similar effects on food intake and body weight, suggesting possible overlapping mechanisms. Intracerebroventricular (icv) injection of leptin results in adipose tissue apoptosis. To determine if CNTF has similar activity, male Sprague Dawley rats implanted with lateral cerebroventricular cannulas were randomly assigned to four treatment groups (N=8), including control (aCSF), 10 μg /day leptin, 1 μg/day CNTF, and 5 μg/day CNTF. Rats received daily icv injections for 4 successive days. Both leptin and CNTF (5 μg) decreased BW (8.6% and 11.77%, respectively, $p<.05$) and cumulative food intake was decreased 43% by leptin ($p<.05$). Leptin and CNTF (5 μg) reduced adipose tissue mass in epididymal adipose (Epi) by 30 and 33.5%, ($p<.05$), in inguinal adipose (Ing) by 51 and 55% ($p<.05$), in retroperitoneal adipose (Rp) by 65 and 64% ($p<.05$), and in intrascapular brown adipose (iBAT) by 34 and 25% ($p<.05$), respectively. Gastrocnemius muscle was not affected. Leptin and CNTF (5 μg) increased apoptosis in Epi by 84 and 150%, respectively ($p<.05$) and in Rp by 121 and 146%, respectively ($p<.05$). Loss of adipocytes by apoptosis may provide an explanation for the unexpected delay in return to initial energy status following CNTF treatments.

Key Words: CNTF, cytokine, leptin, food intake, body weight, fat mass

INTRODUCTION

Ciliary neurotrophic factor (CNTF) is a pluripotent neurocytokine expressed by glial cells in peripheral nerves and in the central nervous system (Manthorpe et al. 1993; Ip et al. 1996). It is a member of the cytokine family that is structurally related to leukemia inhibitory factor, interleukin-6, and other proinflammatory cytokines (Bazan 1991). Peripherally administered CNTF has some actions in common with other cytokines, such as induction of fever, hepatic acute phase protein responses, anorexia, weight loss, muscle wasting and cachexia (Shapiro et al. 1993; Henderson et al. 1994; Espat et al. 1996; Martin et al. 1996). Central administration of CNTF has also produced anorexia and body weight loss, but recent studies suggest that weight loss induced by CNTF is a result of a mechanism different from that of other cytokines, and includes suppression of hypothalamic neuropeptide Y (NPY) expression (Kalra et al. 1998; Xu et al. 1998; Lambert et al. 2001).

Because leptin, a secreted protein from adipocytes, also reduces food intake and body weight and decreases hypothalamic NPY gene expression (Campfield et al. 1995; Sahu 1998), it has been suggested that CNTF actually acts via a leptin-like mechanism (Gloaguen et al. 1997). CNTF and leptin have been shown to activate a similar pattern of signal transducers and activators of transcription (STAT) factors in neuronal cells, and both CNTF and leptin receptors are localized in mouse hypothalamic nuclei involved in the regulation of energy balance (Gloaguen et al. 1997). Both leptin and CNTF induce transcription of several cytokine-inducible inhibitors of signaling, such as suppressor of cytokine signaling 3 (SOCS-3) in the arcuate nucleus of the hypothalamus (Ziotopoulou et al. 2000). Although CNTF shares many similarities with leptin, CNTF reduced body weight and food intake under conditions in which

leptin was ineffective, such as in db/db and diet-induced obese mouse models (Gloaguen et al. 1997; Marsh et al. 1999).

Recent studies have demonstrated that icv injection of leptin in rats causes apoptosis of adipocytes in specific fat pads (Qian et al. 1998; Gullicksen et al. 2003). It has also been shown that leptin directly acts on adipocytes to decrease lipogenesis and increase lipolysis, which results in the mobilization of stored lipid (Fruhbeck et al. 1997; Wang et al. 1999). Although the mechanism for the induction of apoptosis is not yet known, it can be assumed that finding other molecules exhibiting similar apoptotic effects as leptin will be a significant step towards discovering the pathway and mechanism responsible for centrally-induced adipose tissue apoptosis. Although several studies have shown a consistent loss in body weight and reduction in food intake in rodents treated with CNTF (Gloaguen et al. 1997; Kalra et al. 1998; Xu et al. 1998; Ziotopoulou et al. 2000; Kalra 2001; Lambert et al. 2001), there has not been a focus on the mechanisms for reducing adipocyte size and the possible complete elimination of adipocytes. It has been shown that after cessation of CNTF treatment, there is not an immediate rebound in weight gain (Gloaguen et al. 1997; Xu et al. 1998; Lambert et al. 2001). This finding along with many other similarities between CNTF and leptin raises the possibility that CNTF treatment induces the deletion of adipocytes as well. Thus, we compared the effects of CNTF and leptin injected ICV on body weight, fat pad weight and adipose tissue apoptosis.

MATERIALS & METHODS

Animals and diet. Forty male Sprague Dawley rats (250-274 g initial body weight) purchased from Harlan, Inc. (Indianapolis, IN) were housed in hanging plastic shoebox cages in a room with a 12 h illumination, 22 ± 1 ºC ambient temperature, and 50 % humidity. Rats had ad

libitum access to pelleted standard lab chow (5001, PMI Nutritional International, Brentwood, MO) and water throughout the study. Each rat was surgically prepared with a unilateral lateral ventricular guide cannula, as previously described (Choi et al. 2003). Proper placement of the cannulas was determined by the angiotensin drinking test (Choi et al. 2003). All experimental and surgical procedures in this study were approved by the Animal Care and Use Committee for The University of Georgia.

Treatments. Control (aCSF) 10µl/day, leptin 10µg/10µl/day (.625 nmol/day), CNTF 1µg /10µl/day (.04 nmol/day), CNTF 5µg /10µl/day (.22 nmol/day). A previous study used a CNTF dose range of 0.02-0.2 nmol administered ICV and found that the 0.2 nmol dose produced the maximal suppression of food intake and body weight (Kalra et al. 1998).

CNTF (Serologicals Inc.) and recombinant rat leptin (R&D Systems, Minneapolis, MN) were dissolved in an artificial cerebrospinal fluid (aCSF) which consisted of (in g/l): NaCl, 8.66; KCl, 0.224; $CaCl_2 \cdot 2H_2O$, 0.206; $MgCl_2 \cdot 6H_2O$, 0.163; $Na_2HPO_4 \cdot 7H_2O$, 0.214; $NaH_2PO_4 \cdot H_2O$, 0.027. The CNTF and leptin solutions were divided into aliquots for daily injections and stored at –80 C.

Design & Procedures. Following recovery from icv cannulation surgery (Appendix B), rats were weighed, ranked by their body weight from the heaviest to the lightest and randomly allocated to two blocks. Rats in each block were further randomly assigned to the four treatment groups. Rats in block 2 began to receive treatments the day after block 1 rats were killed. Injections were administered once daily for 4 successive days. The experiment was terminated approximately 24 h after the last treatments. On day 5 the rats were placed in a CO_2 chamber prior to decapitation. Trunk blood was collected into glass centrifuge tubes, which were immediately placed on ice and allowed to clot for several hours before centrifuging at 2,400 rpm

at 4C for 20 min. Serum was collected and stored at –20C until assayed by RIA for leptin and insulin concentrations. Intrascapular brown adipose tissue (BAT), retroperitoneal (Rp), epididymal (Epi) and inguinal (Ing) fat pads and gastrocnemius muscle were collected. Both left and right sides of each fat pad and muscle were pooled. These tissues were weighed, and Rp and Epi fat pads were immediately frozen in liquid nitrogen and stored at –80C until they were assayed for apoptosis.

Gel electrophoresis apoptosis assay. Apoptosis was assayed in two ways: DNA isolated from fat tissues was separated into two fractions: fragmented and genomic DNA. First, the fragmented DNA was run on an agarose gel in order to identify a ladder pattern of internucleosomal DNA degradation that is characteristic of apoptosis (Qian et al. 1998). Second, apoptosis was quantified as the ratio of fragmented- to total-DNA, multiplied by 100 (Gullicksen et al. 2004). Briefly, approximately 150 mg of adipose tissues were homogenized in lysis buffer (10 mM Tris-HCL, pH 8.0; 10 mM EDTA, pH 8.0; 0.5% Triton X-100) and centrifuged at 14,000 x g for 15 min to separate fragmented DNA from genomic DNA. The supernatant, containing fragmented DNA, was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and the DNA was precipitated by adding polyacryl carrier (Molecular Research Center, Inc., Cincinnati, OH) and ethanol. Genomic (non-fragmented) DNA was extracted from the pellet with DNAzol and the polyacryl carrier. DNA in each fraction was quantified by the PicoGreen method (Molecular Probes, Inc., Eugene, OR), and fluorescence was measured using a SpectroMax Gemini (Molecular Devices).

Statistical Analysis. Significance of treatment effects was determined by one or two way ANOVA. Significance of differences among means was determined by LSD.

RESULTS

There were significant effects on body weight due to leptin and the 5µg dose CNTF treatments (Figure 5.1). By the end of the 4-day injection period, leptin-treated and CNTF (5µg)-treated rats had lost 29.3 ± 4.4g and 36.0 ± 7.2g body weight, respectively ($p<.05$), while the aCSF-injected rats gained 3.6 ± 4.3g. Leptin decreased cumulative food intake by 43%, but neither dose of CNTF significantly affected cumulative food intake (Table 5.1).

Table 5.1: Cumulative food intake (g; mean ± SEM) in rats treated ICV with aCSF (10µl), leptin (10µg/10µl), CNTF (5µg/10µl), and CNTF (1µg/10µl) once a day for 4 days.

	aCSF	Leptin	CNTF 1µg	CNTF 5µg
Food Intake (g)	146.9 ± 18.8[a]	84.3 ± 7.7[b]	130.5 ± 18.9[a]	102.0 ± 15.1[ab]

[a,b]Within each row, means that do not have a common superscript are different ($p<0.05$).

