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IN VITRO EFFECTS OF OLEIC ACID ON THE RELEASE OF MONOAMINES FROM THE HYPOTHALAMUS OF SPRAGUE DAWLEY AND DIET INDUCED OBESE RATS –EXPLORING POSSIBLE MECHANISMS

presented by

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has been accepted towards fulfillment
of the requirements for the

M.S. degree in Pathobiology

Major Professor's Signature

May 13, 2010

Date

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By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Pathobiology

2010

Abstract

IN VITRO EFFECTS OF OLEIC ACID ON THE RELEASE OF MONOAMINES FROM THE HYPOTHALAMUS OF SPRAGUE DAWLEY AND DIET INDUCED OBESE RATS –EXPLORING POSSIBLE MECHANISMS

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Lakshmikripa Jagannathan

One of the major health concerns that plague the modern world is obesity. Significant proportions of a country's health costs go towards the management of obesity. Recently, understanding the changes that occur in the brain during obesity is viewed as a potential source where the answers to major questions around this disease lie. We were interested in exploring changes in the neurotransmitters in response to fatty acids. Rat hypothalami were incubated in an *in vitro* system, and changes in monoamine release in response to various doses of oleic acid were measured by HPLC-EC. High dose of oleic acid significantly increased the levels of norepinephrine (mean 115.008±22.927pg/mg hypothalamus, $p<0.0001$), dopamine (39.495±13.012pg/mg hypothalamus, p0.0011) and serotonin (mean 47.624±7.370pg/mg hypothalamus, p 0.0001) efflux from the Sprague Dawley rat hypothalamus. This was blocked by the addition of a PPAR alpha antagonist MK 886. In DIO rats there was release of reduced response of norepinephrine and serotonin to high dose of oleic acid, and the response was completely absent with dopamine. Thus free fatty acids could act as potential signals stimulating neurotransmitter release in the hypothalamus which possibly involves PPAR alpha. Differences between Sprague dawley and DIO rats in fatty acid sensing may be a factor in the pathogenesis of this disease.

DEDICATION

"Gnanananda mayam dEvam Nirmala spatikAkrudim!

Aadharam sarva vidhyanAm HayagrIvam upAsmahe!!"

ACKNOWLEGEMENT

I owe my thesis to my mentor Dr P. S. MohanKumar and Dr Sheba MohanKumar. They have been a source of unconditional support and encouragement which has helped me immensely in successfully completing my work. It would not be an exaggeration to say that this would have been an impossible feat without their guidance. Every minute spent with the MohanKumars has been a rich learning experience and it has helped me grow both as a student and as a person. Their positivity and benevolence will continue to be an everlasting source of inspiration to me. I would like to thank my other committee members Dr Tony Nunez and Dr Ioana Sonea for their highly constructive suggestions on my work. Their encouragement has tremendously boosted my confidence which has greatly helped in inching closer towards my graduation. I would like to thank my lab mates Ebony, Madhan and Priya who are also one of my closest friends, for helping me in every possible way both in and outside the lab. Working with them has made my graduate study a memorable and enjoyable experience. My lab manager Katrina Linning has been a fabulous person to work with. She has contributed enormously towards increasing my efficiency as a graduate student and her organizational skills are a huge source of inspiration to me. I would like to thank my other friends Sarguru, Haritha, Nandha, Ninitha, Madhu and Suga whose moral support helped me a long way in finishing my writing effectively. I thank my counselor Christina for keeping me on track. Last but definitely not the least; I thank my family for being there for me every step of the way.

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ABBREVIATIONS

BMI	Body Mass Index
DIO	Diet induced obese
DR	Dietary resistant
NPY	Neuropeptide Y
AGRP	Agouti related protein
POMC	Proopiomelanocortin
CART	Cocaine- and amphetamine-regulated transcript
TRH	Thyrotrophin releasing hormone
CRH	Corticotrophin releasing hormone
ARC	Arcuate nucleus
VMH	Ventromedial hypothalamus
LHA	Lateral hypothalamic area
PVN	Paraventricular hypothalamus
αMSH	αMelanocyte Stimulating Hormone
CCK	Cholecystokinin
FATP	Fatty acid transport protein
FABP	Fatty acid binding protein
GPCR	G protein coupled receptor
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator activated receptor response element
CPT	Carnitine palmitoyl transferase
ACS	Acetyl choline synthetase
FAS	Fatty acid synthase

EDTA	Ethylene diamine tetracetic acid
DHBA	3,4-Dihydroxybenzylamine
KRH	Krebs Ringer Henseleit
OA	Oleic acid
NE	Norepinephrine
DA	Dopamine
5HT	Serotonin
5HIAA	5 Hydoxyindoleacetic acid
DOPAC	3,4-dihydroxyphenylacetic acid

Chapter 1

Introduction

INTRODUCTION

I OBESITY

A. Obesity in the modern world

Obesity is one of the major health concerns in the modern world. Obesity refers to a state of increased Body Mass Index (BMI) according to the National Institute of Health definition. BMI for an individual is determined by dividing the weight in kilograms by the square of the height of an individual in meters. Although revised thoroughly over many decades, there is now consensus among various national and international bodies on the definition of obesity[1]. It is now accepted universally that a BMI $\geq$ 25 is defined as overweight, and BMI $\geq$ 30 is defined as clinically obese. Among obese individuals, the severity is further categorized as class 1(BMI 30-34.9), class2 (BMI 35-39.9) and class3 (BMI $\geq$ 40)[1]. For children and adolescents, the BMI value is not fixed, however, a BMI for age percentile distribution is used to determine whether the child is obese or not [2].

Obesity has been growing in epidemic proportions in the United States over the past three decades. According to the National Health and Nutrition Examination Survey (NHANES) in the year 2007-2008, 33.8% of the adult American population were obese and 68% were overweight[3]. The corresponding prevalence of obesity for the year 1970-74 was only 14.1%[2]. A more disturbing trend was observed with younger Americans where obesity prevalence was 11.9% in children aged between 2 and 19 years in the year 2007-2008[4].This is a

marked increase from a prevalence of 5% in the same age group during the period from 1963-1970[5].

Obesity is seldom noticed as an independent entity and is often associated with a multitude of other disorders which can be grouped together as "metabolic syndrome". Metabolic syndrome can be defined by the presence of obesity concomitantly with dyslipidemia, type 2 diabetes, hypertension and a proinflammatory state[6]. Obesity is also associated with risks for several diseases including certain types of cancers[7], arthritis[8], atherosclerosis[9] etc. Investigating the mechanisms by which obesity is caused and how it leads to the precipitation of other disorders will help us devise strategies to counter obesity and its associated health risks.

Globally, it is estimated that 0.7-2.8% of a country's healthcare expenses go towards the management of obesity[10]. However, in the United States alone the estimated cost for obesity remained at $113.9 billion in the year 2008, which ranged from 5-10% of total healthcare spending during that year[11]. Moreover, other health effects associated with obesity such as cardiovascular disorders, arthritis and cancer compound the economic effect. Thus, there is an urgent need to address the problem of obesity.

B. Modeling obesity in the lab animal

Diet remains one of the foremost factors contributing to the current prevalence of obesity worldwide. This is closely followed by lifestyle changes and genetic factors. There have been many attempts to model the pathophysiological

changes that occur in human obesity. Several animal models of diet induced obesity have been developed to study how genetics predisposes an animal to develop obesity. In human beings, monogenic forms of obesity have been identified, although they form only a small percentage of the various types of obesity. More common forms of human obesity seen clinically are polygenic, which also makes them inherently difficult to study through modeling[12].The monogenic forms of obesity as expected, have been easier to model, with classic examples such as the ob/ob and db/db mice that lack leptin and leptin receptor respectively[13, 14]. These animals are morbidly obese and show other features reminiscent of metabolic syndrome and are popular in obesity research.

The polygenic form of obesity which is more commonly encountered in the human population has also been modeled by dietary interventions using different high-energy and high-fat diets[15]. Typically, common rodent strains like the Sprague Dawley rat are fed obesogenic diets of varying composition and the animals that develop a significant degree of obesity are categorized as diet-induced-obese (DIO) and those which resist weight gain are categorized as dietary-resistant (DR)[16, 17]. This type of obesity was taken to be polygenic as the response to diet was normally distributed in the population. The extreme phenotypes seen in these rodent colonies have been bred over many generations to produce distinct DIO and DR strains[18]. While the former monogenic animals are useful to delineate specific mechanisms of obesity, the polygenic models are better suited for interventional studies as they closely mimic the condition seen in the human population.

C. Brain in the pathogenesis of obesity

Obesity has been researched from the gastrointestinal, metabolic, behavioral and hormonal standpoints. The greatest focus still remains on metabolism, particularly in the intestines, liver, skeletal muscle and adipose tissue[19-21]. Although theses peripheral organs play a crucial role in energy homeostasis, it is the central nervous system that has emerged as a key player in the etiology and pathogenesis of diet-induced obesity[17, 22].

In the central nervous system, the hypothalamus is the primary center which integrates peripheral signals to maintain energy homeostasis and subsequently body weight. The hypothalamus maintains the balance between energy intake and energy expenditure by at least two different mechanisms. One, it senses the metabolic status and regulates feeding behavior through orexigenic and anorexigenic peptides such as Neuropeptide (NPY), Agouti related protein (AGRP), Proopiomelanocortin (POMC) and Cocaine- and amphetamine-regulated transcript CART[23]. Secondly, it directly affects metabolism in the periphery, through autonomic pathways, mainly at the level of the liver to regulate glucose production, fat deposition and weight gain[24].

Apart from these major mechanisms, numerous circuits that influence feeding behavior are also involved like the endocannabinoid system, the catecholaminergic systems, Thyrotrophin releasing hormone (TRH) and Corticotrophin releasing hormone (CRH) [25-27].

D. Neuroendocrine hypothalamus and obesity

The principal hypothalamic regions directly regulating feeding behavior include the arcuate nucleus (ARC), the ventromedial hypothalamus (VMH), the lateral hypothalamic area (LHA), and the paraventricular nucleus (PVN) [28-32]. It is of great interest to understand the regulatory mechanisms that govern these neuronal subtypes and how this regulation is disrupted in obesity.

i. Arcuate nucleus

The arcuate nucleus is one of the most significant players in the hypothalamus with respect to energy homeostasis. Being a circumventricular nucleus, the arcuate nucleus is conveniently positioned outside the bounds of the blood brain barrier; hence can easily access molecules from both the blood and the cerebrospinal fluid[33]. Thus it makes sense that the neurons in the arcuate nucleus act as the first relay point in the hypothalamic sensing of nutrient molecules and hormones, and are termed as first order neurons[34].

The arcuate nucleus has a high density of two major groups of first order neurons involved in energy balance, the AGRP/NPY neurons and the POMC/CART neurons[34]. AGRP and NPY are known as orexigenic peptides as they increase feeding behavior while the POMC, CART, CRH and αMSH are said to be anorexigenic since they inhibit feeding behavior[35]. A fasted state activates the orexigenic AGRP/NPY group of neurons which in turn are inhibitory to the anorexigenic POMC/CART neurons[28]. POMC is a precursor of α-Melanocyte stimulating hormone (αMSH) which acts on its receptor in various

brain regions and is inhibitory to feeding[34]. AGRP also acts as a direct antagonist at the level of αMSH receptors[36]. The brain regions that express the α-MSH receptor include the PVN, VMH and LHA, which also contain second order neurons[28]. Thus a balance between these two systems regulates feed intake and maintains body weight in healthy individuals.

Paraventricular nucleus

The paraventricular nucleus has emerged recently as another important centre in the regulation of feeding and energy metabolism. The paraventricular nucleus is an important relay point in the sympathetic nervous system[37]. In energy homeostasis, sympathetic innervations to the white as well as brown adipose tissue play an important role in controlling energy expenditure and fat mobilization from these organs[9, 37, 38]. Thus the paraventricular nucleus plays a direct role in the autonomic regulation of energy metabolism.

The paraventricular nucleus also contains CRH neurons which play a significant role in regulating feeding behavior. CRH is known to be a direct inhibitor of feeding behavior and is a potent anorectic agent[39]. There is also anatomical evidence of close interaction between the CRH system and the melanocortin system[38]. Above all, CRH is a central molecule in the hypothalamo-pituitary-adrenal or the stress axis, and dysregulation of the stress axis is a phenomenon seen to coexist with obesity[40].

A third functional significance of the paraventricular nucleus lies in the fact that it also contains Thyrotrophin releasing hormone neurons[41]. The thyroid axis

controls many aspects of energy metabolism including metabolism of nutrients, thermogenesis, etc. Thus thyroid hormones also regulate body weight. To summarize, the paraventricular nucleus is involved in energy homeostasis through multiple pathways encompassing the sympathetic nervous system, the hypothalamo-pituitary-adrenal axis and the hypothalamo-pituitary-thyroid axis.