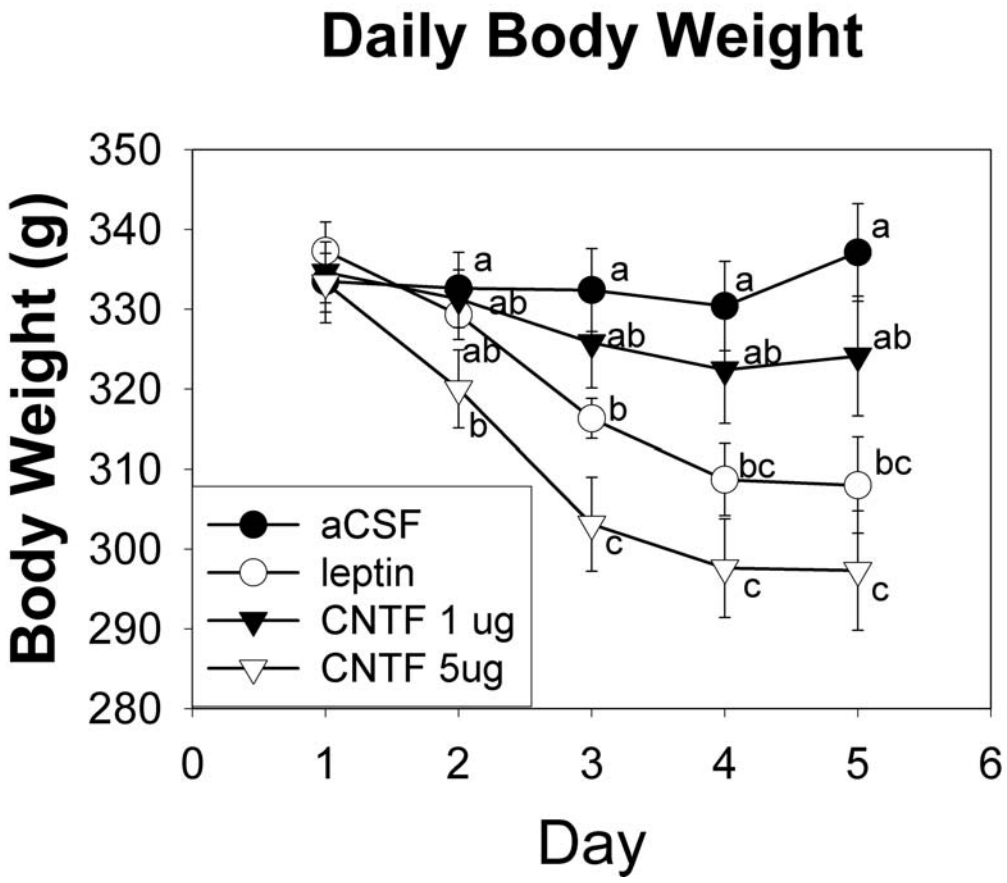


Figure 5.1: Daily body weight (g; mean +SEM) in rats injected ICV daily for 4 days with control (aCSF), leptin (10 µg), CNTF (1 µg) and CNTF (5µg). [a,b,c] Within each day, means without a common letter are different, $p<.05$.

Both leptin and CNTF (5µg) treatments caused significant reductions in BAT, Epi, Ing and Rp fat pads. CNTF (1 µg) significantly reduced BAT and RP mass (Table 5.2). Gastrocnemius muscle mass was not changed by any of the treatments.

Table 5.2: Tissue weight (mg: mean ± SEM) in rats treated ICV with aCSF (10µl), leptin (10µg/10µl), CNTF (5µg/10µl), and CNTF (1µg/10µl) once a day for 4 days. Serum leptin and insulin concentrations (ng/ml; mean ± SEM) 24 h after the last injection in rats treated with the above doses.

	Acsf	Leptin	CNTF 1µg	CNTF 5µg
BAT	218.3 ± 17.1 [b]	143.0 ± 12.8 [a]	174.5 ± 12.6 [a]	164.5 ± 11.4 [a]
Epi	2509.9 ± 102.6 [b]	1757.7 ± 138.1 [a]	2424.3 ± 169.1 [b]	1669.0 ± 198.7 [a]
Ing	2449.1 ± 260.3 [b]	1197.0 ± 202.4 [a]	1879.6 ± 300.3 [b]	1094.4 ± 192.5 [a]
Rp	919.9 ± 132.1 [b]	322.7 ± 55.5 [a]	613. 8 ± 123.3 [a]	333.1 ± 114.7 [a]
GC	1894.0 ± 249.6 [a]	2058.1 ± 48.4 [a]	2061.2 ± 47.3 [a]	2000.0 ± 68.9 [a]
Insulin (ng/ml)	2.00 ± .28 [b]	.59 ± .21 [a]	.81 ± .22 [a]	.58 ± .11 [a]
Leptin (ng/ml)	4.52 ± 1.23 [b]	1.33 ± .54 [a]	1.18 ± .53 [a]	.94 ± .41 [a]

[a,b]Within each row, means that do not have a common superscript are different ($p<0.05$).
Abbreviations: BAT, brown adipose tissue; Epi, epididymal white adipose tissue (WAT); Ing, inguinal WAT; Rp, retroperitoneal WAT; GC, gastrocnemius muscle.

Leptin and CNTF (5µg) significantly increased apoptosis in Epi by 84 and 150%, respectively, $p<.05$. Apoptosis of the Rp fat pad was also increased 121 and 146 % by leptin and CNTF (5µg), respectively, $p<.05$ (Figure 5.2).

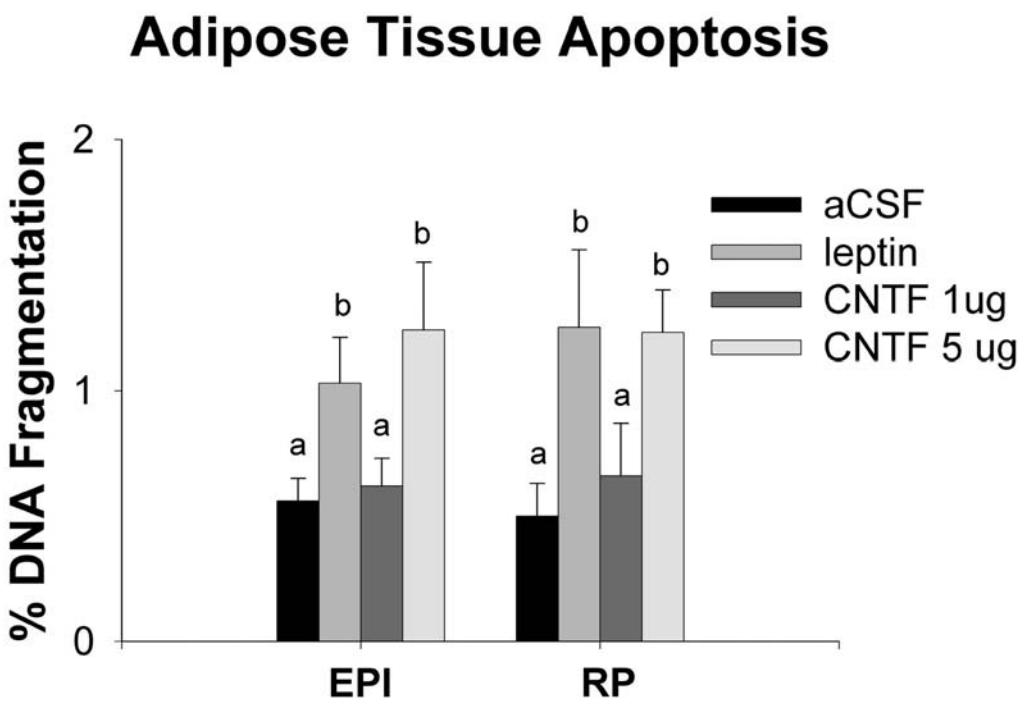


Figure 5.2: Apoptosis (% DNA Fragmentation; mean+SEM) of epididymal (Epi) and retroperitoneal (Rp) fat pads 24 h after the last injection in rats injected ICV daily for 4 days with control (aCSF), leptin (10 µg), CNTF (1 µg) and CNTF (5µg).

[a,b] Within each fat pad, means without a common letter are different, $p<.05$.

The formation of distinct DNA fragments, a biochemical hallmark of apoptosis, can be visualized on a gel as a distinct ladder of bands. Figure 5.3 shows gel electrophoresis of DNA from epididymal adipose tissue. The ladder pattern is evident for both 5 µg CNTF and 10 µg leptin treatments.

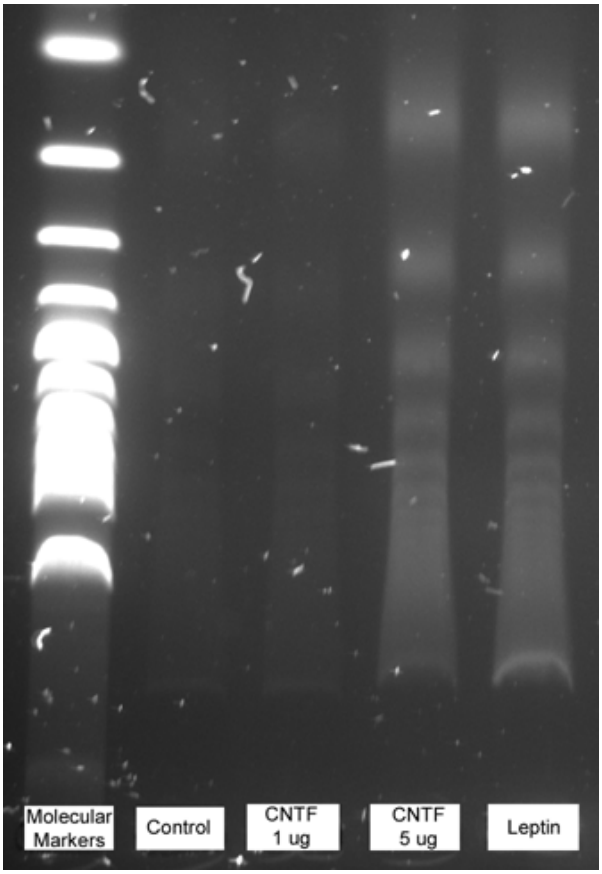


Figure 5.3: Gel showing fragmentation of DNA (ladder pattern) from epididymal (Epi) fat pads of ICV CNTF 5 μg and leptin treated rats.

Leptin and both doses of CNTF caused significant decreases in serum concentrations of leptin and insulin (Table 5.2). There were no differences among the three treatments, however.

DISCUSSION

Consistent with previous studies (Pelleymounter et al. 1995; Gloaguen et al. 1997; Kalra et al. 1998; Xu et al. 1998; Ziotopoulou et al. 2000; Lambert et al. 2001; Gullicksen et al. 2003), our results showed that both leptin and CNTF caused a significant loss in body weight, and in addition, reduction in fat pad weight. Because leptin and CNTF have been shown to have similar actions on adipose tissue mass, we hypothesized that CNTF treatments would increase adipose tissue apoptosis, a significant characteristic of leptin-treated rats (Qian et al. 1998; Della-Fera et

al. 2001; Gullicksen et al. 2003). CNTF significantly increased apoptosis in epididymal and retroperitoneal adipose tissue, thus confirming our prediction that centrally administered CNTF causes adipose tissue apoptosis. Although the degree of apoptosis occurring in these fat pads appeared to be small, apoptosis is a dynamic process, and the half life in adipocytes of the biomarkers of apoptosis is not known. Thus, it is difficult to predict the impact on adiposity or body weight of a small increase in apoptosis measured at one point in time.

Although the mechanisms involved in either leptin or CNTF-induced adipose tissue apoptosis are not yet known, both similarities and differences between these two peptides are beginning to suggest a likely CNS pathway. Two important CNS peptides that act as downstream effectors of leptin are α-melanocortin stimulating hormone (αMSH) and NPY (Inui 1999). Leptin and CNTF both activate STAT-3 in areas of the hypothalamus involved in feeding behavior and body weight regulation (Sleeman et al. 2000; Lambert et al. 2001). However, CNTF causes weight loss in animal models that are resistant to the effects of leptin, including mice lacking leptin receptors (db/db), mice with diet-induced obesity (DIO) and mice with melanocortin-4 receptor deficiency (Gloaguen et al. 1997; Marsh et al. 1999). It is of interest to note that mice with DIO have enhanced sensitivity to the anorectic effects of melanocortins, suggesting that DIO may involve reduced melanocortin signaling (Hansen et al. 2001). Thus, the effect of CNTF on food intake and body weight appears to be mediated independently of melanocortin receptors. Administration of either leptin or CNTF resulted in decreased serum levels of leptin and insulin, most likely a result of the decrease in fat mass caused by these peptides.

Both leptin and CNTF have been shown to suppress NPY expression, and their effects on food intake and body weight can be reversed by concurrent NPY administration (Jang et al.

1998; Kotz et al. 1998; Yokosuka et al. 1998; Lambert et al. 2001). Likewise, the lack of rebound hyperphagia after CNTF or leptin treatments are terminated has been suggested to be a result of the decrease in NPY levels, compared to the increase that occurs with food deprivation (Lambert et al. 2001). We have shown that leptin's effects on food intake and weight loss, but not its effect on adipose tissue apoptosis, could be blocked by a melanocortin receptor antagonist, providing further evidence that melanocortin receptors were not involved in leptin-mediated apoptosis (Choi et al. 2003). In addition, adipose apoptosis was increased by icv injection of NPY-Y5 receptor antisense oligonucleotides in obese rats (Gong et al. 2003) and systemic administration of an NPY antagonist stimulated adipose tissue apoptosis in high fat-fed rats (Margareto et al. 2000).

We have hypothesized that the sympathetic nervous system (SNS) is the link between the brain and adipose tissue that mediates leptin-induced adipose tissue apoptosis. Both NPY and leptin have been shown to influence SNS activity. Leptin acts centrally to increase SNS activity in brown and white adipose tissue (Shiraishi et al. 1999), while intracerebroventricular injection of NPY suppresses SNS stimulation of brown adipose tissue (BAT) (Egawa et al. 1991). Furthermore, we showed that β-adrenergic receptor agonists increased white adipose tissue apoptosis in mice (Page et al. 2004). Whether CNTF might also act via the SNS is not known, but its suppressive effects on NPY could result in increased SNS output.

As noted earlier, both leptin and CNTF are cytokines, and in a recent paper Prima et al (Prima et al. 2004) showed that constitutive expression of cytokines in the brain induced changes in gene expression characteristic of chronic inflammation leading to weight loss. Induction of heat shock proteins, which are involved in the regulation of apoptosis, can be a result of inflammation induced by cytokines. Although induction of heat shock proteins in the

hypothalamus occurred following CNTF administration in rats (Suzuki et al. 2001), it is unclear whether this was in sufficient amounts to affect peripheral tissues, such as adipose tissue. Moreover, food deprivation has also been shown to induce heat shock protein synthesis in the hypothalamus (Aly et al. 1994), and we have shown that food deprivation does not cause adipose tissue apoptosis (Qian et al. 1998).

In conclusion, our findings confirm our hypothesis that CNTF, like leptin, acts centrally to induce apoptosis of adipose tissue. The fact that the weight-reducing effects of CNTF are seen in both leptin-deficient (*ob/ob)* and leptin-resistant (*db/db)* models suggests that its effects are not mediated by the release of leptin or activation of leptin receptors (Gloaguen et al. 1997). Thus, the effects of these two cytokines on body weight, adipose tissue and energy expenditure may involve distinct but converging hypothalamic neuronal pathways.

CHAPTER 6

EFFECT OF IN VITRO INCUBATION WITH CNTF ON APOPTOSIS OF 3T3-L1 ADIPOCYTES

INTRODUCTION

Ciliary Neurotrophic Factor (CNTF) has typically been regarded for its role as a trophic factor in nervous system development. However, the cloning and sequencing of CNTF revealed that it is not related to neurotrophins but is instead a member of the family of cytokines including leukemia inhibitory factor (LIF), interleukin-6 (IL-6), oncostatin M (OSM), and interleukin-1 (IL-1) (Boulton et al. 1994; Kishimoto et al. 1994; Pennica et al. 1995). The action of CNTF is mediated in part through its binding to the CNTF receptor, (CNTFRα). This receptor binding is responsible for the subsequent interaction between CNTF and gp130 and LIFR, which results in the activation of the JAK/STAT pathways. In addition to the similar effects on food intake and weight loss in rodent models, CNTF has been compared to leptin because of the findings that CNTF's receptors have characteristics similar to those of the leptin receptors, including their distribution within hypothalamic nuclei involved in feeding (Lambert et al. 2001). Several studies have shown that the leptin receptor (OB-Rb) is predominantly expressed in brain regions associated with food intake and energy expenditure, including the arcuate, ventromedial, and paraventricular hypothalamic nuclei. To support that CNTF similarly targets hypothalamic satiety centers, *in situ* hybridization was performed, and the results showed that the arcuate and paraventricular nuclei of the mouse hypothalamus express mRNAs for CNTF receptor subunits (Gloaguen et al. 1997).

Although CNTFRα was initially described as being distributed primarily within neural tissues (Davis et al. 1991), it has since been reported in skeletal muscle, adrenal gland, sciatic nerve, skin, kidney, and testes (Ip et al. 1992). CNTFRα can be cleaved from the cell surface and exist and act in soluble form. An important observation is that in contrast to CNTF- or LIF-deficient animals, mice lacking CNTFRα or LIFR display deficiency in neuronal activity, which suggests the presence of additional receptor ligands (Gloaguen et al. 1997).

The weight loss caused by CNTF administration has previously been attributed to the preferential loss of fat which is believed to occur by resetting the hypothalamic weight set point, such that cessation of CNTF treatment does not result in overeating and rebound weight gain (Henderson et al. 1994; Lambert et al. 2001). While this may be true, there are likely other mechanisms involved in the reduced food intake and body weight loss involved with CNTF administration. For instance, it has been shown that intracerebroventricular (icv) administration of CNTF in rats caused adipose tissue to undergo apoptosis in addition to activating lipolysis (Duff et al. 2004). These findings support a novel mechanism involving adipocyte deletion during CNTF treatment. Both morphological and cellular data are consistent with this observation. Adipose tissue from CNTF-treated rats demonstrated a loss of adipocytes and the appearance of adipocytes with condensed chromatin, a characteristic feature of apoptotic cells. Total fat pad weights and DNA content decreased in CNTF-treated rats as compared with control rats (Duff et al. 2004).

The mechanisms for CNTF-induced adipose apoptosis are yet known although recent findings suggest that the apoptosis is a centrally-mediated effect (Duff et al. 2004). While there have been several *in vivo* studies involving rodent models to determine the effects of CNTF on the physiology of metabolism and obesity, few *in vitro* experiments have been performed to date.

Therefore, central effects have been confirmed but direct and peripheral effects of CNTF need to be explored more. A recent study examined the regulation and activation of STATs and proteins by CNTF in adipocytes with the objective of determining whether CNTF could have effects on peripheral tissues such as white adipose tissue (Zvonic et al. 2003). In this experiment, the researchers demonstrated that CNTF administration results in the activation of STAT3 in preadipocytes, cultured 3T3-L1 adipocytes and in rodent adipose tissue. The effects of CNTF on cytosolic and nuclear extracts prepared from fully differentiated 3T3-L1 adipocytes were dose-dependent. The dose range spanned 0 to 3.2 nM of CNTF with 0, 0.1, 0.4, 0.8, 1.6, and 3.2 nM as the full range of doses.

Two of the three CNTF receptor complex components, CNTFRα and LIFR, decrease during adipocyte differentiation (Zvonic et al. 2003). Although these results demonstrate that fully differentiated 3T3-L1 adipocytes do not express CNTFRα, it has been demonstrated previously that CNTF can signal via gp130 and LIFR in the absence of CNTFRα (Touw et al. 2000). Although LIFR and CNTFRα protein levels are reduced in cultured adipocytes, as compared with preadipocytes, adipocytes were still responsive to CNTF.

Despite decreased levels of CNTFRα expression in fully differentiated 3T3-L1 adipocytes, CNTF treatment of these cells resulted in a time-dependent activation of STAT 3. Treating fully differentiated 3T3-L1 adipocytes with an acute treatment of 0.8nM CNTF or 0.8nM LIF demonstrated their expression of STAT 3, as evident by increased tyrosine phosphorylation. When fully differentiated 3T3-L1 adipocytes were treated for a 12-h period, whole cell extracts were isolated and this acute CNTF treatment resulted in a time-dependent activation of STAT 3 and MAPK but not Akt. In addition, fully differentiated 3T3-L1

adipocytes were treated for 24 h with CNTF and resulted in a notable increase (25-50%) in GLUT 4 levels.

The Zvonic study also provided the first evidence that CNTFRα is expressed in adipose tissue. This research supports the notion that the ability of CNTF to result in weight loss may not be solely mediated by the central nervous system (Zvonic et al. 2003).

The objective of this study was to determine whether CNTF can induce adipocyte apoptosis of 3T3-L1 cells *in vitro* using the laser scanning cytometry (LSC), a new method shown to detect adipocyte apoptosis over time (Lin et al. 2004). Three doses of CNTF (.2, .8, 3.2 nM) were used as treatement based on a previous study in which CNTF was shown to effectively act on CNTFRα located in adipocytes (Zvonic et al. 2003). To show that CNTF is in fact acting on the cells, Western Blot was used to show the expression of phosphorylated STAT3 which is known to be activated by CNTF (Zvonic et al. 2003). A media only treatment was used as a control whereas a treatment of 6nM tumor necrosis factor-alpha (TNFα), a known apoptosis inducer, was used as a positive control.

MATERIALS AND METHODS

Materials: Dulbecco's modified Eagle's media (DMEM), calf and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Rat CNTF was purchased from Serologicals, Inc. (Norcross, GA). TNFα was obtained from Sigma (St. Louis, MO). The non-phospho-STAT antibodies were monoclonal IgGs purchased from Transduction Laboratories (San Jose, CA). The highly phospho-specific polyclonal antibody for STAT 3 (Tyr^{705}) was purchased from BD Biosciences (San Jose, CA). The secondary antibodies were horseradish peroxidase-conjugated and were obtained from Sigma.

***Cell Culture**:* 3T3-L1 mouse fibroblast cells (American Type Culture Collection (ATCC), Manassas, VA) were cultured with a density of 5 x 10^4cells / 2 ml 10% calf serum (CS) medium/per 35mm dish and incubated until confluence was reached. Cells were allowed to continue to grow in 10% CS medium for 2 days post confluence (Day 0). Cells were then cultured with induction media consisting of 10% fetal bovine serum FBS/DMEM culture medium, supplemented with 1 mM insulin (final concentration: 1μM), 0.5 mM isobutylmethylxanthine (IBMX) (final concentration: .5 μM), and 1 mM dexamethasone (final concentration: 1μM), for two days (Day 2) before switching to a culture medium with 1 mM insulin supplement (final concentration: 1μM) for another two days (Day 4). Cells were then cultured with 10% FBS/DMEM medium **(without insulin)** for an additional 4 days with the medium change every two days, and were challenged with specified doses of CNTF or TNFα once 90% of the cells were matured adipocytes (See Appendix B). All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. All media contained 100 U/ml of penicillin, 100 μg/ml of streptomycin and 292 μg of L-glutamine/ml (Invitrogen, Carlsbad, CA).

CNTF incubation: In addition to the medium only control group, three doses of CNTF (.2nM, .8nM, 3.2nM) were added to the culture medium (10% FBS) of matured adipocytes accordingly. TNFα (6nM) was used as a positive control. Media only (10% FBS) treatment was used as a positive control. Cells were removed at 0, 24, 48 and 72 hr for LSC testing. For the 72 h treatment group, all medium was changed at 48 h and fresh medium replenished accordingly. For the protein extraction, cells were subjected to a chronic CNTF treatment (.8nM) and removed at time periods 0, 24, 48, 72, and 96 hr. There was also an acute treatment of CNTF (.8nM) at time periods 0, .08, .5, 2, 6, and 12 hr.