Ventromedial hypothalamus

The ventromedial hypothalamus was once the most prominent nucleus of the hypothalamus thought to be involved in energy homeostasis[42]. This was on account of a number of reproducible experiments in which lesions in and around the ventromedial hypothalamus produced obesity in laboratory rats[42]. However, soon it was refuted with the hypothesis that obesity due to VMH lesions occurred due to metabolic consequences of damage to autonomic neurons rather than due to change in feeding behavior and the exact nature of the pathogenesis of hypothalamic obesity was a matter of much debate[42, 43]. But recently, the ventromedial hypothalamus has regained its role as a centre for regulation of feeding following studies that demonstrated specialized glucose sensing neurons[44] as well as various neurons that respond to feeding related neuropeptides[45]. Presently, there is little doubt that the ventromedial hypothalamus has a significant role to play in modulating feeding and is probably mostly inhibitory to feeding.

Lateral hypothalamic area

The lateral hypothalamic area is supposed to have an effect quite opposite to the ventromedial hypothalamus, i.e., it mainly stimulates feeding behavior[46]. The lateral hypothalamic area also contains glucose sensing neurons which are predominantly inhibited by increases in extracellular glucose concentrations[47]. These neurons respond to reduced glucose levels in circulation with the expression of the peptide orexin, which as the name indicates is orexigenic in nature[48].

Notwithstanding the wealth of information gathered on how distinct areas of the brain regulate feeding, better understanding of neuronal networks and processes has lead to a shift in the focus from anatomical regions to functional systems like the melanocortin system, dopaminergic, serotoninergic systems and so on. More often than not, neurons belonging to feeding related circuits overlap with more than one of the aforementioned nuclei and also extend into other regions of the brain like the brain stem[49].

II HYPOTHALAMUS IN THE MAINTENANCE OF ENERGY HOMEOSTASIS

A. Energy intake versus energy expenditure

The balance between energy intake and energy expenditure determines whether an animal is obese or not. This intricate balance is maintained by a number of physiological systems of which the brain acts as the highest centre for integration. Like any balancing system, the hypothalamus senses the metabolic status of the animal from a variety of peripheral signals. These signals are then integrated and appropriate efferent pathways are activated or humoral responses are instituted to maintain equilibrium.

B. Peripheral Signals

The brain receives information on the nutrient status from a variety of sources. These types of peripheral signals encompass hormones like insulin, leptin, cholecystokinin (CCK) and ghrelin, nutrient signals such as glucose, fatty acids and amino acids. Besides these, the brain also receives afferent inputs from the autonomic nervous system from the gastrointestinal tract[49].

i. Hormonal signals to the hypothalamus

Of all the metabolic signals that influence the activity of the neuroendocrine feeding circuits, two major hormones have held the interest of researchers for a long period of time, namely leptin and insulin [49-51]. Leptin is an adipose tissue derived hormone whose gene, the ob locus was first isolated in mice that carried mutations in this gene[13] .Leptin is a pleiotrophic hormone which

acts mainly on the hypothalamus but also has receptors localized in many other tissues like the lungs, liver, kidneys, hematopoietic cells and reproductive organs[52]. In the hypothalamus, leptin exerts its effects on the hypothalamo-pituitary adrenal axis[53], hypothalamo-pituitary gonadal axis[54] and the hypothalamo-pituitary-thyroid axis[55]. Of great interest in energy homeostasis are the effects that leptin has on the neuroendocrine neurons in the arcuate nucleus. The arcuate nucleus as mentioned earlier, has a number of peptidergic neurons that are critical for feeding behavior.

The second most important hormone regulating energy metabolism is insulin[56]. Insulin is known to affect the melanocortin system in the hypothalamus[57]. Insulin action in the hypothalamus is also known to affect peripheral insulin sensitivity and glucose production[56].

Other molecules that signal the hypothalamus include gut hormones such as Cholecystokinin (CCK), glucagon like peptide (GLP-1) and ghrelin which act as satiety signals and modulate feeding behavior [27].

ii. Nutrient signals to the hypothalamus

The hypothalamus also gets direct cues on the metabolic status of the animal by actually sensing the nutrient molecules in the circulation[58]. Two principal nutrient signals that regulate feed intake and body weight include glucose and fatty acids[58].

Glucose

Glucose sensing by the hypothalamus has been widely studied and is of great significance in obesity and diabetes research[59]. Hypothalamic neurons, particularly those in the arcuate nucleus are known to respond to extracellular glucose concentrations by either excitation or inhibition[60]. Those groups of neurons that respond by excitation are known as 'glucose excited' and those that are inhibited are known as 'glucose inhibited' neurons. These two groups of neurons have been characterized through electrophysiological and molecular studies. One would expect that increase in extracellular glucose concentrations would signal the brain in a way that feeding is inhibited, in other words the orexigenic NPY neurons should be inhibited and the anorexigenic POMC neurons should be excited[61].These differential effects may contribute to the regulation of feeding behavior and subsequently body weight. However, evidence to support this is still being gathered and much of the electrophysiological studies in isolated neurons have produced uncertain results[58]. Glucose sensing at the hypothalamus is also a key event in peripheral glucose homeostasis and is impaired in type 2 diabetes and metabolic syndrome[62].

Free fatty acids

While glucose signaling at the hypothalamus is only beginning to be properly understood, information on fatty acid sensing by the hypothalamus is even scarcer. The brain does not use free fatty acids as a source for energy but at the same time, free fatty acids have been demonstrated to readily cross the

blood brain barrier and reach significant concentrations in the brain[63]. Hypothalamic free fatty acid levels have been shown to change significantly with feeding, but they are not a source of energy for the brain. This has raised the interest in fatty acids as possible signaling molecules at the level of the brain rather than a nutrient molecule[64]. Moreover, studies have shown that the same groups of neurons that respond to glucose also respond to free fatty acids[65] and extracellular glucose concentration was shown in another study to affect fatty acid signaling in hypothalamic arcuate nucleus neurons[66]. Thus both fatty acids and glucose appear to play a concerted role at the level of the hypothalamus in the regulation of energy homeostasis. Impaired fatty acid signaling, just like glucose and other hormone mediated signaling, can be explored as another etiological factor in the development of diet induced obesity and the apparent 'anergy' of the hypothalamus to respond appropriately to metabolic signals.

B. Integration of various metabolic signals

It is now realized that the brain circuits that respond to metabolic cues and influence feeding behavior and metabolism are highly complex, and the list of molecules and regulatory pathways keep growing[67]. The major relay centers that process this information include the hypothalamus and the brain stem[68]. A wide variety of neuronal networks are involved in regulating feeding behavior[49]. The most prominent ones are those in the hypothalamus, although numerous other brain regions are also involved. Some other brain regions directly or indirectly involved in modulating feeding related behavior include but are not restricted to the brain stem[68], the cortex and the limbic pathways[69]. The hypothalamic regions

controlling energy homeostasis have been discussed before (I D). Hypothalamic neurons are known to project into the nucleus accumbens and ventral tegmental area where reward calculation is made and motivation to eat arises[70]. Also in higher animals, the cortical centers are also involved in voluntary regulation of feeding and voluntary exercise behaviors[71]. These regions integrate the information on the metabolic status of the animal to recruit appropriate effectors.

C. Efferent pathways

Based on the metabolic signals the hypothalamus senses a fed versus fasted state and recruits a variety of efferent pathways and also secretes various effector substances that ultimately alter either feeding behavior or metabolism or both.

i. Autonomic pathways

Autonomic projections from the brain and the spinal cord innervate peripheral organs and control metabolism. Classical examples are the sympathetic innervations to adipose tissue[37, 38], vagal efferents to the liver and gut[72]. While sympathetic innervations to adipose tissue may play a role in lipolysis and lipid mobilization, vagal efferents to the liver and gut may promote gluconeogenesis and increased fat deposition.

ii. Behavioral pathways

Behavioral pathways can be considered in two different ways. First is the physiological feeding behavior that includes hunger and satiety. These behaviors are vital to the survival of the organism and are direct responses to the metabolic status. Specific areas of the hypothalamus and the brain stem regulate this kind of feeding behavior[49]. The second type of behavior is the motivation to eat even in the absence of the need or in some cases even when there is energy surplus in the body. Brain circuits controlling reward and motivational behavior like the mesolimbic dopaminergic system are involved in this kind of behavior [73].

iii. Endocrine pathways

Classical hormones such as cortisol, thyroxin, estrogen, testosterone, etc control metabolism via established neuroendocrine axes. Examples include the hypothalamo-pituitary-adrenal axis and the hypothalamo-pituitary-thyroid axis. The target endocrine organ secretes the hormones in response to brain derived releasing factors, and act on other visceral organs to regulate metabolism. A number of other hormone-like substances are also secreted by peripheral organs as a result of stimulation from the nervous system. Sometimes, the molecules that act as signals also act as effectors. A good example of this is leptin, which is shown to have a number of peripheral effects and central effects[74]. Insulin secretion is again controlled by nervous efferents.

III IMPAIRED HYPOTHALAMIC SIGNALING IN OBESITY – FOCUS ON FREE FATTY ACIDS

A. Impaired hypothalamic signaling mechanisms in obesity

The complex nature of central nervous system feeding circuits gives way to an equally complex variety of defects that could contribute to obesity. Thus it has become extremely difficult to pin point a single mechanism in the pathogenesis of obesity. However, defective signaling of the peripheral nutritional status to brain regions regulating energy homeostasis has been a recurring motif in many forms of obesity in laboratory rodents as well as humans[28]. Leptin signaling has been widely studied and impaired sensitivity of the brain to leptin is seen as one of the major events in the pathogenesis of obesity[75]. Free fatty acid is another peripheral signal that could possible play a role in the pathogenesis of obesity in the brain, however its role in the onset and development of obesity have not been fully explored.

B. Free fatty acids and metabolism

The adipose tissue is a rich source of fatty acids in the complexed form of triglycerides. Breakdown of triglycerides in the adipose tissue and other peripheral organs contributes to the free fatty acids in circulation[76]. Elevated free fatty acid levels are noticed transiently in fasted states, and persistently in obesity and diabetes. Elevated free fatty acid level seen in obesity is highly correlated with insulin resistance and subsequent diabetes[77]. A sustained elevation in free fatty acids is seen in obese individuals with and without diabetes. Moreover these

individuals are more likely to develop insulin resistance later which in turn contributes to increased lipolysis and an increase in circulating free fatty acids[78]. In laboratory animals, free fatty acids are found to be highly elevated in obese animals, more so with high fat feeding (our unpublished data).

C. Types of free fatty acids

Biochemically, fatty acids are categorized based on the length of their carbon chain and the presence of double bonds in their structure. Saturated fatty acids are free of double bonds, and the common ones found in circulation include palmitic acid, stearic acid and myristic acid. Unsaturated fatty acids contain one or more double bonds. Monounsaturated fatty acids contain one double bond and oleic acid is a classic example. Polyunsaturated fatty acids contain more than two double bonds and include linoleic acid, linolenic acid and arachidonic acid[79]. Various fatty acids have different physiological functions and hence are also different in their intracellular signaling mechanisms. Oleic acid is commonly studied as a representative fatty acid particularly as a nutrient signal[66, 80, 81]. Oleic acid levels are one of the most prominently elevated following high fat feeding. Impaired lipid metabolism and subsequent elevation in various plasma lipids including free fatty acids, is a hallmark of obesity and metabolic syndrome[6].

E. Hypothalamic sensing of fatty acids

Fatty acids have been demonstrated to readily traverse the blood brain barrier [63]. Free fatty acids are known to signal at the level of the brain particularly the hypothalamus [65, 66]. The elevated levels of free fatty acid seen in obesity most

probably alter the hypothalamic neuronal environment significantly to cause various pathological changes seen in obesity.

Hypothalamic fatty acid sensing has been studied under physiological concentrations both in vitro and in vivo[82-84]. Fatty acid levels have a profound influence on feeding behavior and central administration of oleic acid was found to inhibit feeding behavior[84]. It is also interesting to note that free fatty acid signaling at the hypothalamus can have direct peripheral effects. One study showed that central fatty acid signaling was important in the regulation of hepatic glucose fluxes and glucose homeostasis[85]. Thus there appears to be considerable evidence to support the role of central free fatty acids in energy metabolism.

Neuronal responses to free fatty acids have also been demonstrated directly. Isolated groups of neurons from the arcuate nucleus and the ventromedial hypothalamus respond to extracellular free fatty acid levels[66, 82]. Moreover, neurons important in the feeding circuitry like POMC neurons were shown to be directly modulated by free fatty acids[86]. However, the mechanism of fatty acid signaling in the hypothalamus has not been explored completely.

F. Cellular mechanisms of fatty acid signaling

Fatty acid signaling has been well characterized in the periphery. Numerous protein acceptor molecules have been identified that can bind to free fatty acids. At the level of the plasma membrane, fatty acid binding receptors belonging to the G protein coupled receptor family have been identified of which

GPCR40 has gained attention[87]. Fatty acid transport proteins (FATPs) and fatty acid binding proteins (FABPs) are also recognized as crucial to the transmembrane signaling of free fatty acids[88]. But the most widely studied molecules are the nuclear receptors belonging to the peroxisome proliferated receptor (PPAR) group, mainly because of their direct effect on transcription of various genes. The latter are a group of nuclear receptors that are further classified into alpha, beta/delta and gamma subtypes. PPAR alpha, beta/delta are expressed in a wide variety of tissue and their function is mainly regulation of energy homeostasis. PPAR gamma is almost exclusively expressed in the adipose tissue and is involved in adipocyte differentiation and other adipocyte functions[89].