Basic Design:

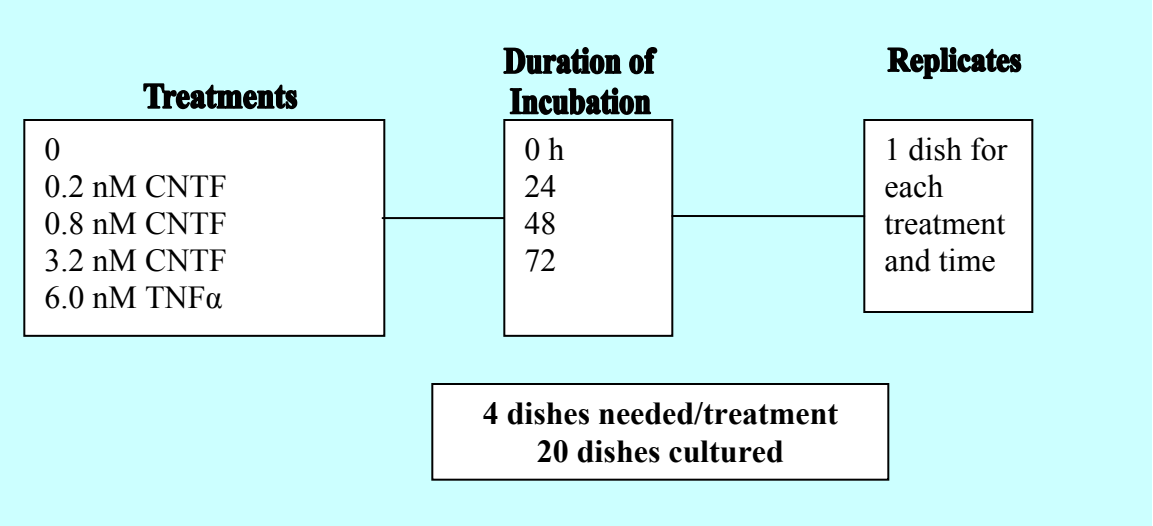


Figure 6.1: LSC Cell Culture - Six blocks of this design were conducted for LSC analysis.

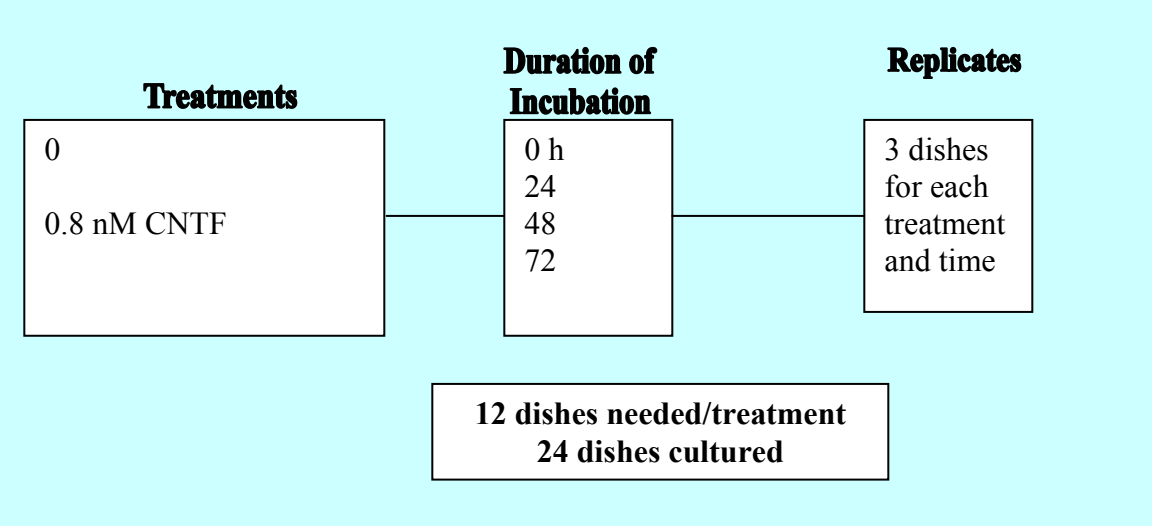


Figure 6.2: Chronic CNTF treatment for protein extraction – this experiment was independently performed three times.

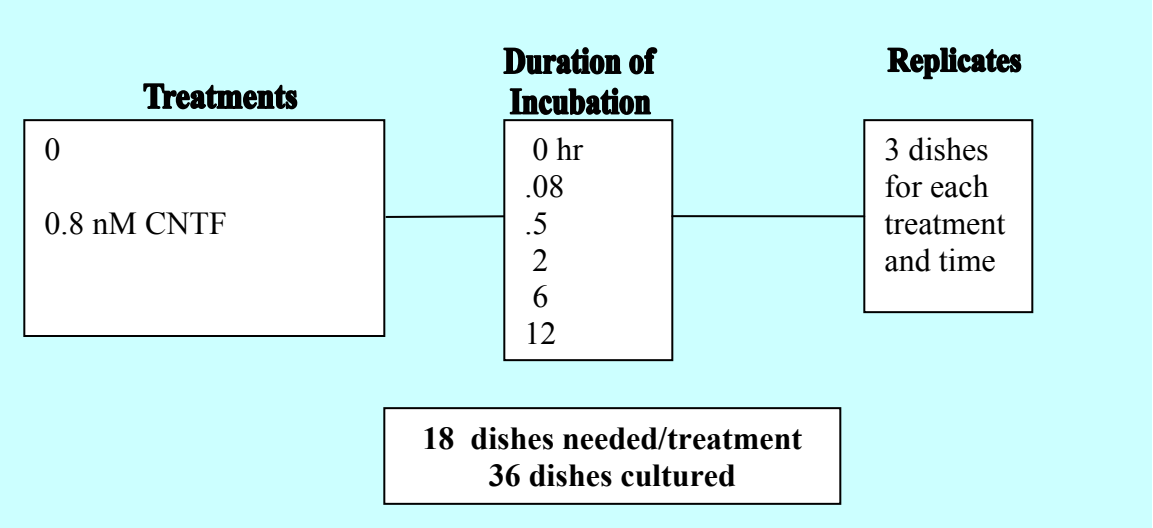


Figure 6.3: Acute CNTF treatment for protein extraction – this experiment was independently performed three times.

Laser Scanning Cytometry (LSC): Laser scanning cytometry (CompuCyte, Cambridge, MA) is a slide-based solid phase cytofluorometer, which allows relocalization and visualization of measured events, thus providing quantitative flow fluorescence data together with morphological information. LSC was used to determine changes in biochemical markers of apoptosis over time. Fluorescence of gated cell populations was measured by LSC. According to designated time periods, Annexin V and Propidium Iodide (Pharmingen) were used to stain the cells on each treatment dish to identify cells with early apoptosis from non-apoptotic cells. Monolayer cells with each of the treatments were washed twice with 2 ml ice cold PBS and once in binding buffer. Next, the cells were incubated for 10 minutes with 5 µl Annexin V-FITC (AV) and 5 µl Propidium Iodide (PI) in 450 µl binding buffer (BD Biosciences, San Diego, CA) at ambient temperature in the dark with gentle agitation for 10 minutes. The dish was then trimmed to fit the LSC stage. The slides (trimmed dishes) were then positioned on the microscope stage, excited by an argon laser at 488 nm and scanned stepwise using a 20x

objective lens. Fluorescence emitted by the cells was collected through green (for AV/FITC or TUNEL/Alexa-Fluor) and long red (for PI) filters. Cell population data (fluorescent intensity, expressed as maximum pixel value of fluorescence) were analyzed by WinCyte and CompuSort software for sorting and morphological relocation. The scanning process on the user-defined region of interest took about 30 minutes to finish or stopped automatically when 5000 events were collected.

Whole Cell Protein Extraction and Protein Quantification: Cell groups were removed from culture at specified time periods for protein extraction using a Mammalian Protein Extraction Reagent kit (Pierce, Rockford, IL). Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit (Pierce, Rockford, IL) according to manufacturer's instruction.

Western Blot Analysis: Proteins were separated on NUPAGE 10% precast gels (Invitrogen) in MOPS SDS running buffer and transferred to a PVDF membrane (Invitrogen) in NUPAGE transfer buffer. Following transfer, the membrane was blocked in 5% milk for 1 h or overnight at 4ºC. After blocking, membranes were incubated, with agitation, with the specified primary antibody. Results were visualized with the horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (ECL Plus kit; Amersham Biosciences).

Statistical analysis: Data was analyzed by two-way ANOVA according to the general linear model procedure using SAS.

RESULTS

The Annexin V/Propidium iodide (AV/PI) combination assay detected apoptotic cell membrane phosphatidyl serine (PS) externalization and served as a measure of adipocyte viability. Utilizing these two stains, the LSC system can provide information about the progression from early apoptotic events to end stage apoptosis by measuring the relative changes

in percent of cells stained with AV and PI over time (Lin et al. 2004). In each scattergraph, cells in the lower left quadrant (Q1) have a low intensity of staining with either AV or PI, and are considered to be normal cells. Cells in the upper left quadrant (Q2) are stained primarily with AV, indicating early apoptosis. Cells with both AV and PI staining are entering a later stage of apoptosis and appear in the upper right quadrant (Q3). Cells with only PI staining appear in the lower right quadrant (Q4). These cells are dead, but at this stage, death by necrosis cannot be differentiated from death by apoptosis. It is the progression of changes over time that indicates that cells are becoming apoptotic and dying. In this study, the number of apoptotic cells at each time point was determined by the total number of cells in quadrants 2, 3 and 4. Quadrant settings are determined by the software, using intensity value criteria for each channel that are set by the user.

There were no statistically significant differences between the CNTF treatment groups, media only control, and TNFα according to LSC data analysis. This is lack of difference was observed for all time periods periods, as well as treatments (Figure 6.4, Figure 6.5).