G. PPAR alpha

Long chain fatty acids, including oleic acid, have been demonstrated to activate PPAR alpha[90, 91]. PPAR alpha expression has also been demonstrated in the brain[92]. PPAR alpha is a transcriptional regulator of a number of genes involved in lipid oxidation, fatty acid transport and many other metabolic processes[93, 94]. It plays a prominent role in the liver as well as the intestines[94] in the regulation of fat metabolism. Brain PPAR alpha activation is known to be impaired when glucose levels are altered and also in PPAR knockout animals that are subjected to fasting[95]. Thus there is a huge potential for PPAR alpha to act as a signaling molecule for fatty acids in the brain.

H. Downstream pathways of fatty acid signaling

Free fatty acid binding molecules described above activate a variety of other downstream molecules to bring about their physiological effects. At the level of the pancreas, the K_{ATP} channels are important targets for free fatty acid-induced regulation of insulin secretion. This channel has been demonstrated in the central nervous system and possibly is a key player in the central regulation of glucose metabolism[85]. For the PPARs, which are nuclear transcription factors, the downstream targets are the regions of DNA that bind to these molecules and regulate transcription. These regions are termed as PPRE or Peroxisome Proliferator Activated Receptor Response Elements[90].

The most widely known functions of PPAR alpha are similar to other nuclear receptors. These receptors when activated by endogenous or exogenous ligands heterodimerize with retinoid X receptor (RXR); this dimer subsequently modulates transcription of specific genes via binding to PPREs. This is the classical genomic effect of PPARs[89, 96]. Some of the genes up regulated by PPAR alpha activation include the Carnitine palmitoyl transferase I and II (CPT), Acyl-CoA synthetase (ACS), Fatty acid binding protein (FABP), fatty acid translocase (CD36) and mitochondrial βoxidation enzymes[97]. These proteins ultimately lead to the uptake and utilization of free fatty acids by cells. Some of the genes down regulated by PPAR alpha include Fatty acid synthase (FAS) and acetyl-CoA carboxylase, which also leads to an overall decrease in free fatty acids[97]. Thus PPAR alpha plays an important role in fat mobilization and utilization.

IV REGULATION OF HYPOTHALAMIC NEURONS BY CATECHOLAMINES

A. Norepinephrine

Norepinephrine effects in the hypothalamus are most popularly associated with two neuroendocrine systems, the hypothalamo-pituitary-adrenal axis and the hypothalamo-pituitary-gonadal axis[98, 99]. Noradrenergic cell bodies are mainly located in the nucleus tractus solitaries (A1/A2) and locus ceruleus (A6) regions of the brain stem[98]. The effects of norepinephrine on hypothalamic neurons can best be described as modulatory rather than directly stimulatory or inhibitory[100]. The stress axis and CRH are intricately connected with the neuronal networks that regulate feeding behavior. Norepinephrine injection into the hypothalamus has been demonstrated to modulate feeding behavior[101]. The effects of norepinephrine on feeding have been stimulatory or inhibitory depending on the site of injection as well as the type of receptor stimulated. Moreover, norepinephrine also affects feeding behavior while interacting with other neurotransmitters such as dopamine[102]. Thus norepinephrine exerts a significant albeit complex effect on the neuroendocrine hypothalamus in the control of feeding behavior. This is also reflected in the fact that many antiobesity drugs also contain a norepinephrine reuptake inhibitor.

B. Dopamine

Dopamine is another significant neurotransmitter in the brain having a wide range of localization and functions. The mesolimbic dopaminergic system which is the centre for motivational behavior is of great interest in the study of the

behavioral changes leading to diet-induced obesity[73]. Altered responses in this region would explain why obese individuals are motivated to ingest highly palatable foods irrespective of the satiety status of the body[71]. Hypothalamic dopamine is also an important player in feeding behavior. In a diet-induced obese mouse model, high fat diet increased the expression of genes that increased dopamine availability in the hypothalamus. Therefore, a sustained reward effect to feeding might exist in these mice leading to over-consumption of feed and increased weight gain[103]. Differential expression of dopamine receptor levels were also seen in various brain regions, including the hypothalamus in diet-induced obese mice[104]. Although not as well characterized as the mesolimbic dopaminergic system, hypothalamic dopamine also appears to play a crucial role in regulating feeding.

C. Serotonin

Serotonin reuptake inhibitors are one of the most common types of antiobesity drugs available. Serotonin thus possibly plays a crucial role in regulating feeding behavior at the level of the brain. Serotonin agonists and pharmacological compounds that increase the bioavailability of serotonin are known to inhibit feeding behavior and reduce body weight [49]. Specific subtypes of serotonin receptors localized in the satiety related centers of the brain have been implicated in the effect of serotonin on feeding behavior and energy balance[105]. Serotonin has been shown to exert its effects in hypothalamic regions of the PVN, VMH, suprachiasmatic nucleus and the dorsomedial nucleus[106]. Thus

changes in serotonin levels in the hypothalamus can substantially affect feeding behavior.

D. Potential regulatory role of monoamines in feeding circuits

Monoamine neurotransmitters, which include both catecholamines, (norepinephrine and dopamine) and indoleamines (serotonin) in the brain, have pleiotrophic effects. The hypothalamus like the other brain systems is also a site for the actions of these regulatory molecules. In general, norepinephrine and serotonin in the brain induce satiety and therefore the drugs that inhibit their reuptake have been a good choice for the treatment of obesity[107]. Dopamine is slightly different in its effects in that it affects feeding behavior based on motivation and reward characteristics[107]. In general, dopamine reuptake inhibitors are also used to treat obesity. However, it may not be safe to generalize their effects as their actions probably vary among various brain regions and different physiological conditions. Monoamines have been demonstrated to induce satiety in the perifornical region of the hypothalamus which is also expected to be a downstream target for the first order neurons in the arcuate nucleus[108].

Hypothesis behind this study

Considering the significant role that monoamines play in the brain as a whole and specifically the hypothalamus, we were interested in studying the changes in monoamine neurotransmitters in the hypothalamus in response to free fatty acids. We were also interested in understanding if there were significant

changes in this response in diet-induced obesity. In order to get a more complete picture, we also wanted to explore the possible cellular mechanisms by which free fatty acids could induce changes in neurotransmission. The brain PPAR alpha appeared to be a good candidate for this purpose. PPAR alpha binds to free fatty acids, particularly with high affinity to oleic acid. Moreover, PPAR alpha has been demonstrated at the level of the hypothalamus to regulate feeding behavior in concert with the fatty acid responsive enzymes[92]. One possible mechanism by which these fatty acid responsive systems can affect feeding behavior is through changes in monoamine neurotransmitters. Therefore, we hypothesized that free fatty acids, oleic acid in particular can stimulate the release of monoamines from the hypothalamus in vitro. We chose to use oleic acid in our experiments because serum levels of oleic acid are significantly elevated after feeding a high fat diet when compared to any other free fatty acid. We also hypothesized that PPAR alpha plays a role in mediating the effect of oleic acid on hypothalamic neurotransmitter release.

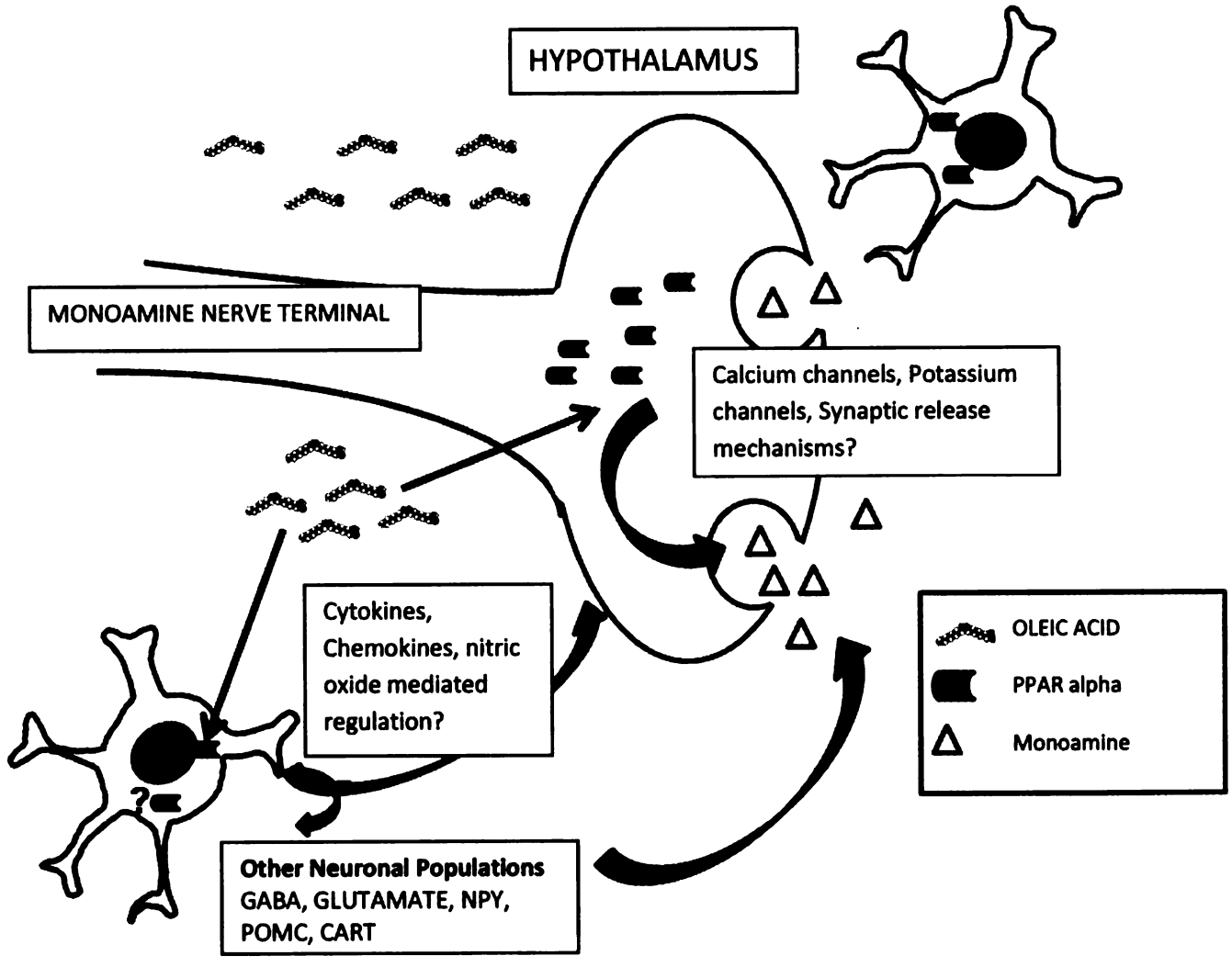

Fig 1-1. Hypothesis

Chapter 2

Materials and Methods

Animals

Adult male Sprague Dawley rats obtained from Harlan Sprague-Dawley, Indianapolis, IN were used in experiment 1. Diet induced obese (DIO) were purchased from Charles River Laboratories, Wilmington, MA, USA. They were bred in our colony. Male DIO rats were used in experiment 2 and were compared with Sprague Dawley rats. All animals were housed in temperature controlled (23±2°C) and light controlled (lights on from 0700 to 1900 hours) environment and maintained on *ad libitum* water and regular rat chow. Animals were used in the experiments in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee at MSU. Oleic acid complexed with BSA was obtained from Sigma Aldrich (St. Louis, MO) and MK 886 (PPAR alpha specific antagonist) was obtained from Cayman Chemicals (Ann Arbor, MI).

On the day of the experiment, the animals were weighed and randomly allocated to different treatment groups. The animals were sacrificed between 1000 to 1100 hours by rapid decapitation and their hypothalami were quickly dissected out. The boundaries of the hypothalamus included the optic chiasm as the anterior limit and the mamillary bodies as the posterior limit and the lateral hypothalamic sulci as the lateral limits as previously described [109].

In vitro incubation of hypothalamus

The hypothalamic tissue was weighed and incubated in a physiological solution of Krebs's Ringers Henseleit (KRH) in an *in vitro* incubation system as described before [109]. The hypothalamus was bisected sagitally into two halves and incubated in a plastic tube containing 300 µl of KRH. The incubation medium, KRH, consisted of 117m NaCl, 4.7 mM KCl, 1.2 mM of MgSO4, 1.2 mM of KH2PO4, 2.5 mM of CaCl2, 24.8 mM NaHCO3, 11.1 mM of glucose; pH 7.4 which was maintained at 37 °C in a water bath. A mixture of 95% O_2 and 5% CO_2 was bubbled through the tubes throughout the incubation. The incubation medium was collected every hour for 5 consecutive periods of time. During the first hour the medium was discarded as a prewash. The incubation medium from the second hour was collected to measure the basal neurotransmitter release. At the end of the second hour, the KRH solution was replaced with KRH containing 0.00132, 0.132 and 1.32 mM oleic acid, 50 µM MK 886 or 1.32mM oleic acid + 50 µM MK 886. The medium was then collected after one hour. The fourth incubation was again done using KRH alone to look for residual effects of treatment. During the final hour of incubation, the medium was replaced with high K^+ KRH to check the viability of the tissue. The media collected from various incubations were stored with the addition of 15µl of 0.1 M perchloric acid at -70°C until further analysis using High performance liquid chromatography with electrochemical detection (HPLC-EC).

HPLC with Electrochemical Detection

The monoamines in the incubation medium were measured using HPLC-EC method which has been described before [110]. The HPLC system consisted of a LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA), a phase II, 5 mm DS reverse phase, C-18 column (Phenomenex, Torrance, CA, USA), a glassy carbon electrode, a CTO-10 AT/VP column oven, and an LC-10 AT VP pump (Shimadzu,Columbia, MD, USA). The composition of the mobile phase was monochloroacetic acid (14.14g/L), octane sulfonic acid disodium salt (0.3 g/L), sodium hydroxide (4.675g/L), ultrapure EDTA (0.25g/L) , acetonitrile (35ml/L) and Tetrahydrofuran (5ml/L) in nanopure water. The solution was filtered through a 0.22µm filter through a Milli-Q purification system ((Millipore Co., Bedford, MA, USA) and degassed for 30 minutes. The mobile phase was pumped through the HPLC system at a constant flow rate of 1.8 ml/min. The sensitivity of the electrode was 1 nA and applied potential was 0.65 V. The column oven was maintained at a temperature of 37°C in which both the column and the working electrode were placed. Dihydroxybenzylamine (DHBA; 0.05M) was used as an internal standard for measuring the monoamines. At the time of analysis, 30 µl of DHBA standard was added to 60 µl of the incubation medium diluted with another 30 µl of 0.1 M perchloric acid. 100 µl of this mixture was injected into the HPLC system by the autoinjector during each run. Chromatograms were analyzed using the Chromatopac Class VP software (version 7.2) (Shimadzu, Columbia, MD). Neurotransmitter levels were expressed as pg/mg hypothalamus wet weight.

Statistical Analysis

To evaluate the viability of the hypothalamic tissue, it was first determined using ANOVA if high potassium KRH caused a significant increase in neurotransmitter release when compared to other incubations. The difference in neurotransmitter release between different periods and also between different treatment groups were analyzed using repeated measures ANOVA followed by Fisher's Least Significant Difference (LSD) test.

Chapter 3

In Vitro Effects of Oleic Acid on Neurotransmitter Release from the Rat Hypothalamus – Possible Mechanisms

I. Introduction

An elevated level of circulating free fatty acids is a common feature of obesity and the metabolic syndrome[78]. These free fatty acids are known to cross the blood brain barrier and exert their effects on brain regions such as the hypothalamus[63]. It is of great interest to understand how high levels of circulating free fatty acids affect neuronal circuits, particularly ones that regulate energy metabolism. Oleic acid administered centrally has the potential to inhibit feeding[84]. Thus under normal physiological conditions, high levels of circulating lipids probably act as a signal to the brain to inhibit further feeding and utilize the existing energy sources appropriately. However, when this homeostatic mechanism is impaired it may contribute to pathologies like obesity and metabolic syndrome.

The hypothalamus is the seat of major neuronal networks controlling energy homeostasis[27], although other brain regions are also involved in the process. Free fatty acids have been demonstrated to have direct effects at the level of the hypothalamus[111]. Thus there appears to be necessary molecular mechanisms in the hypothalamus to bind to circulating free fatty acids and sense the changes in circulating free fatty acid levels. This is particularly interesting because free fatty acids are not an important source of energy for the brain. Thus apart from being an integral part of the cellular structures in the brain, free fatty acids have a great potential to act as signaling molecules.

Oleic acid is one of the most highly elevated fatty acid in the circulation. It is also one of the most highly abundant fatty acid in the human diet[79]. The effects of oleic acid on the hypothalamus have been studied to some extent [66, 83, 84, 86]. However, the effects of free fatty acids on monoamines have not been explored at the level of the hypothalamus. Hence, we were interested in studying the possible effects of various doses of oleic acid on norepinephrine, dopamine and serotonin levels in the hypothalamus.

II. Results

Effect of different doses of oleic acid on Norepinephrine Release from the rat hypothalamus *in vitro*

The effect of oleic acid on norepinephrine efflux from the hypothalamus is shown in fig 3-1. There was a significant increase in norepinephrine release during the 4th incubation period where hypothalami were exposed to high K+ KRH. This has a depolarizing effect on the hypothalamus and causes a complete release of neurotransmitter release from the terminals. The marked increase in norepinephrine release that was observed during the 4th incubation period ($p<0.0001$) suggests that the hypothalami were viable throughout the experiment.

There was no significant difference in norepinephrine release during the first incubation period between hypothalami from different treatment groups. In

period 2, addition of 1.32 mM oleic acid stimulated norepinephrine release (Mean±S.E; pg/mg hypothalamus) significantly into the incubation medium (115.008±22.927; $p<0.0001$) when compared to the control (8.718±1.888), 0.00132mM Oleic acid (7.296±1.685) and the 0.132mM oleic acid (38.542±19.892) groups. There was a trend for norepinephrine release to increase with higher doses of oleic acid however this was not statistically significant. Incubation with high K+ KRH, produced a much lower release of norepinephrine in the high dose oleic acid group (130.624±17.245) compared to the rest of the groups.

Effect of different doses of oleic acid on Dopamine Release from rat hypothalamus

Dopamine release from the rat hypothalamus in response to different doses of oleic acid is shown in fig 3-3. Dopamine release was increased significantly after stimulation with high K+KRH indicating that the hypothalami were viable during the previous 3 incubation. There was no change in dopamine release between the different incubations in the control group. High dose of oleic acid (1.32mM) produced a significant increase in dopamine release (pg/mg hypothalamus; Mean±S.E) of 39.495±13.012 during the second incubation which was significantly higher than the control (5.263±1.663; $p<0.0011$), low dose (2.951±1.796) and the medium dose (2.587±1.019) of oleic acid. There were no residual effects on dopamine release during the third incubation period.

Effect of different doses of oleic acid on Serotonin Release from the rat hypothalamus

Serotonin release followed a pattern similar to the other monoamines and increased significantly after high K^+ stimulation when compared to the first 3 periods ($p<0.001$; Fig 3-5). During period 2 among various treatments, high dose of oleic acid stimulated serotonin release (Mean±S.E; pg/mg hypothalamus; 47.624±7.370) when compared to the control group ($p<0.0001$). In this group stimulation with high K^+KRH produced only a modest increase in serotonin release (16.223±0.745) when compared with the control ($p<0.0462$). During period 3 there was no significant change in serotonin release among different groups.

DOPAC and 5HIAA levels in response to various doses of oleic acid

Although stimulation with high K^+ KRH produced a significant increase in DOPAC release (Mean±S.E.; pg/mg hypothalamus) in all the treatment groups, incubation with the high dose of oleic acid in the second period, lead to significantly lower efflux of DOPAC into the incubation medium (47.464±12.265) when compared to the control group (190.063±59.897; $p<0.04$). There were no significant changes in the release of DOPAC with the other 2 doses of oleic acid (fig 3-7).

5HIAA release appeared to be higher compared to DOPAC during all four incubations (fig 3-8). During the second period, high dose of Oleic acid caused a

significant increase in 5HIAA release (Mean±S.E; pg/mg hypothalamus; 1016.652±192.929) when compared to the control group (306.668±78.747; $p<0.0013$). During high K^+ KRH stimulation, the release of 5HIAA into the medium was highly variable between the different groups.

Effect of PPAR alpha antagonist on neurotransmitter release from rat hypothalamus

To explore the possible mechanism of oleic acid-induced changes in neurotransmitter release in vitro, the hypothalami were treated with the PPAR alpha antagonist MK 886. Any effects of MK 886 by itself were studied by incubating the hypothalamus with the antagonist alone. MK 886 alone did not cause any change in the release of norepinephrine, dopamine, serotonin, DOPAC or 5HIAA from the hypothalamus during the second incubation period. In contrast, when the high dose of oleic acid was co-incubated with MK 886, norepinephrine release was reduced to control levels (Fig 3-2). Norepinephrine release (Mean±S.E.; pg/mg hypothalamus) during the second period with the high dose of oleic acid was 115.008±22.927 and this effect was blocked by co-treatment with MK 886 (13.232±3.207; $p < 0.0001$).

Dopamine release (Mean±S.E.; pg/mg hypothalamus) was also significantly reduced ($p<0.0045$) by the combination of oleic acid and MK 886 treatment (10.862±5.623) when compared with high dose oleic acid alone (39.495±13.012; Fig 3-4). Addition of MK 886 to the medium brought down serotonin release (Mean±S.E.; pg/mg hypothalamus) following high dose of oleic acid from

47.624±7.370 to 5.648±2.066 ($p < 0.0001$; Fig 3-6). There were no significant changes in the release of 5-HIAA between the different treatment groups (Fig 3-8). While high dose of Oleic acid significantly reduced DOPAC concentrations, treatment with the combination of Oleic acid and MK886, prevented this decrease, bringing oleic acid levels back up to that of the control group (Fig 3-7).

Fig 3-9 depicts the differences in the release of the three monoamines, norepinephrine, dopamine and serotonin in response to incubation with the different doses of oleic acid. The most dramatic changes were observed in norepinephrine efflux compared to serotonin and dopamine. The effects of these three neurotransmitters were completely blocked by incubation with MK 886, the PPARαantagonist.

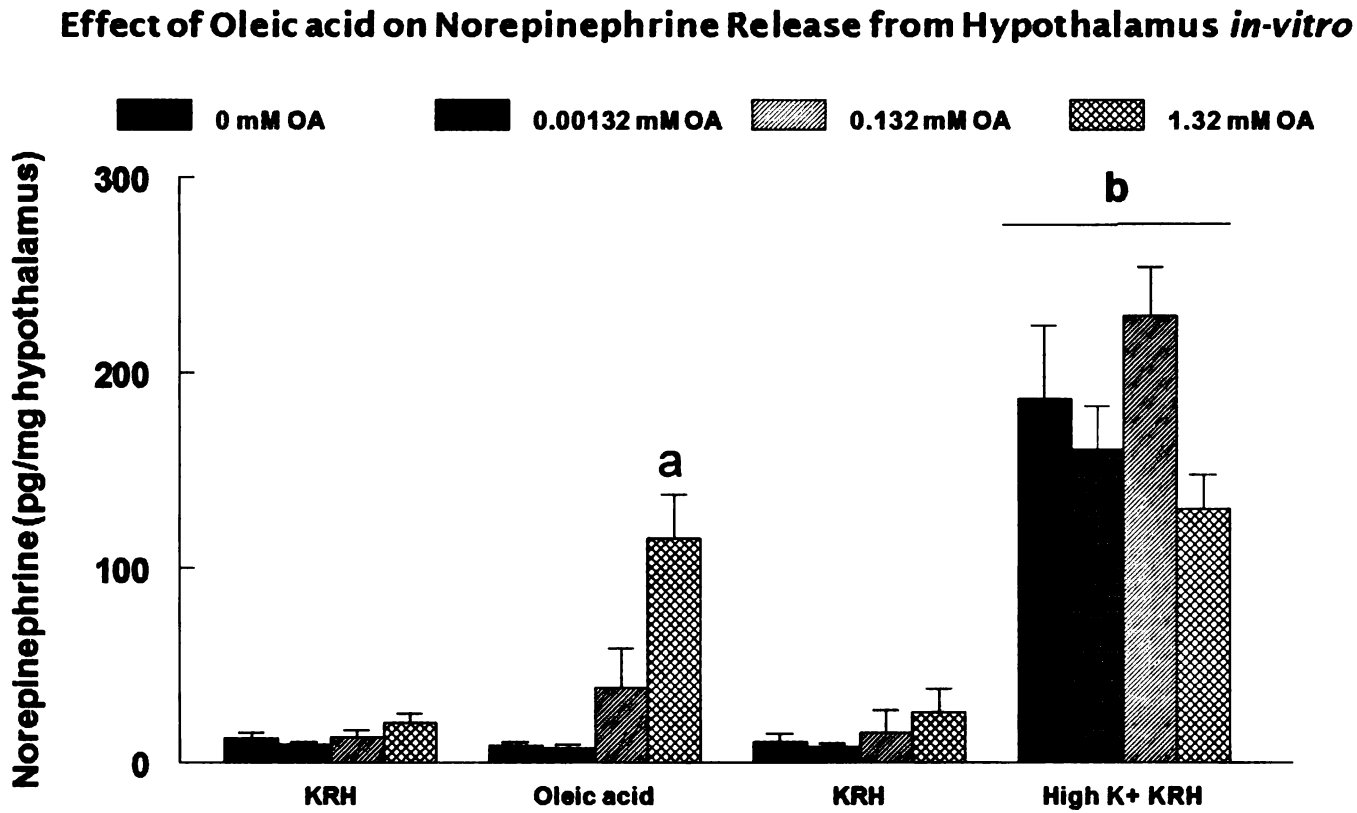

Fig. 3-1. Effects of incubation of hypothalami with different doses of oleic acid (OA) on Norepinephrine release. Hypothalami were incubated in KRH in an atmosphere of 95% O2 and 5% CO2 for 1-hr incubation periods. Hypothalami were incubated with KRH; 0, 0.00132, 0.132 or 1.32 mM/L of oleic acid; KRH alone and high potassium KRH in that order. As shown in the figure, OA increased NE efflux in a dose-dependent manner. **a** $p<0.01$ compared to KRH alone , **b** $p<0.01$ compared to other incubations with KRH alone. (n=4-5)