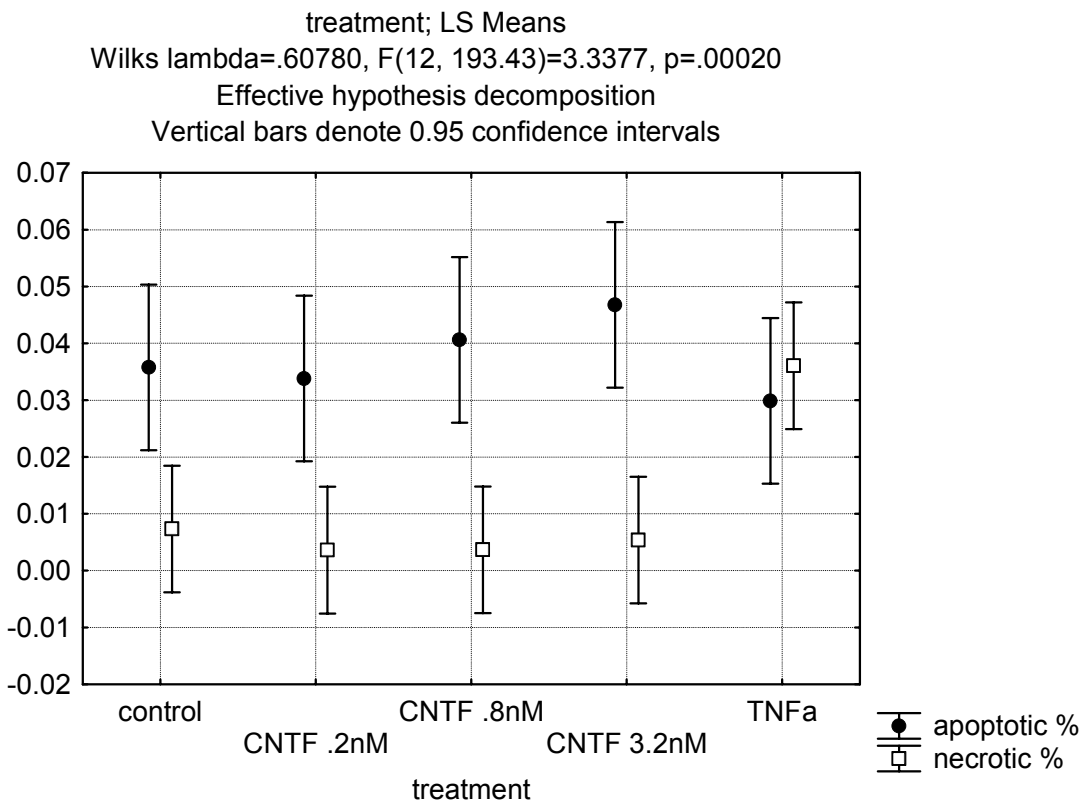


Figure 6.4: Percentages of apoptosis and necrosis among the five treatment groups.

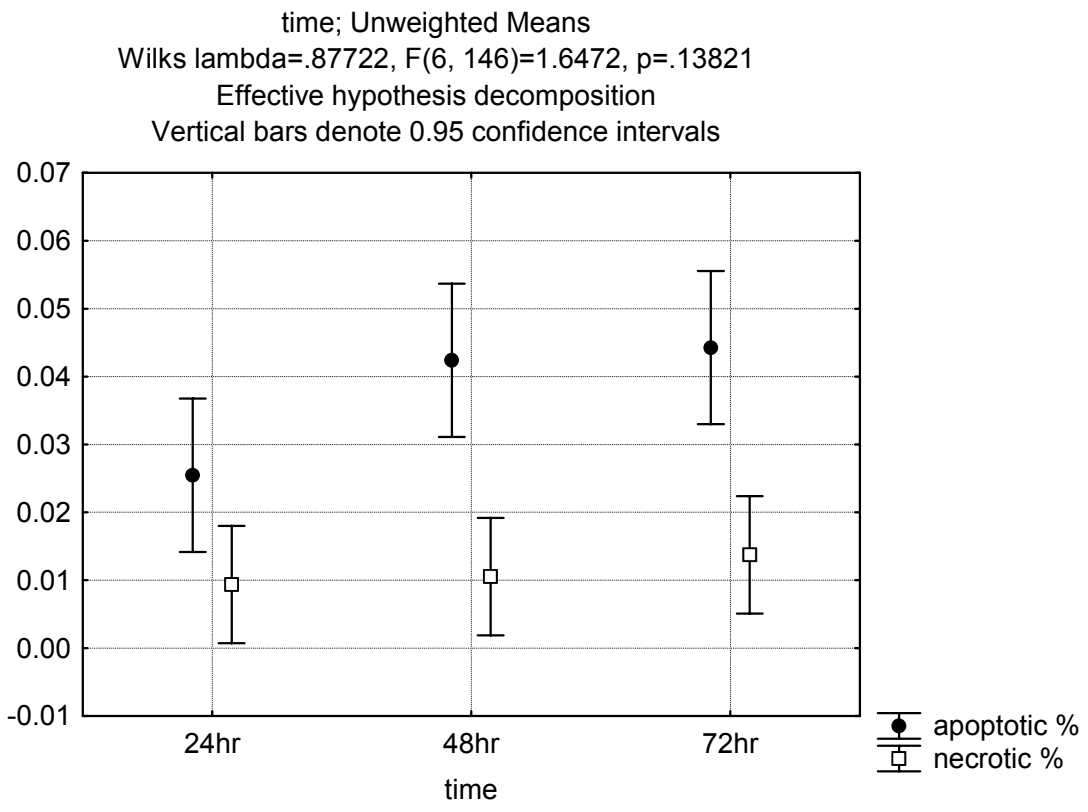
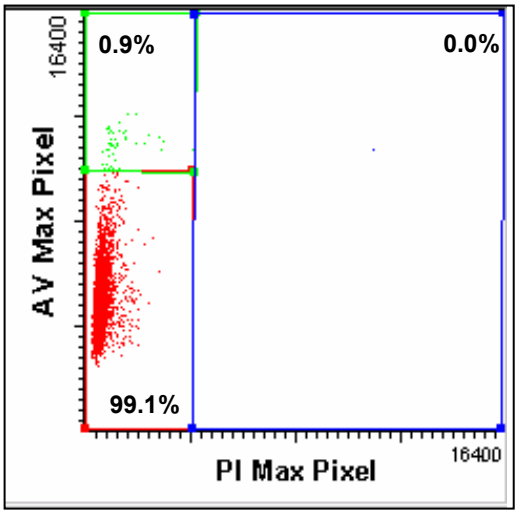


Figure 6.5: Percentages of apoptosis and necrosis among the three time periods.

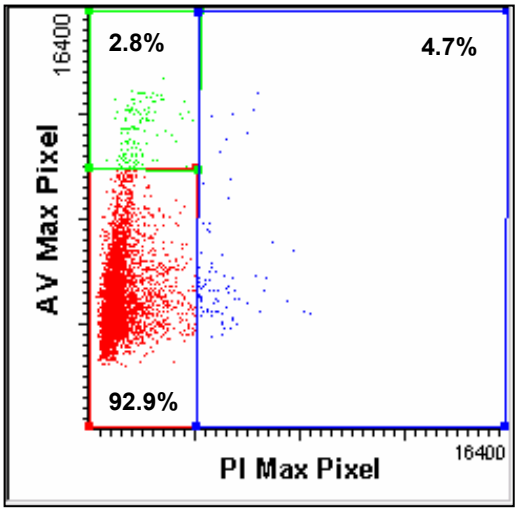
The control cells exhibited approximately 3.6% apoptosis. The percentage of apoptosis for the treatment groups are as follows: .2 nM CNTF = ~3.5%, .8 nM CNTF = ~4.0%, 3.2 nM CNTF = ~4.8%, and 6 nM TNFα = 3.0%. It is evident from these figures that there were no significant differences between the groups. The control cells showed approximately .8% necrosis. The treated cells had the following necrotic percentages: .2 nM CNTF = ~.40%, .8 nM CNTF = ~.40%, 3.2 nM CNTF = ~.50%, and 6 nM TNFα = 3.6%. There was a slight, but non-significant, increase in necrosis for the TNFα cells compared to controls and CNTF treatments. Time-dependent effects, depicted in Figure 6.5, were also not significantly different.

The following figures depict what a potential apoptotic effect may look like as specific blocks did appear to have a time and dose-dependent effect according to treatment. There are

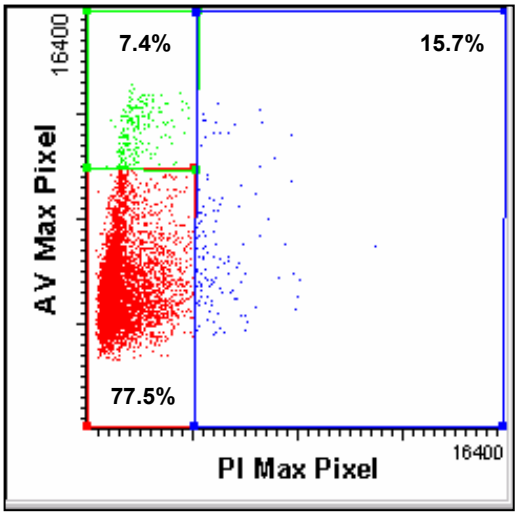
only three sections shown on each scattergraph, rather than the four quadrants described above. The bottom left section displays the percentage of viable cells. The top left section contains apoptotic cells and the entire right section displays the necrotic cell population.



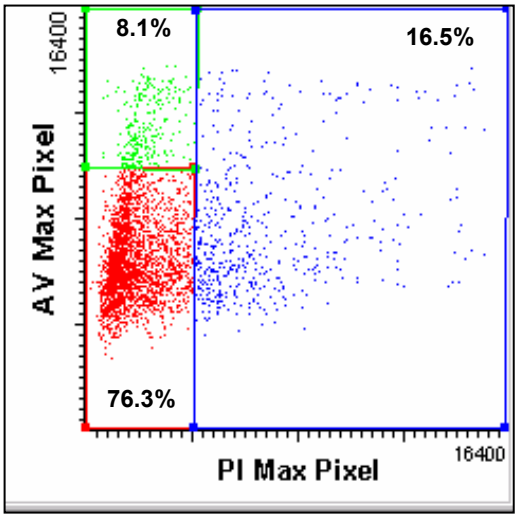
0 hr control (FBS media)



24 hr TNFα (6 nM)



48 hr TNFα (6 nM)



72 hr TNFα (6 nM)

Figure 6.6: LSC data for one block of 6 nM TNFα treatment including 0, 24, 48, and 72 hour time periods. There are three sections of interest. The bottom left section contains percentage of viable cells. The top left section contains early apoptotic percentage. The entire right section depicts the necrotic population of cells.