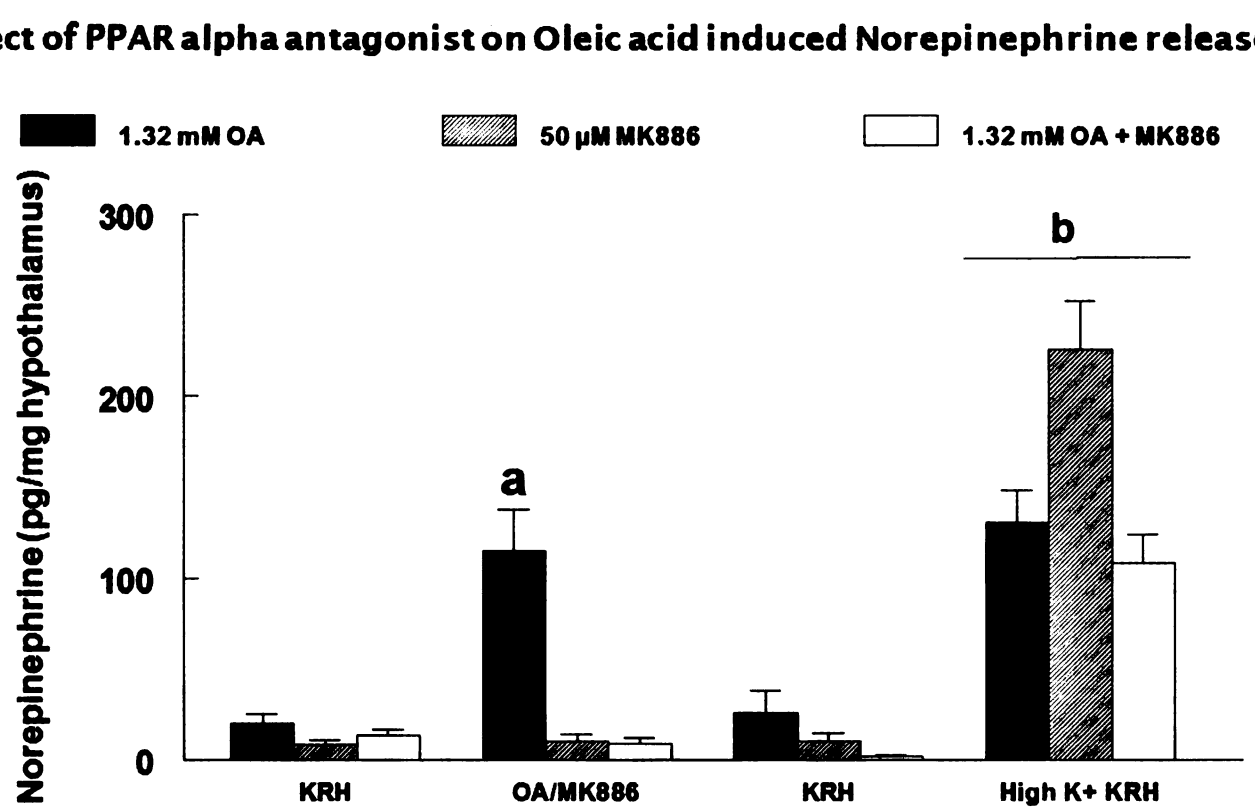

Fig. 3-2. Effects of incubation of hypothalami with MK886, a PPARαantagonist and 1.32 mM of oleic acid. Hypothalami were incubated in KRH in an atmosphere of 95% O_2 and 5% CO_2 for 1-hr incubation periods. Incubations included KRH; 1.32 mM/L of oleic acid or MK 886 (50 µM) or a combination of MK886 (50 µM) and 1.32 mM of oleic acid and high potassium KRH. As shown in the figure, MK 886 by itself did not affect NE efflux, incubation of hypothalami with a combination of MK886 (50 µM) and 1.32 mM of oleic acid completely blocked OA acid-induced increase in NE efflux, $p<0.01$ compared to KRH alone (n=4-5)

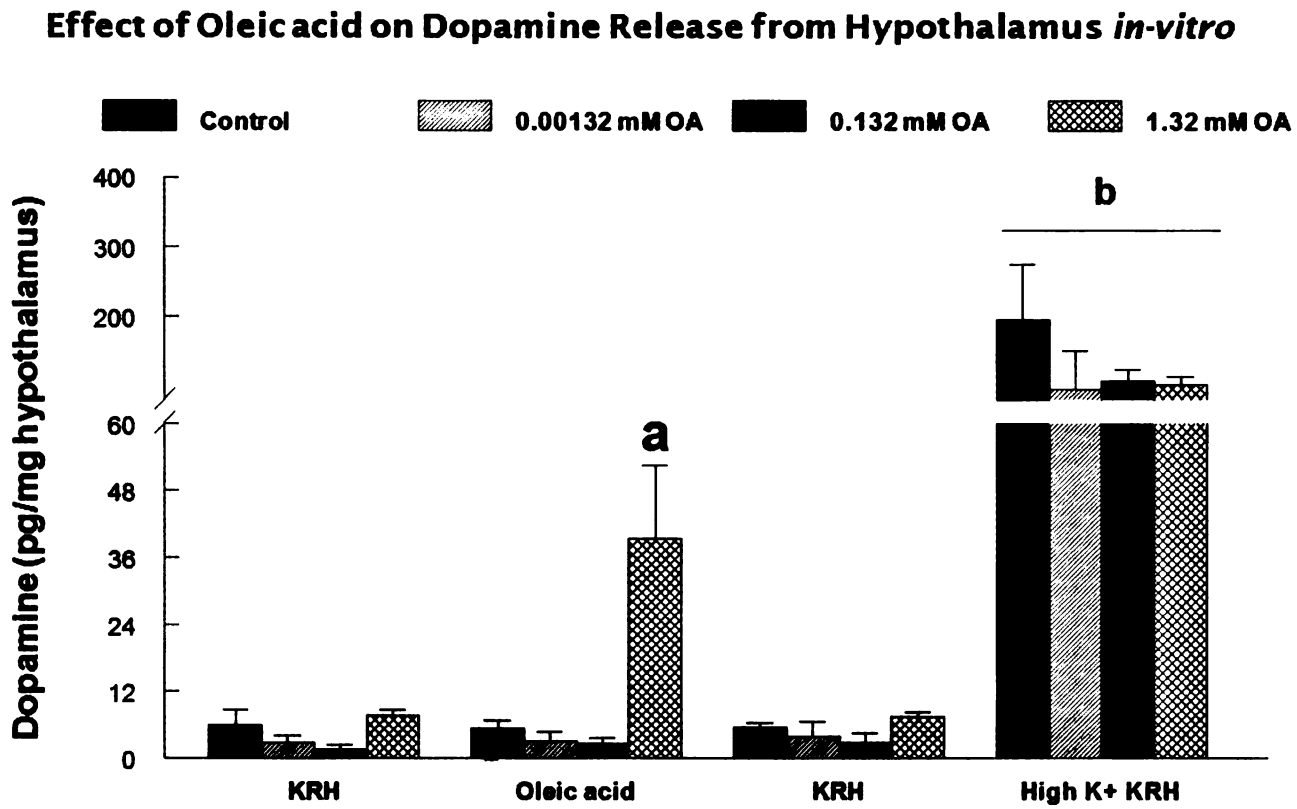

Fig. 3-3. Effects of incubation of hypothalami with different doses of oleic acid (OA) on release. Hypothalami were incubated in KRH in an atomosphere of 95% O2 and 5% CO2 in 4 1-hr incubation periods. Hypothalami were incubated with KRH; 0, 0.00132, 0.132 or 1.32 mM/L of oleic acid; KRH alone and high potassium KRH in that order. As shown in the figure OA increased NE efflux in a dose-dependent manner. **a** $p<0.01$ compared to KRH alone , **b** $p<0.01$ compared to other incubation 1 with KRH alone. (n=4-5)

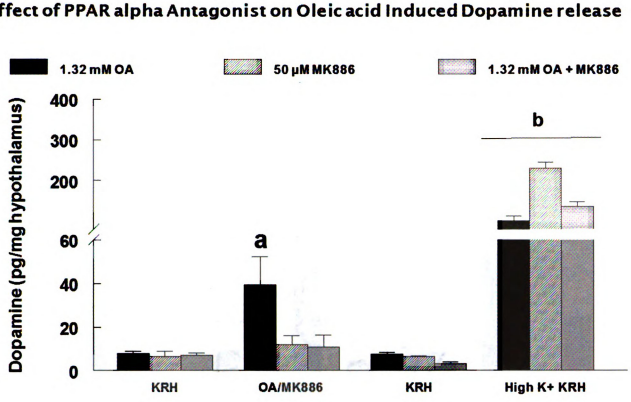

Fig. 3-4. Effects of incubation of hypothalami with MK886, a PPARαantagonist and 1.32 mM of oleic acid. Hypothalami were incubated in KRH in an atmosphere of 95% O_2 and 5% CO_2 for 1-hr incubation periods. Incubations included KRH; 1.32 mM/L of oleic acid or MK 886 (50 µM) or a combination of MK886 (50 µM) and 1.32 mM of oleic acid and high potassium KRH. As shown in the figure, MK 886 by itself did not affect DA efflux, incubation of hypothalami with a combination of MK886 (50 µM) and 1.32 mM of oleic acid completely inhibited OA acid-induced increase

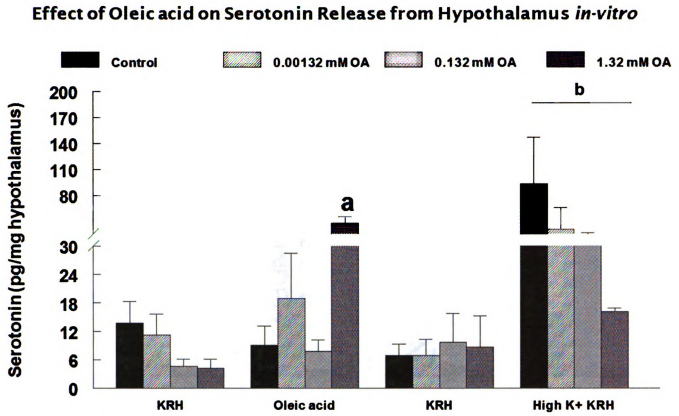

Fig. 3-5. Effects of incubation of hypothalami with different doses of oleic acid (OA) on Serotonin release. Hypothalami were incubated in KRH in an atmosphere of 95% O2 and 5% CO2 for four 1-hr incubation periods. Hypothalami were incubated with KRH; 0, 0.00132, 0.132 or 1.32 mM/L of oleic acid; KRH alone and high potassium KRH in that order. As shown in the figure, OA increased 5HT efflux at high doses. **a** $p<0.01$ compared to KRH alone . **b** $p<0.01$ compared to other incubations with KRH alone. (n=4-5)

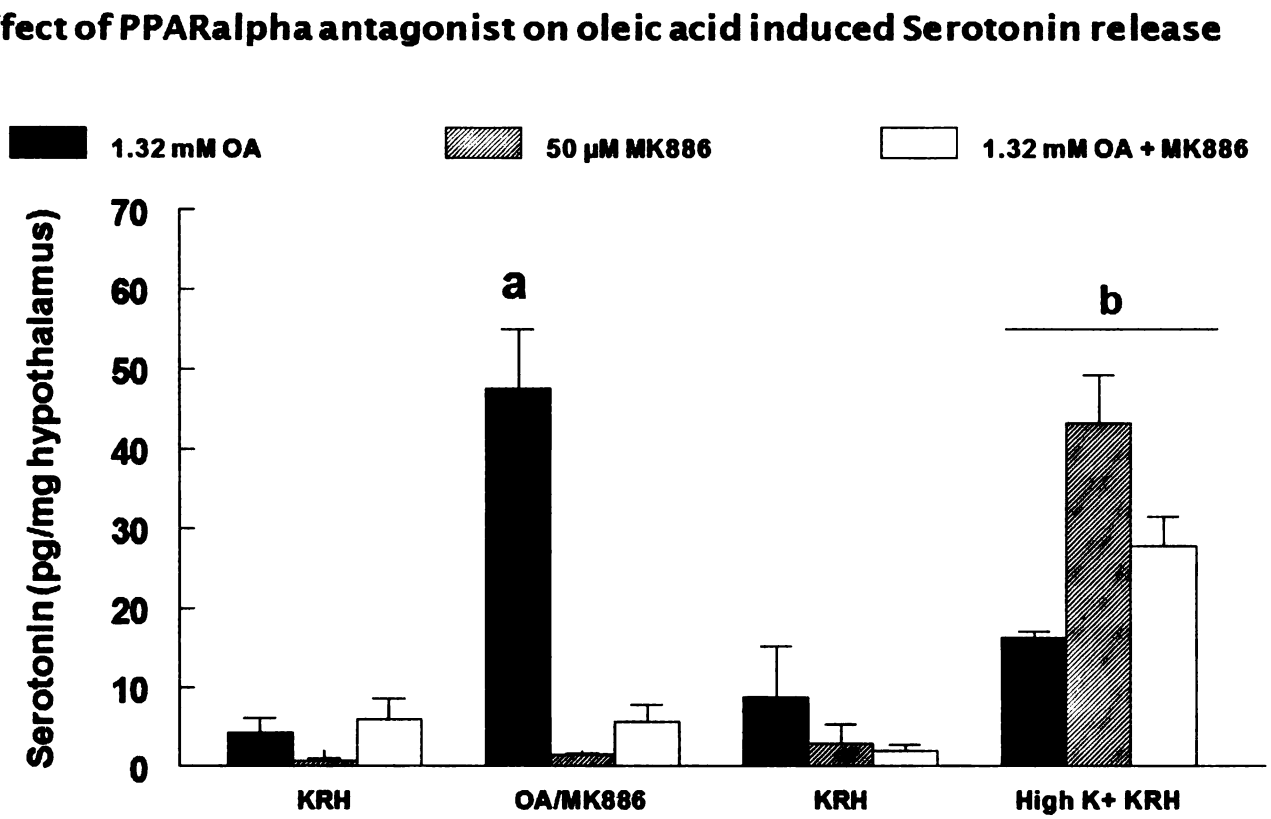

Fig.3-6. Effects of incubation of hypothalami with MK886, a PPARαantagonist and 1.32 mM of oleic acid on serotonin efflux. Hypothalami were incubated in KRH in an atomosphere of 95% O_2 and 5% CO_2 in 4 1-hr incubation periods. Incubations included KRH; 1.32 mM/L of oleic acid or MK 886 (50 µM) or a combination of MK886 (50 µM) and 1.32 mM of oleic acid and high potassium KRH. As shown in the figure MK 886 by itself did not affect 5HT efflux, incubation of hypothalami with a combination of MK886 (50 µM) and 1.32 mM of oleic acid completely inhibited OA

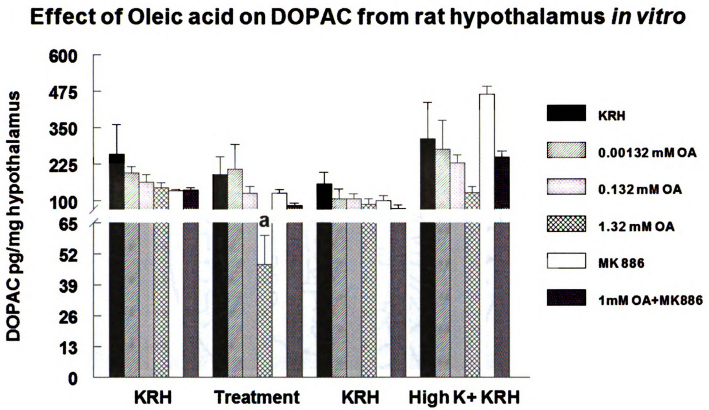

Fig.3-7. Effects of incubation of hypothalami with different doses of oleic acid (OA) on DOPAC levels in the incubation medium. Hypothalami were incubated in KRH in an atomosphere of 95% O2 and 5% CO2 for four consecutive 1-hr incubation periods. Hypothalami were incubated with KRH; 0, 0.00132, 0.132 or 1.32 mM/L of oleic acid; KRH alone and high potassium KRH in that order. **a** $p<0.01$ compared to KRH alone (n=4-5)

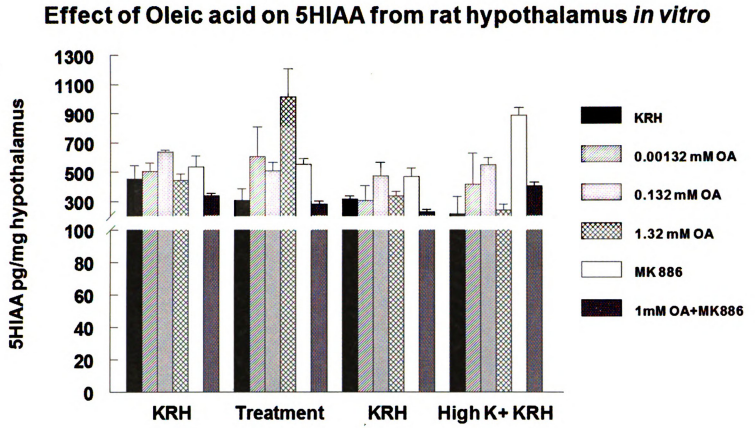

Fig. 3-8. Effects of incubation of hypothalami with different doses of oleic acid (OA) on 5HIAA levels in the incubation medium. Hypothalami were incubated in KRH in an atmosphere of 95% O2 and 5% CO2 for four 1-hr incubation periods. Hypothalami were incubated with KRH; 0, 0.00132, 0.132 or 1.32 mM/L of oleic acid; KRH alone and high potassium KRH in that order.

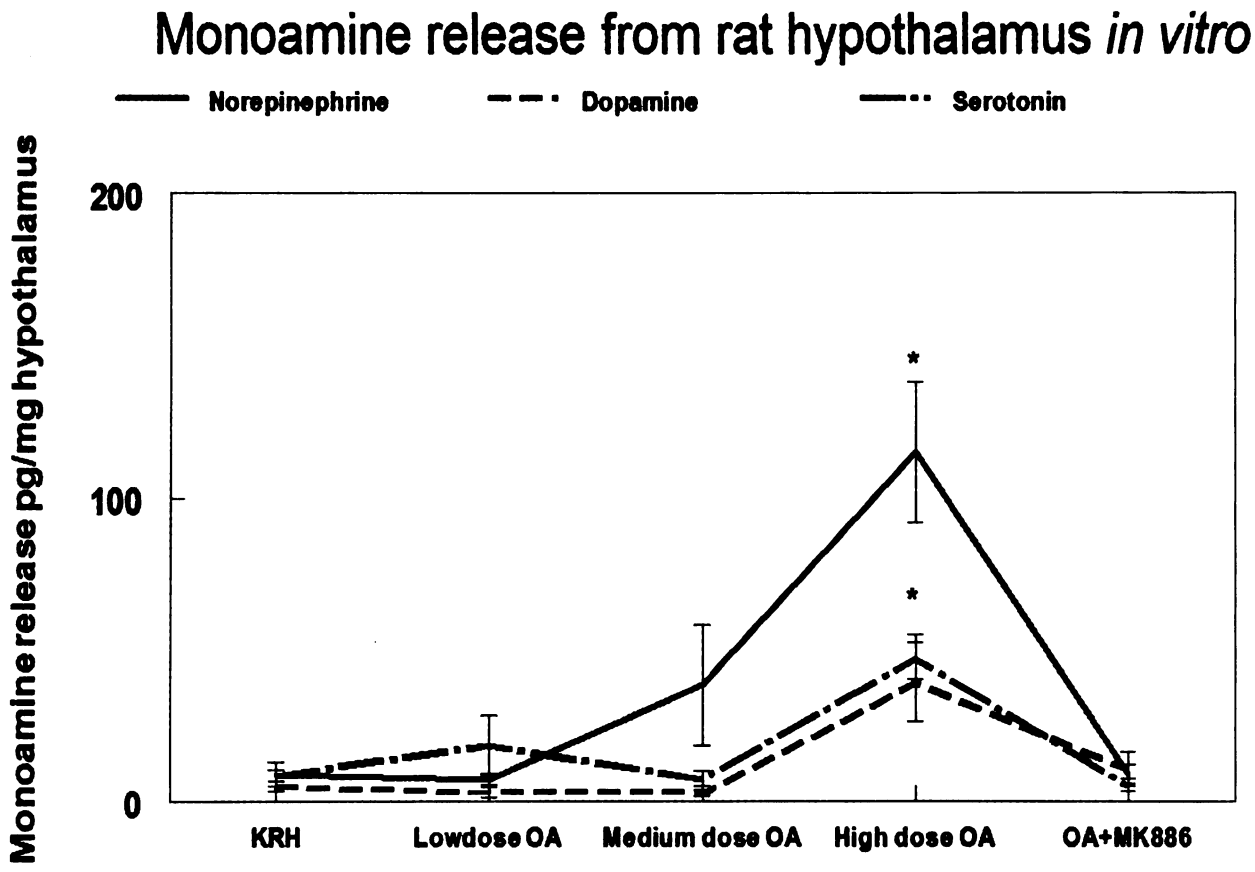

Fig 3-9. Comparison of the dose response of monoamine neurotransmitters released *in vitro* to different doses of oleic acid, the incubation medium KRH alone and a combination of high dose of oleic acid and MK886. As shown, maximal release was evident with norepinephrine. * indicates

III Discussion

Among the monoamine neurotransmitters, the release of norepinephrine from the isolated rat hypothalamus *in vitro* showed the greatest change in response to oleic acid treatment. High dose of oleic acid produced a significant increase in norepinephrine release when compared to controls group treated with KRH alone. Although medium dose did not produce a significant change compared to controls, there was an increasing trend from the low dose to the high dose indicative of a dose response. Catecholamines injected into regions of the hypothalamus including perifornical hypothalamus were found to be potent suppressors of feed intake even in hungry rats[112]. Interestingly, the same catecholamines injected into the paraventricular nucleus of the hypothalamus were shown to increase feed intake[101]. However, the well known anorectic agent, amphetamine is a potent activator of catecholamine release[113, 114]. Hence it is possible that the overall effect of catecholamine release in the hypothalamus would be in fact to reduce feed intake than increase it. In that context the increased norepinephrine release into seen following oleic acid possibly in a dose dependent manner, could act as a homeostatic mechanism to indicate a surplus of energy. This role of fatty acids has been demonstrated before [64, 84] and fatty acids are considered as important signals for energy metabolism next to glucose. However, fatty acid induced change in monoamine release in the brain is a scarcely explored area.

With high K^+ KRH stimulation, there was a significant decrease in the norepinephrine release from the hypothalamus that was treated with high dose of oleic acid. This could be explained in the view of the neurotransmitter already released during the previous incubation. The *in vitro* incubation system we used provides for the mere maintenance of the tissue and cannot provide raw material for biosynthesis of norepinephrine in this case.

It was interesting to note that responses in dopamine and serotonin at high doses of oleic acid were similar to norepinephrine. Central dopamine is another important regulator of feed intake[115]. The dopamine neurons extending from the ventral tegmental area to the nucleus accumbens are the most widely studied with respect to feeding behavior as they are established centers for motivational behavior[73]. Dopamine deficient mice are aphagic and starve to death in 4 weeks[116]. But interestingly, in these animals, restoration of dopamine in the nigrostriatal dopaminergic system, rather than the mesolimbic dopaminergic system restored normal feeding[116]. Thus the exact mechanism of how dopamine regulates feeding is still not completely delineated. In the hypothalamus, the most prominent dopaminergic system is the tuberoinfundibular dopaminergic neurons that regulate prolactin secretion[117]. However, dopamine in the hypothalamus is also known to be involved in multiple other pathways including the feeding circuits[103]. Some studies clearly show that dopamine signaling in the hypothalamus is necessary for regulation of feeding and that this is impaired in obesity. Feeding was followed by a spontaneous decrease in monoamine levels in the hypothalamus which was blunted in the

obese animals[118]. Our study shows a similar increase in dopamine and furthermore it shows that these changes are in response to free fatty acids in circulation.

Serotonin is another important monoamine neurotransmitter involved in feeding behavior. It is generally thought of as a satiety promoting neurotransmitter like norepinephrine and to some extent dopamine. Serotonin levels in the hypothalamus like dopamine were also found to change spontaneously following feeding which is a natural mechanism promoting satiety[118]. Depletion of serotonin centrally produced profound hyperphagia and obesity in rats[119]. Conversely, the antiobesity drug sibutramine acts by enhancing the availability of serotonin centrally, particularly at the level of the hypothalamus[120]. The increases in serotonin seen in this study may hence act as a satiety signal in combination with norepinephrine and dopamine at the level of the hypothalamus. This may further be involved in inhibiting feed intake. However, live animal studies measuring feeding behavior must be performed to ascertain the role of fatty acid induced increases in hypothalamic monoamines in the control of feeding.

The metabolite of dopamine, DOPAC was significantly reduced in the incubation medium following high dose of oleic acid. However, changes in serotonin metabolite 5 HIAA did not show a consistent pattern worthy of interpretation. This is in concordance with another study in which the monoamines and their metabolites were measured from microdialysis in the hypothalamus of rats to look at the spontaneous response to feeding [121]. In this

study also, while dopamine increased with feeding, DOPAC levels reduced with feeding. The converse was true for 5 HIAA responses, which also showed an increased trend along with serotonin. Thus this pattern may be associated with satiety at the level of the hypothalamus, and free fatty acids may play a significant role in the whole process.

The release of all three monoamines was inhibited when high dose of oleic acid was combined with a PPAR alpha specific antagonist MK886[122]. Free fatty acids have been shown to act at the level of the hypothalamus through the activation of PPAR alpha in regulating feeding behavior[92]. Oleic acid in particular binds avidly to the PPAR alpha subtype of nuclear receptors than the other PPARs[123]. Hence it is highly likely that oleic acid induced effects involve the recruitment of the nuclear receptor PPAR alpha. However, there still remains a huge gap in our understanding of how the nuclear receptor could possibly affect neurotransmitter release. A number of possibilities can happen in this scenario. The first thing to consider is the localization of the PPAR molecules in this scene. PPAR alpha could be located in a neuronal site or even in other cells such as the glial cells. PPAR alpha has been demonstrated in cultured neurons[124] and also in microglial cells[125]. Thus PPAR alpha could directly act in the neurons synthesizing the neurotransmitters or can act on glial cells which are also known for their modulatory effect on the synaptic transmission in many regions of the brain[126].