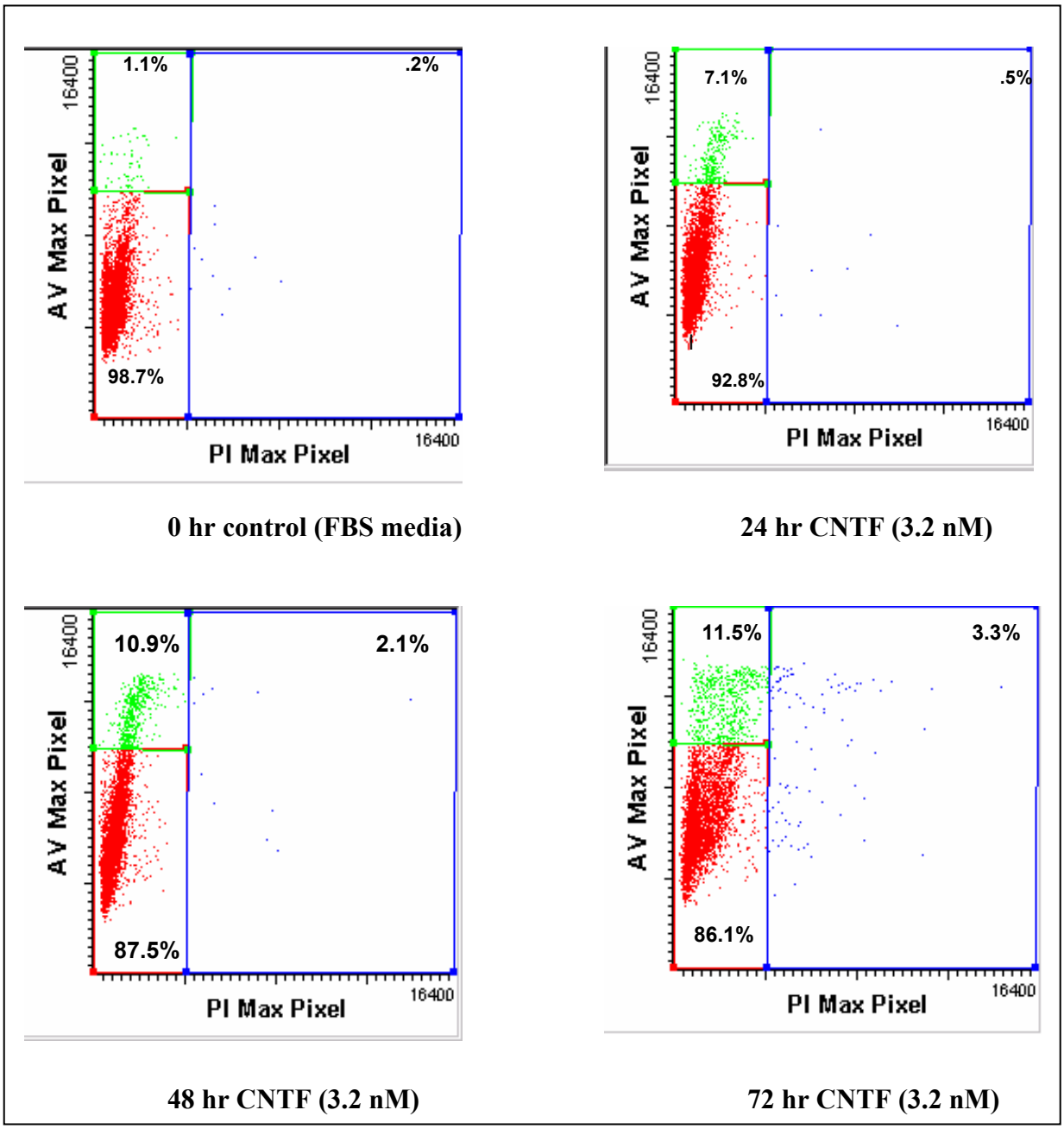


Figure 6.7: LSC data for one block of 3.2 nM CNTF treatment including 0, 24, 48, and 72 hour time periods. There are three sections of interest. The bottom left section contains percentage of viable cells. The top left section contains early apoptotic percentage. The entire right section depicts the necrotic population of cells.

The expression of the non-phosphorylated STAT3 was expected to be seen across all treatment and time periods. The non-phosphorylated STAT3 was expected to be present only in the acute CNTF treatments with a decrease in expression as time progresses. The results for both

the acute and chronic CNTF treatments are inconclusive. There were bands of interest, indicating the possible activation of STAT3, however, the results are inconsistent.

DISCUSSION

This study was designed to test the effects of CNTF on adipocyte apoptosis *in vitro* using the new technology, laser scanning cytometry (LSC). There are several techniques that have been developed to detect apoptosis that are based on morphological, biochemical or molecular mechanisms of the apoptosis process, including light and electron microscopy, laser scanning confocal microscopy, flow cytometry, gel analysis of DNA fragmentation, and certain apoptotic factor assays (Watanabe et al. 2002). Laser scanning cytometry has recently been developed and is a microscope-based cytofluorometry method (Bacso et al. 2001; Smolewski et al. 2001; Bollmann et al. 2002; Verdaguer et al. 2002) The LSC includes a digital microscope and an image processor to automatically segment cells and measure stoichiometric quantitative and morphological features of each cell. The LSC applies user-defined sets of rules to segment cells, and the image processing software extracts measurements for a variety of event features, including total fluorescence at each detector wavelength, peak brightness of each detector's signal, absolute position of the event, nuclear vs. peripheral fluorescence, etc. A unique feature of LSC is that every event (cell) recorded with fluorescence can be visualized after the completion of the analysis using Compusort software; thus LSC allows the analysis of a cell population quantitatively and with morphological evidence (Lin et al. 2004).

Using LSC in conjunction with AV/PI, allowed for the development of a method to evaluate apoptotic progress in adipocyte cultures. Annexin V is a Ca^{2+} dependent phospholipid binding protein that has a high affinity for PS, a membrane phospholipid. Under normal conditions, PS is distributed towards the intracellular leaflet of the cell membrane. During

apoptosis, PS is translocated to the outer leaflet of the membrane, where it is exposed to AV. The externalization of PS requires the activation of caspase 3 and calcium flux (Martin et al. 1996; Bratton et al. 1997), both important factors in the apoptotic process (Lin et al. 2004). PI, a fluorescent dye that binds to DNA, is not able to permeate normal live cells; thus, it was used to distinguish end stage apoptotic cells from normal viable cells in live cell cultures.

In this study, it was hypthothesized that the percent of AV and PI positive cells would increase with increased CNTF doses and treatment time as well as TNF-α incubation time, indicating that more adipocytes would be undergoing the apoptotic process. These results were not obtained in the current study. There were no statistically significant differences between treatment and time groups and the rate of apoptosis was relatively low across the six different blocks.

In addition, the STAT 3 results are not reliable, so it cannot be confirmed that the doses of CNTF applied to the cultured cells, were in fact acting on the CNTF receptor, CNTFRα. The Zvonic et al. paper performed a comparable experiment in which they treated fully differentiated 3T3-L1 adipocytes for a 12-h period and isolated whole cell extracts at various time periods. The acute CNTF treatment resulted in a time-dependent activation of STAT 3 (phosphorylated). They also examine chronic CNTF administration on the expression of STAT3 in adipocytes and exposed fully differentiated 3T3-L1 adipocytes to CNTF over a 96 hour period. Chronic CNTF treatment did not alter the expression of adipocyte expressed STAT3 (Zvonic et al. 2003). The experiments performed in the Zvonic experiment were closely repeated in this current study. Without this confirmation of positive CNTF activity, CNTF's direct effects on adipocyte apoptosis can not be determined based on the results of this study. It can be assumed that CNTF may not act directly on adipocytes. A likely explanation suggests that because CNTF has been

shown to induce apoptosis when administered centrally, it is probable that there are signaling pathways in the hypothalamus that modulate the apoptotic mechanism. Eliminating one mode of action by CNTF may help to clarify the role that it plays in the pathway associated with apoptosis. Gaining more insight into the apoptotic process of adipose tissue opens up new avenues for future anti-obesity therapeutics.

CHAPTER 7

CILIARY NEUROTROPHIC FACTOR - A ROLE IN OBESITY?[1]

[1] Duff, E. and C. A. Baile (2003). "Ciliary neurotrophic factor: a role in obesity?" Nutr Rev **61**(12): 423-6.

Abstract

Ciliary neurotrophic factor (CNTF) is a neurocytokine expressed by glial cells in peripheral nerves and the central nervous system. CNTF is generally recognized for its function in support and survival of non-neuronal and neuronal cell types. Following a serendipitous finding, CNTF was recently acknowledged for its potential role in the control of obesity.
Key words: ciliary neurotrophic factor, neurocytokine, obesity, energy balance

Cytokines are crucial components in the regulation of immunity, inflammation, tissue repair, cell growth, and other important physiologic processes. Cytokines are typically proteins or peptides secreted by one cell as regulators of neighboring cells. Ciliary neurotrophic factor (CNTF) is one such cytokine that plays a role among several processes within the human body from endogenous neuroprotection to regulation of energy expenditure. CNTF is a pluripotent neurocytokine expressed by glial cells in peripheral nerves and in the central nervous system (CNS). It is implicated in the differentiation and survival of non-neuronal and neuronal cell types, including sensory, sympathetic, ciliary, and motor neurons (Gloaguen et al. 1997; Sleeman et al. 2000) . The non-neuronal effects include initiating an acute-phase response in liver cells, maintaining embryonic stem cells in an undifferentiated state, and producing a myotrophic effect on denervated skeletal muscles of mice.

CNTF was initially identified more than 20 years ago for its ability to support the in vitro survival of chick ciliary ganglion neurons at different developmental stages (Adler et al. 1979). Purified CNTF also proved to support the survival of cultured neurons from certain chick and rodent sensory and sympathetic ganglia. In a later experiment, CNTF was purified from rabbit sciatic nerves and found to have a unique sequence that allowed the cloning of a full-length

cDNA for CNTF and the subsequent determination of its primary structure. The crystal structure of human CNTF has been determined; it is dimeric, consisting of a unique antiparallel arrangement of the subunits. The individual subunits contain a double crossover four-helix bundle fold, in which the two helices contain kinks that contribute to its dimer appearance. These findings provide a platform for defining the actions and functions of CNTF at the molecular level within the nervous system.

CNTF, a protein with a molecular weight of 22 kD, is a member of a cytokine family that is structurally and functionally related to leukemia inhibitory factor (LIF), interleukin-6 (IL-6), oncostatin M (OSM), and interleukin-1 (IL-1). This cytokine family is known for its pleiotropic effects and its involvement in cachexia characterized by anorexia, weight loss, and metabolic breakdown. Experimental evidence has shown that these deleterious symptoms cannot be attributed to just one cytokine, however, but rather to interactions among several cytokines. Experimental evidence indicates an increased level of CNTF synthesis in the CNS as a response to CNS injury, trauma, sepsis, and cancer, a set of clinical conditions all characterized by loss of appetite (Kalra et al. 1998).

Each member of this distantly related cytokine family acts to initiate receptor signaling by either homo-or heterodimerization of shared β subunits. IL-6 requires the homodimerization of gp130, while CNTF, LIF, and OSM receptor activation depends on heterodimerization between gp130 and leukemia inhibitory factor receptor-β (LIFRβ). Although the sequence of CNTF lacks exact homology with other related cytokines, studies have shown that receptor recognition sites of cytokines are organized as exchangeable modules between the cytokines. IL-6 signals via a gp130 homodimer, whereas CNTF and LIF signal by induction of gp130 and

LIFR. CNTF also signals via the CNTF receptor (CNTFRα), which is expressed exclusively within the nervous system and skeletal muscle (Davis et al. 1991).

The binding of CNTF to its receptor CNTFRα is responsible for the subsequent interaction between CNTF and gp130 and LIFR, which results in the activation of the JAK/STAT pathways.2 CNTF's possible mechanism of action introduces the possibility that other related molecules, such as a suppositional second ligand for CNTFRα or LIF, might also play a crucial role in the regulation of food intake and energy expenditure. An important related observation is that in contrast to CNTF-or LIF-deficient animals, mice lacking CNTFRα or LIFR display deficient neuronal activity, which suggests the presence of additional receptor ligands (Gloaguen et al. 1997).

CNTF was first clinically recognized for its profound effects on amyotrophic lateral sclerosis (ALS), the most experimentally tractable of the neurodegenerative diseases. Administration of CNTF reduces motor neuron cell death, which is characteristic of ALS. During a clinical trial in which recombinant CNTF was used to treat ALS for neurotrophic benefits, researchers serendipitously stumbled on the discovery that the treatment produced severe anorexia and weight loss (Miller et al. 1996). After a similar outcome was reproduced in experiments using rodents (Martin et al. 1996), CNTF quickly became associated with its potential effects on metabolic pathways and was recognized as a new target for therapeutic applications for obesity.