Direct effects of PPAR alpha in an acute time frame as seen in this experiment possibly involve rapid mechanisms apart from the conventional

nuclear signaling. Indeed, there have been studies where a rapid non-genomic effect of PPAR alpha agonists have been noticed[96]. These have been mainly through the direct modulation of ion channels to influence neuronal activity. A similar modulation of monoamine release could be effected by PPAR alpha in the terminals. The modulation of neurotransmitter at the terminal makes more sense in this experiment as the effects were seen in isolated hypothalami. The cell bodies of most of the monoamine neurons studied are located well outside the hypothalamus. Whichever may be the mechanism of action, PPAR alpha in the regulation of monoamine neurotransmission in the hypothalamus presents an exciting new possibility in the field of obesity research.

Thus free fatty acids particularly oleic acid, can act at the level of the hypothalamus directly to modulate serotonin, dopamine and norepinephrine release in Sprague Dawley rats. This effect appears to be rapid and could possibly be mediated by the intracellular fatty acid binding receptor PPAR alpha, as specific antagonist to this receptor obliterated the response. Free fatty acid induced elevation in the hypothalamic monoaminergic system could act as an integral part of the complex events culminating in the maintenance of energy homeostasis.

Chapter 4

Effects of oleic acid on neurotransmitter release from hypothalamus of Diet induced obese (DIO) rat

I. Introduction

Obesity as seen in the modern world is mainly attributed to dietary factors. Hence diet-induced obesity has been the most studied problem in metabolic research. Diet-induced obesity also happens to be one of the most complex of all metabolic pathologies and still continues to develop new unforeseen dimensions giving opportunity for more research in this area. Diet-induced obese models in laboratory animals have been extremely helpful in understanding the pathophysiology of obesity. Initial models of diet-induced obesity were developed in mice but later on, the rat models have gained popularity. Different methods have been utilized to model obesity in rodents [16]. Single gene mutations occurring spontaneously in genes that are crucial in maintaining body weight have given rise to obesity models like the ob/ob, db/db and fa/fa deficient animals. The ob/ob is a leptin deficient mouse and the db/db is a leptin receptor deficient mouse model of obesity[127, 128]. These have been used widely to study the functions of the adipose tissue derived hormone, leptin [52]. The fa/fa rat is also known as the zucker rat, is deficient in a form of leptin receptor and shows many features resembling metabolic syndrome in humans[16].

Another type of laboratory animal model involves creating artificial mutations in rodents to produce obesity which include irradiation-induced changes as well as transgenic attempts to develop mutant mouse models[16]. Again, such models are more useful to study functions of a single protein rather than the evaluation of a complex pathology such as metabolic syndrome. The model that is considered closest to mimicking human diet-induced obesity is the

polygenic model of obese rats developed from common laboratory strains such as Wistar and Sprague Dawley rats[17].

A number of metabolic disturbances have been observed in these models of obesity both centrally and peripherally. Diet-induced obese rats and diet resistant rats differ in many metabolic features including feed intake, body weight, thermogenesis, motor activity and insulin sensitivity[18]. The pathophysiological bases for these are yet to be delineated clearly. There have however been some important clues from some experiments done with these animals. Obesity prone rats were found to have significant differences in their monoamine turnovers particularly that of serotonin in the hypothalamus[129]. Hypothalamic neurons from these animals characteristically have reduced leptin sensitivity[130]. There was also a significant reduction in neuronal projections from the arcuate nucleus to the PVN and also decreases in feeding-related peptide neurotransmitters[131]. Although research into the possible mechanisms of development of this phenotype has continued, the condition still remains poorly understood.

These diet induced obese animals tend to gain more weight than control animals when placed on a chow diet. A high fat diet produces a greater increase in weight over and above that produced in response to a chow diet. The diet resistant animals on the other hand fail to put on weight significantly even after a high fat diet, and require highly concentrated forms of high fat diet to actually make them gain significant amount of fat tissue[18]. The diet induced obese animals demonstrate an increase in circulating leptin levels both when on a chow

diet as well as when they are fed with a high fat diet. They also develop insulin resistance and have elevated circulating insulin levels. Other metabolic abnormalities include increased blood glucose, plasma triglycerides, plasma free fatty acids and cholesterol. They also showed had increased corticosterone levels in the circulation and have a hyperactive stress axis [53].

Although the name implies that DIO rats become obese upon feeding, changes in their neuronal responses have been demonstrated even before the animals are fed on a high fat diet[132]. Thus it is possible that anatomical and molecular differences in the brain circuitry and subsequently variable brain responses to nutrient molecules exist in obesity prone rats when compared to normal rats. Hence we replicated the same conditions under which the Sprague Dawley rat hypothalami were treated except that only the high dose of oleic acid was used since it showed the maximal response with respect to change in neurotransmitter release. We were interested in exploring the possibility of impaired fatty acid signaling at the level of hypothalamus as a potential factor in the pathogenesis of diet induced obesity.

II. Results

Diet induced obese (DIO) rats were found to be significantly heavier than the Sprague Dawley rats even on a chow diet. Body weight (Mean±S.E.; g) of DIO rats was 632.2±0.5 and was significantly higher than that of the Sprague Dawley rats used in the experiment (479.4±14.7; $p<0.05$).

Effects of high dose of oleic acid on norepinephrine release from the DIO rat hypothalamus *in vitro*

Effects of oleic acid on norepinephrine release from the DIO hypothalamus are shown in Fig 4-2. As in the previous experiment, the hypothalami were found to be viable at the end of the experiment by measuring the release of norepinephrine following high K^+ KRH. This was significantly higher than any of the other incubations ($p < 0.0001$). High dose of oleic acid (1.32mM) stimulated norepinephrine release (Mean±S.E.; pg/mg hypothalamus) from the DIO hypothalamus (43.715±8.839) when compared with incubation with KRH alone (27.050±13.25). When the hypothalami were incubated with the PPAR alpha antagonist MK 886 alone, norepinephrine release was 12.183±1.494. When oleic acid was combined with the antagonist, the response was inhibited significantly (20.667±5.592; $p<0.045$). There were no residual effects during the third incubation period. Norepinephrine release following high K^+ KRH stimulation was modest from hypothalami that were exposed to high dose of oleic acid in the second incubation period.

Changes in norepinephrine release in both DIO and Sprague Dawley rats are compared in Fig 4-7. While oleic acid produced a marked increase in norepinephrine release in both groups, it was much attenuated in DIO animals ($p<0.05$).

Effects of high dose of oleic acid on the release of Dopamine from DIO rat hypothalamus *in vitro*

Effects of oleic acid on dopamine release from DIO rat hypothalami are shown in fig 4-3. The DIO rat hypothalami were confirmed to be viable as there was increased DA release (Mean±S.E.; pg/mg hypothalamus) following high potassium KRH ($p<0.0001$). During period 2, there was no significant difference between dopamine released from control hypothalami (12.080±6.620) and hypothalami treated with high dose of oleic acid (7.035±2.466). Similarly, there was no change in dopamine release following treatment with MK 886 or a combination of MK886 and oleic acid.

Fig 4-8 shows the differences in dopamine release between Sprague Dawley and DIO rats. While oleic acid stimulated dopamine release from the hypothalami of Sprague Dawley rats, it did not affect dopamine release from the hypothalami of DIO rats.

Effects of high dose of oleic acid on the release of Serotonin from DIO rat hypothalamus *in vitro*

Fig 4-4 demonstrates the effects of oleic acid on serotonin release from the hypothalami of DIO rats. As with norepinephrine, high dose of oleic acid produced a significant increase in serotonin release (Mean±S.E.; pg/mg hypothalamus) from the hypothalamus during the second incubation period (21.135±11.765) when compared to KRH treatment alone (1.683±.673; $p<0.0295$). While MK 886 alone did not produce any significant difference in serotonin release (2.237±1.324) when compared with control animals, the combination of oleic acid and MK 886 increased serotonin release to 19.2±6.036. These levels were comparable to levels stimulated by the high dose of oleic acid and was significantly different from control animals ($p<0.0287$). There were no residual effects of different treatments in the third incubation period.

Fig 4-9 shows the effects of oleic acid from the hypothalami of DIO and Sprague Dawley rats. While it increased serotonin release from the hypothalami of Sprague Dawley rats, it did not have any effect on DIO rats. While the effect observed in Sprague Dawley rats was completely blocked by MK 886, it appeared to have no effect on DIO rats.

Effect of high dose of oleic acid on DOPAC and 5HIAA release from the hypothalamus in vitro.

There were no significant changes in DOPAC and 5HIAA levels in response to the different treatments in the second incubation period. Moreover,

the release of both DOPAC and 5HIAA remained higher compared to other neurotransmitters and therefore, there was no significant increase in the release of these metabolites after stimulation by high K^+ KRH (Figs. 4-5 and 4-6).

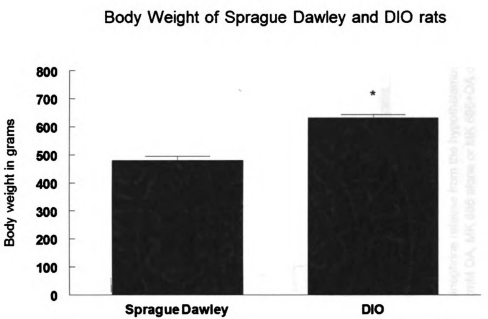

Fig 4-1 Body weight of animals used in the *in vitro* experiment. DIO animals were significantly heavier than Sprague Dawley animals. *p<0.01

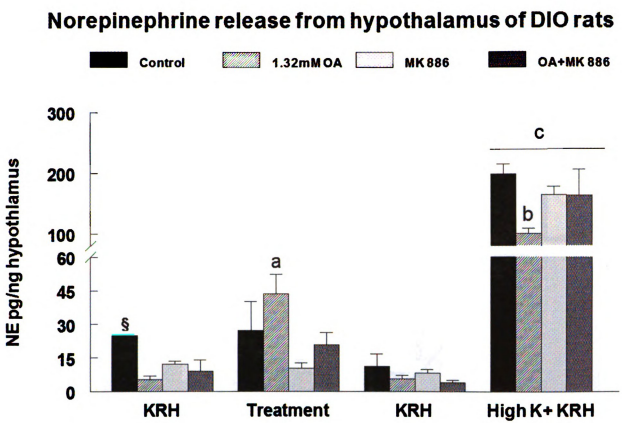

Fig 4-2. Effects of high dose of oleic acid on norepinephrine release from the hypothalamus of diet-induced obese rats. DIO rat hypothalami were incubated with 1.32mM OA, MK 886 alone or MK 886+OA during treatment period in an *in vitro* incubation system. As shown in the figure, high dose of oleic acid stimulated NE efflux which was reversed by the addition of the antagonist. a $p<0.01$ compared to MK886 and OA+MK886 groups, b $p<0.05$ compared to control during period 4, c $p<0.01$ compared to all other incubation periods. (n=4) § significantly different from other groups