Initial concerns were related to the possibility that CNTF's mode of action is similar to those of cachectic cytokines that induce fever, hepatic acute-phase protein responses, anorexia,

weight loss, and muscle wasting (Lambert et al. 2001). Studies have shown that supraphysiologic doses of CNTF resulted in a rapid wasting syndrome characterized by weight loss, breakdown of fat tissue and skeletal muscle protein, and reduction of food and fluid intake. However, other findings showed that although CNTF is closely related to a group of cytokines displaying unfavorable effects, CNTF is unlike prototypical cytokines. When administered at lower doses, CNTF can induce weight loss without causing the typical deleterious effects of other cytokines. IL-1 is a typical cytokine that has often been compared with CNTF to demonstrate how CNTF differs from most cachectic cytokines. CNTF does not induce the muscle wasting, proinflammatory responses, conditioned taste aversion, or corticosterone release seen with doses of IL-1 that cause comparable weight loss (Lambert et al. 2001). In a study involving CNTF administration in both *db/db* (mutated leptin receptor) and *ob/ob* (leptin deficient) mice models, CNTF did not induce toxicity, malaise, illness, or taste aversion (Gloaguen et al. 1997). These findings support the suggestion that CNTF acts via a leptin-like pathway.

Leptin is an adipocyte-derived cytokine involved in body weight homeostasis (Gloaguen et al. 1997). Leptin's discovery in 1994 was a huge breakthrough for obesity research. It was found to act through a negative feedback mechanism that relayed a signal between the body's fat stores and the hypothalamic networks, thus providing a mechanism for maintaining body weight (Kalra et al. 1998). Leptin is released by fat cells and is found in the blood in proportion to the amount of stored energy.

The comparison of CNTF's actions to those of leptin is based on the findings that CNTF's receptors have characteristics similar to those of the leptin receptors, including their distribution within hypothalamic nuclei involved in feeding (Lambert et al. 2001). A plausible explanation for the overlapping biologic activity of leptin and CNTF is that they stimulate

common signaling pathways in brain areas involved in the regulation of energy intake and expenditure (Gloaguen et al. 1997). Several studies have shown that the leptin receptor (OB-Rb) is predominantly expressed in brain regions associated with food intake and energy expenditure, including the arcuate, ventromedial, and paraventricular hypothalamic nuclei. To support the notion that CNTF also targeted hypothalamic satiety centers, in situ hybridization was performed; the results showed that the arcuate and paraventricular nuclei of the mouse hypothalamus express mRNAs for CNTF receptor subunits (Gloaguen et al. 1997). The physiologic and behavioral effects that CNTF and leptin produce when administered exogenously include weight loss induction and food intake suppression in *ob/ob* mice, a strain characterized by leptin deficiency (Kalra et al. 1998; Xu et al. 1998).

Recent studies showed that CNTF and leptin negatively regulate the appetite-stimulating signals NPY (neuropeptide Y), AGRP (agouti-related protein), and GABA (gamma-aminobutyric acid) in the arcuate nucleus of the hypothalamus. In addition, they positively regulate CART (cocaine-amphetamine-related transcript) and POMC (pro-opiomelanocortin), which produces the appetite-inhibiting peptide, α -MSH (melano-cyte-stimulating hormone) (Figure 7.1) (Kalra et al. 1998).

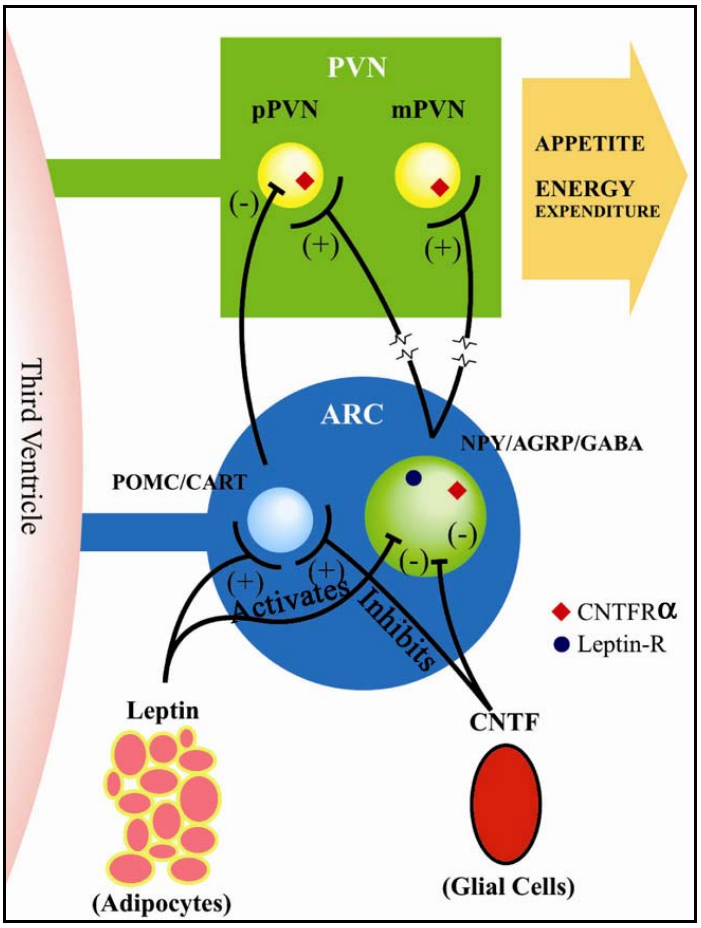


Figure 7.1: Ciliary neurotrophic factor (CNTF) and leptin act on the arcuate and paraventricular nuclei of the hypothalamus. Both cytokines down-regulate the activity of the orexigenic (appetite-stimulating) signals, neuropeptide Y (NPY), agouti-related peptide (AGRP), and gamma amino butyric acid (GABA), while concurrently up-regulating the activity of the anorexigenic signals (appetite-suppressing), pro-opiomelano-cortin (POMC) and cocaine and amphetamine–related transcript (CART) (Duff, 2003, original design).

Daily icv administration of 0.5 μ g CNTF in rats was shown to prevent the increase in hypothalamic NPY expression that occurred during food deprivation, and a higher dose of CNTF (5 μ g/day) actually suppressed NPY expression (Xu et al. 1998). In the same study, daily icv injections of leptin also suppressed hypothalamic NPY expression (Xu et al. 1998). Another

interesting finding related to CNTF and leptin's interaction with NPY involves a system that controls luteinizing hormone (LH) secretion. Both leptin and CNTF have been shown to prevent food deprivation induced suppression of LH secretion, an effect that is mediated by their suppression of NPY expression (Sleeman et al. 2000). This implies that the NPY system is a central target of CNTF and leptin action. These findings support the hypothesis that up-regulation of hypothalamic NPY results in a decrease in pituitary gonadotropin secretion, and that this pathway may act as a communication link between the neural processes involved in reproduction and those that regulate energy balance (Kalra et al. 1998). Other findings have demonstrated that when NPY is centrally infused with CNTF, the anorexic and weight-reducing effects of CNTF are completely reversed (Pu et al. 2000). This not only reemphasizes the safety and specificity of CNTF but also exhibits CNTF's interactive position among appetite-stimulating and appetite-suppressing signals.

Administration of leptin has been shown to attenuate the fasting-induced increase in corticosterone secretion, which is consistent with the finding that leptin receptors are found in corticotrophin-releasing hormone (CRH)–containing neurons. Leptin's effect on food intake may be mediated by altered CRH-mRNA expression in the paraventricular nucleus (Ettinger et al. 2003). In contrast with leptin's effect, CNTF administration does not significantly change the fasting-induced increased levels of corticosterone. Although the CNTF receptor has been found in the para-ventricular nucleus (Gloaguen et al. 1997), these findings indicate that CNTF receptors are not involved in regulation of CRH secretion (Ziotopoulou et al. 2000), which suggests that the actions of CNTF and leptin bypass each other at certain points on the pathways involved in food intake control and energy balance regulation. Another discrepancy between the actions of leptin and CNTF is in their effects on hypothalamic AGRP, which is normally

increased with food deprivation. In a study in which mice deprived of food for 48 hours showed increased AGRP mRNA expression, the administration of leptin reversed the increase, whereas CNTF administration had no effect on AGRP expression (Ziotopoulou et al. 2000). This extensive regulation complex provides a representation of the often concurrent attenuation of appetite-stimulating signals and augmentation of appetite-suppressing signals, thus explaining the resulting decrease in food intake with a consequent decrease in body weight.

Whereas the similarities between CNTF and leptin have spawned great interest in their possible overlapping mechanisms, it is their differences that have researchers extremely curious about the possible implications that CNTF's actions could have for pharmaceutical applications for obesity in the near future. Having two well-researched mechanisms (i.e., those of CNTF and leptin) that are very similar but that possess unique aspects will make it easier to discover the overall neuronal pathway responsible for food intake and energy expenditure. The premature excitement about leptin's potential as an obesity treatment was quickly quelled after the finding that leptin resistance is very common in diet-induced obese rodent models (Kalra et al. 1998). This type of obesity is readily compared with the ever-rising obesity epidemic found in humans, which is attributed to a self-inflicted lifestyle rather than an unpreventable genetic disorder. In the case of leptin resistance, even the administration of supraphysiologic doses of leptin is largely ineffective in reducing body weight in clinically obese patients (Kalra 2001). CNTF appears to be more efficacious than leptin because body weight was reduced in conditions in which leptin was ineffective, most often in leptin-resistant models. Whereas leptin has been successful in normalizing the obese phenotype in *ob/ob* (leptin-deficient) rodent models, CNTF has done the same in *ob/ob*, *db/db*, MC-4 receptor–deficient mice, and diet-induced obese mice (Martin et al. 1996; Gloaguen et al. 1997; Xu et al. 1998; Lambert et al. 2001). CNTF appears to

activate hypothalamic pathways that are downstream of leptin in diet-induced obese models (more representative of human obesity) that are unresponsive to leptin treatment. CNTF is also noted for its ability to cause weight loss without post-treatment overeating and immediate rebound weight gain. Experimental evidence suggests these results are due to CNTF's ability to reduce food intake without triggering the typical hunger signals associated with stress responses. This suggests the possible modification of a set body weight point encoded by the brain (Lambert et al. 2001).

After CNTF proved to have a profound effect on appetite and energy expenditure, it became a promising prospect as a leptin-like cytokine. After a chance discovery within an ALS clinical trial, CNTF was quickly recognized by the pharmaceutical industry as a hopeful candidate for a much anticipated obesity drug. By early 2000, a re-engineered analog of CNTF, named AXOKINE®, produced by traditional biotechnology processes, was already in the first stages of clinical trials at Regeneron Pharmaceuticals. In March of 2000, investigators initiated a Phase-II dose-ranging trial to study the safety and efficacy of AXOKINE (Ettinger et al. 2003). By July of 2001, a press release announced that Regeneron had initiated a Phase-III clinical program of AXOKINE treatment for obesity. On April l4th, 2003, another press release announced the initial results of its Phase-II study of AXOKINE. The study involved a 12-week trial testing AXOKINE on overweight and obese people with type 2 diabetes at doses 1 μg/kg and 0.5 $\mu g \cdot kg^{-1} \cdot day^{-1}$. At the end of the trial period, subjects who were treated with the 1 μg/kg dose of AXOKINE lost 6.5 pounds on average, whereas those treated with placebo and dietary counseling lost only 2.5 pounds. There were also improvements in blood glucose and other metabolic parameters seen in the AXOKINE-treated patients (unpublished data, 3/31/2003,

available at: http://www.regeneron.com/investor/press_detail.asp?v_c_id=169).

Although the initial results of the AXOKINE clinical trials appear promising, the most recent finding indicates an adverse side effect was reported by many of the participants. From the phase-II results, approximately one third of the 1 μ g/kg AXOKINE–treated subjects developed antibodies to AXOKINE. In the phase-III study involving overweight and obese non-diabetic participants, approximately half of the AXOKINE-treated subjects developed AXOKINE-neutralizing antibodies after 12 weeks. The higher incidence of antibody development in non-diabetic subjects will be explored further throughout the phase-III study. Further weight loss appeared to be limited in those people who had developed antibodies (unpublished data, 3/31/2003; available at: http://www.regeneron.com/investor/press_detail.asp?v_ c_id=169). AXOKINE, a modified version of natural CNTF, could be sensitizing the immune system to produce the neutralizing antibodies as a result of the amino acid modifications, because of impurities in the formulation being used, or through direct CNTF stimulation of the immune system, etc. It is likely that the cause for the immunogenicity of AXOKINE will have to be identified and eliminated before it is approved as an antiobesity therapeutic agent.

The discovery that linked CNTF with decreased food intake and weight loss promises a potential therapeutic application for obesity. However, there is still much more to be learned about its mechanisms of action and how it is integrated into the neural pathways that control energy expenditure and food intake. The growing obesity problem is no longer believed to be predominantly a genetic mystery but is becoming more accepted as an issue of unhealthy lifestyle choices. Although the discovery of leptin was a landmark for metabolic research, there are many obstacles yet to be overcome because of leptin resistance, which is a characteristic of diet-induced obesity. The leptin-like effects of CNTF do not appear to be hindered in models

characterized by leptin resistance, a finding that is encouraging enthusiasm for finding a treatment for diet-induced obesity.

CHAPTER 8

SUMMARY

The review of scientific literature found in Chapters 1-4 focuses on the objective of providing all relevant background on the main thesis topic, Ciliary Neurotrophic Factor (CNTF) and the role it plays in obesity and adipose tissue apoptosis. Previous studies have shown that CNTF reduces food intake and body weight in subjects that are leptin deficient as well as leptin resistant which include *ob/ob, db/db,* and diet-induced obesity (DIO) rodent models. A recent report showed that leptin induces adipose tissue apoptosis when administered centrally in the lateral ventricle of the hypothalamus of rats (Qian et al. 1998). The first study was designed to determine whether CNTF, which is known to have similar effects to leptin on energy homeostasis, induces adipose tissue apoptosis when delivered intracerebroventricular (icv) in rats. The exogenous application of CNTF resulted in adipose tissue apoptosis as well as a reduction in food intake and a significant decrease in body weight. These results confirm yet another complementary action that CNTF has to leptin, the induction of adipose tissue apoptosis. CNTF and leptin's ability to induce adipose tissue apoptosis after central administration could be attributed to a hypothalamic signaling pathway that includes the induction of apoptotic proteins Bax and Bcl-2 in the adipose tissue. It is also a possibility that their similar ability to induce apoptosis centrally may involve the NPYergic pathway, and is worth exploring this concurrent signaling mechanism further. By determining another factor involved in the deletion of fat cells, these findings will contribute to discovering the overall mechanism involved in adipose tissue apoptosis which has future implication in the treatment of obesity.

The design of the *in vitro* study was based on a recent study in which CNTFRα was found to be expressed by adipocytes in culture (Zvonic et al. 2003). The results of this study indicate that while CNTF may act directly on adipocytes, clearly evident by the activation of STAT 3 *in vitro* (Zvonic et al. 2003), its ability to induce adipose tissue apoptosis is likely limited to signaling through the hypothalamic CNTF receptor.

The broad implications of this research can be applied to the ever-rising obesity epidemic and a major need for more safe and effective medicinal products to treat obesity and associated diseases. The recent acquisition of knowledge of the regulation of energy homeostasis is promising for the development of new drugs to aid the clinically overweight. Understanding the pathogenesis of obesity and related disorders presents an important opportunity to create strategies for treatments involving the activation of anorexigenic factors and/or the down-regulation of orexigenic pathways. Although the mechanisms involved in either leptin or CNTF-induced adipose tissue apoptosis are not yet known, both similarities and differences between these two peptides suggest a likely CNS pathway. It is to be expected that the rapidly progressing knowledge on apoptosis will create new avenues for pharmacological intervention and the development of pro- and anti-apoptotic drugs.

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APPENDICES

APPENDIX A

Intracerebroventricular (ICV) Cannulation of Rats

Time: ~45 minutes/animal.

Prepare for surgery

1. Administer anesthetic IP: 1 ml/kg of 3:2:1 v/v/v ketamine HCL/acepromazine maleate/xylazine. Record amount, time, and placement.

2. Shave rat and vacuum fur gently. Lubricate eyes with Artificial Tears. Wipe surgery site with sterile solution 4 times.

3. Place animal on stereotaxic instrument. Adjust and tighten ear bars. Place teeth over bar and clamp nose.

4. Affix cannula to stereotaxic apparatus.

Incision

5. Disinfect the skin with chlorhexidine.

6. Make incision from slightly lateral of midline from just behind eyes to back of skull. Scrape exposed skull with back of scalpel and wipe with sterile gauze. Use retractor to hold open incision.

7. Level skull using bubble level.

   Coordinates: A-P, 0.8 mm and M-L, 1.4 mm with respect to bregma

8. Drill 2 or 3 holes for screws: one directly behind cannula, one aft and lateral, and one fore and lateral. Remove bone fragments and insert screws.

9. Re-check level and center cannula over bregma. Take measurement: anterior-posterior, medial-lateral, and then move to correct coordinate.

10. Verify cannula is vertical, or zero on stereotaxic device. Mark skull at intended cannula insertion point.

11. Drill hole for cannula. Make sure cannula will not touch bone upon insertion. Remove any bone fragments with tweezers.

12. Re-check coordinates and position. Place cannula just touching the cortex. Take reading and calculate proper depth.

    Coordinate: D-V, -3.5 mm from the skull surface

13. Lower cannula to proper depth.

Cementing cannula

14. Apply cranioplastic cement by measuring out equal parts of powder and liquid. Mix together–not over 30 seconds–and apply.

15. When material is in position, allow 20-30 minutes for hardening.

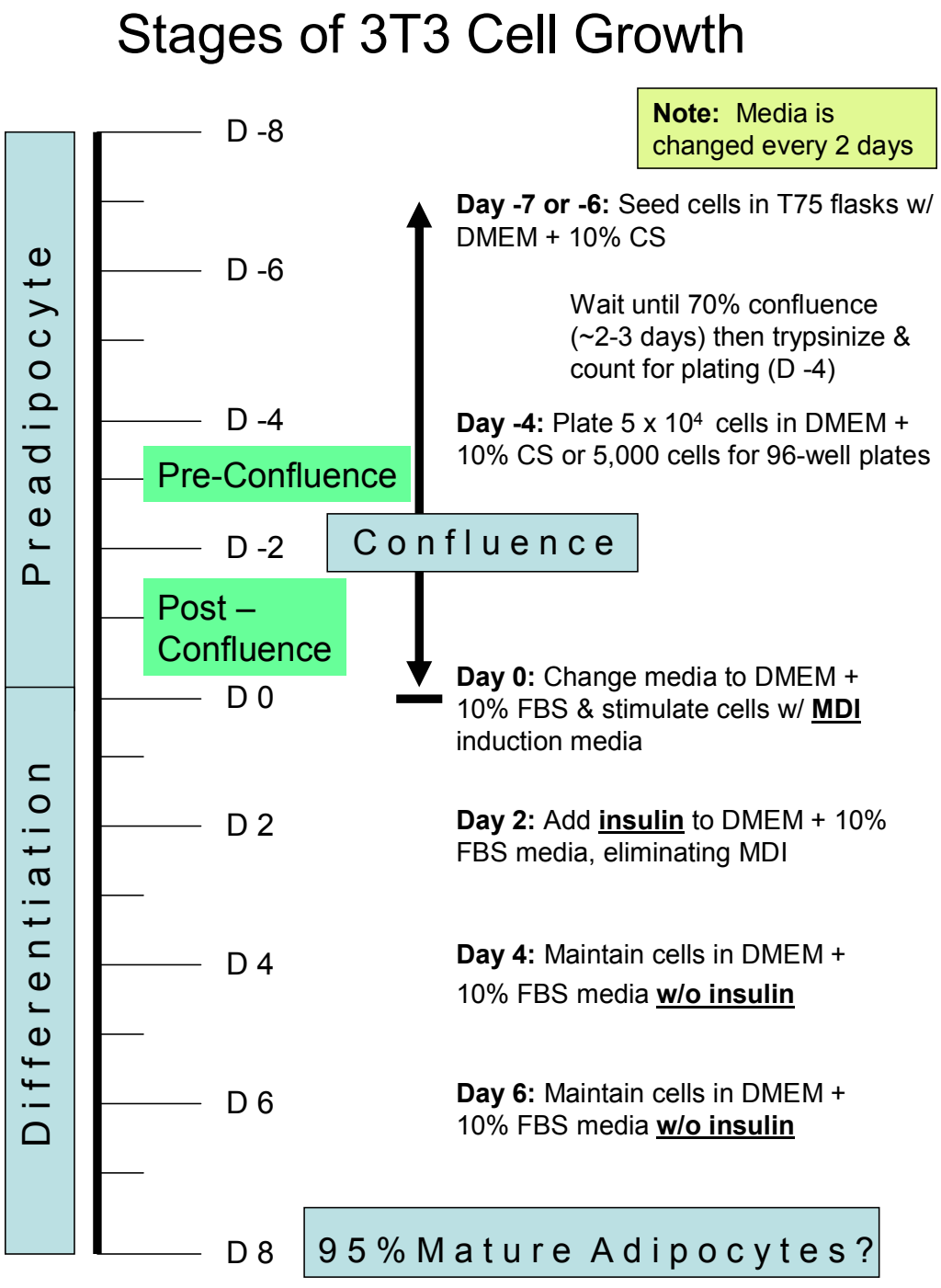
16. Detach the cannula from the stereotaxic device.

17. Insert a 28-gauge stylet into the guide cannula. This will remain inserted when the rat is not receiving an injection.

18. Suture loose skin, if necessary, using loose square knots 3 times.

Recovery

19. Let the animal recover in a shoe-box cage with soft bedding. Place a warmed isothermal pad under the cage.


Stages of 3T3 Cell Growth
Note: Media is changed every 2 days
Preadipocyte
Differentiation
D -8
D -6
D -4
D -2
D 0
D 2
D 4
D 6
D 8
Day -7 or -6: Seed cells in T75 flasks w/ DMEM + 10% CS
Wait until 70% confluence (~2-3 days) then trypsinize & count for plating (D -4)
Day -4: Plate 5 x 10⁴ cells in DMEM + 10% CS or 5,000 cells for 96-well plates
Pre-Confluence
Confluence
Post – Confluence
Day 0: Change media to DMEM + 10% FBS & stimulate cells w/ MDI induction media
Day 2: Add insulin to DMEM + 10% FBS media, eliminating MDI
Day 4: Maintain cells in DMEM + 10% FBS media w/o insulin
Day 6: Maintain cells in DMEM + 10% FBS media w/o insulin
95% Mature Adipocytes?