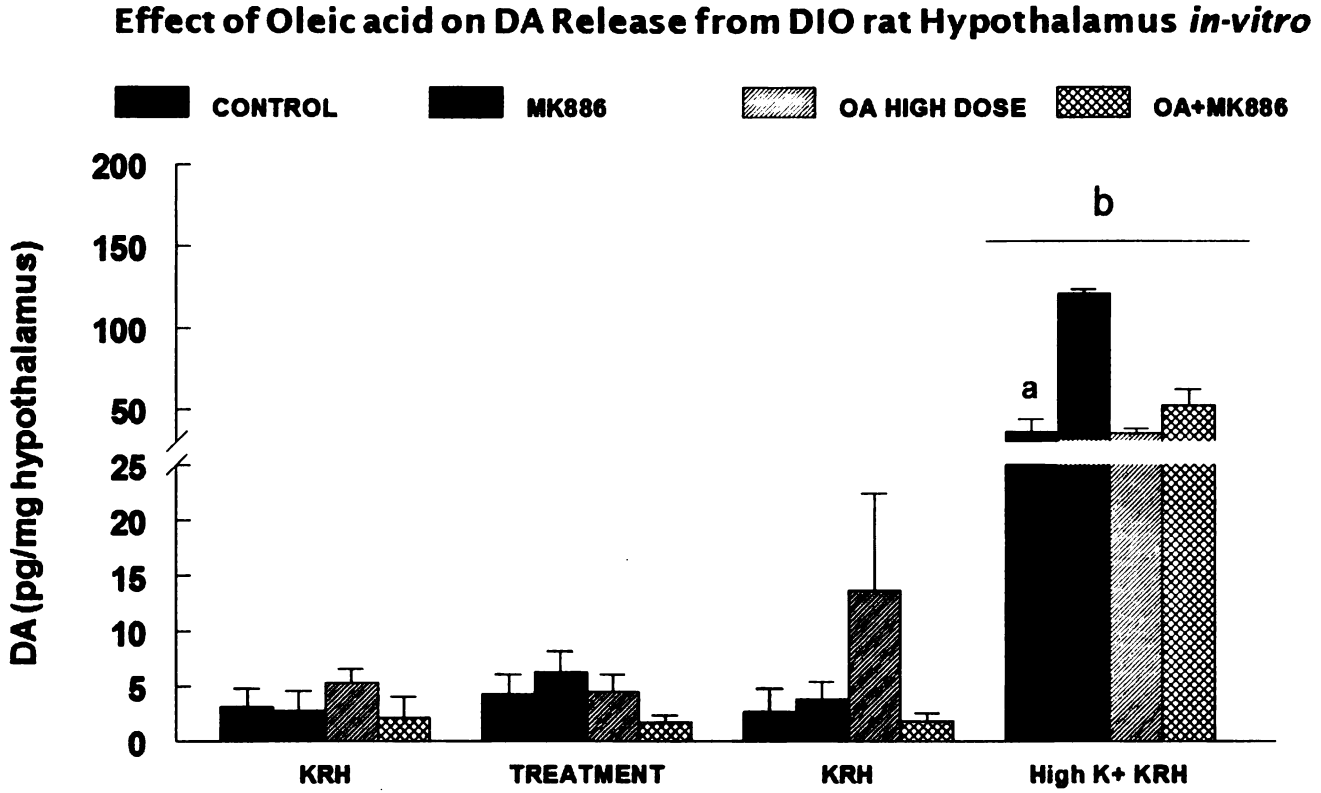

Fig 4-3. Effects of high dose of oleic acid on dopamine release from the hypothalamus of diet induced obese rats. DIO rat hypothalami were incubated with 1.32mM OA, MK 886 alone or MK 886+OA during treatment period in the *in vitro* incubation system. As shown in the figure, high dose of oleic acid failed to stimulate DA efflux significantly. **a** $p<0.01$ compared to KRH alone, **b** $p<0.01$ compared to all other incubation periods. (n=4)

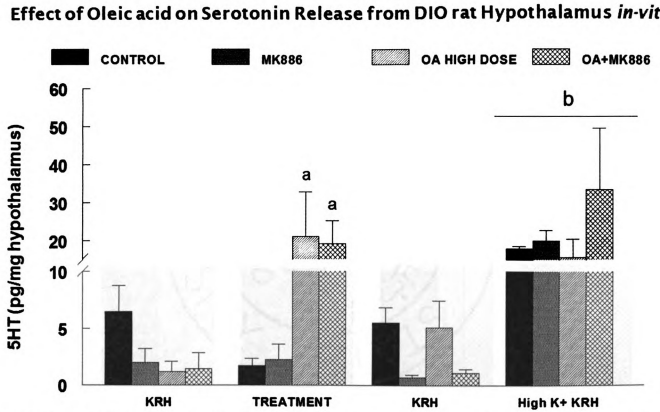

Fig 4-4. Effects of high dose of oleic acid on Serotonin release from the hypothalamus of diet induced obese rats. DIO rat hypothalami were incubated with 1.32mM OA, MK 886 alone or MK 886+OA during treatment period in the *in vitro* incubation system. As shown in the figure, high dose of oleic acid stimulated 5HT efflux which was however not reversed by the addition of the antagonist. **a** $p<0.01$ compared to control and MK886 groups, **b** $p<0.01$ compared to all other incubation periods. (n=4)

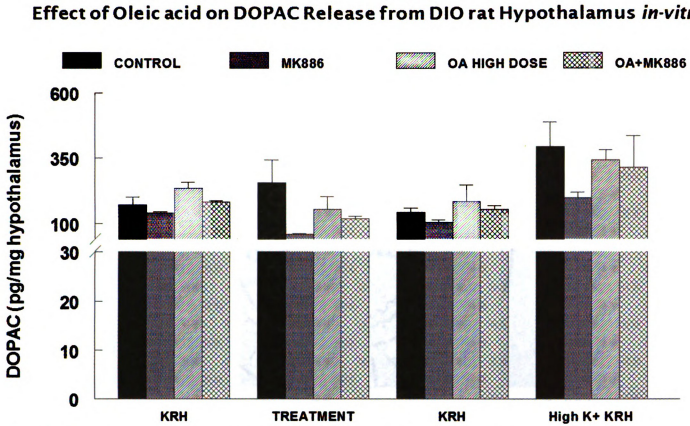

Fig 4-5. Effects of high dose of oleic acid on DOPAC release from DIO hypothalami. DIO rat hypothalami were incubated with 1.32mM OA, MK 886 alone or MK 886+OA during treatment period in the *in vitro* incubation system.

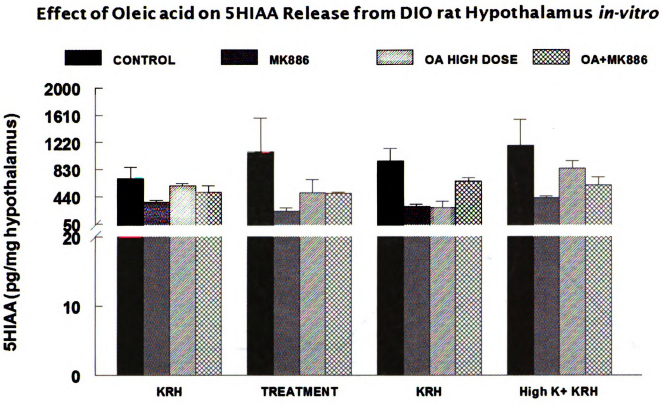

Fig 4-6. Effects of high dose of oleic acid on 5HIAA release following the incubation of hypothalami from diet induced obese rats. DIO rat hypothalami were incubated with 1.32mM OA, MK 886 alone or MK 886+OA during treatment period in the *in vitro* incubation system.

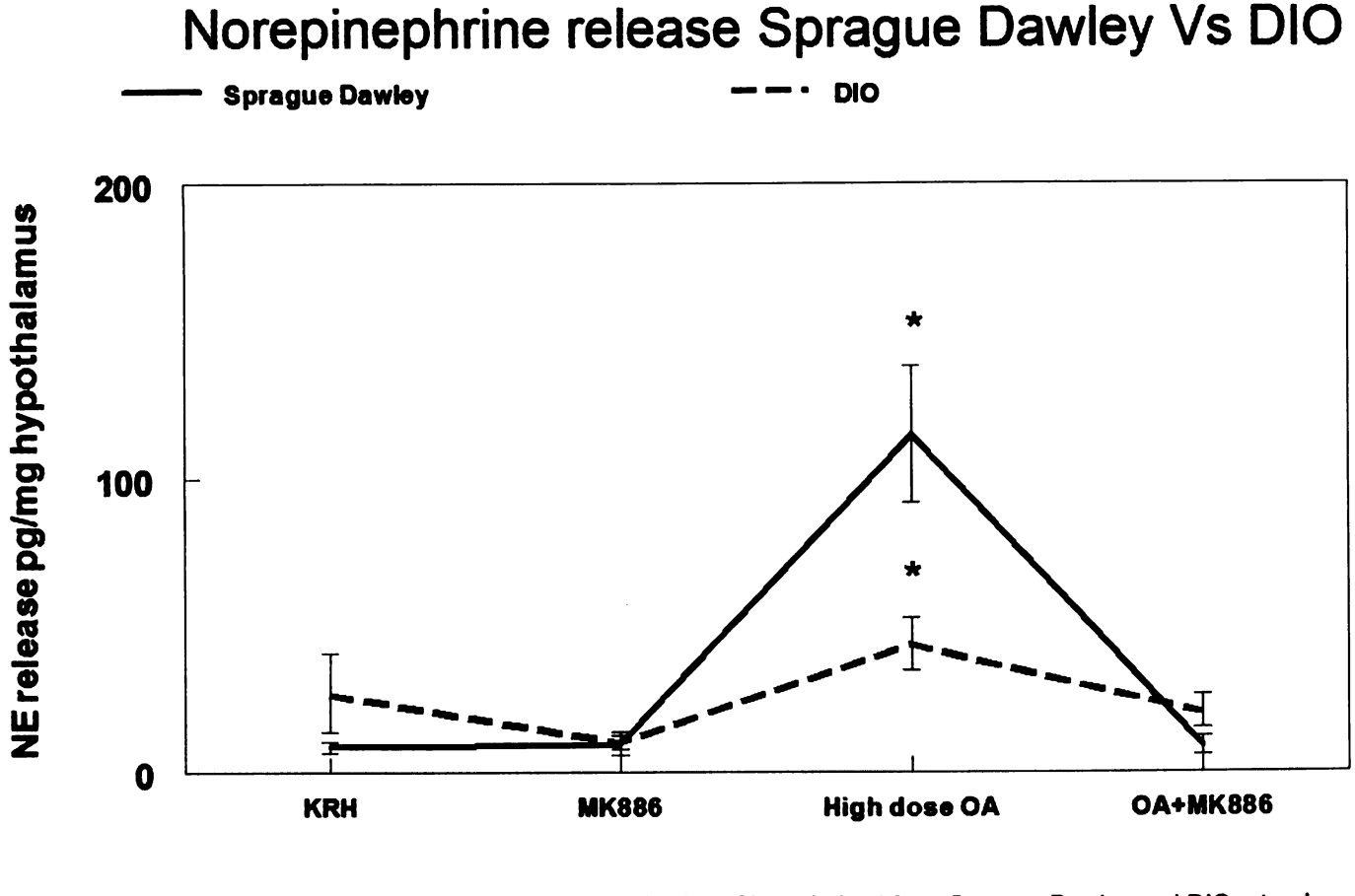

Fig 4-7. Comparison of the norepinephrine response *in vitro* of hypothalami from Sprague Dawley and DIO rats when incubated with KRH, MK 886, high dose of oleic acid and OA+MK886. As shown, the NE release was greater in Sprague dawley rats than in DIO rats. * $p<0.01$ when compared to KRH incubation

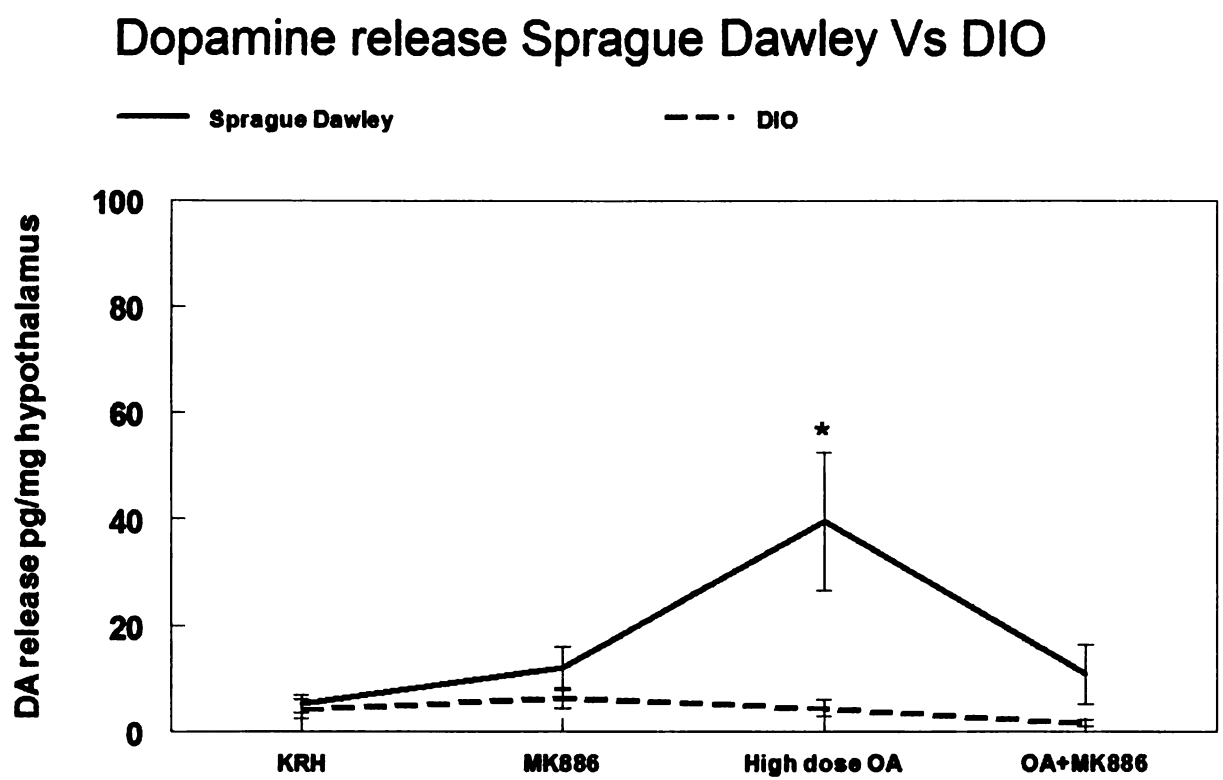

Fig 4-8. Comparison of the Dopamine response *in vitro* of hypothalami from Sprague Dawley and DIO rats when incubated with KRH, MK 886, high dose of oleic acid and OA+MK886. As shown, the DA release was greater in Sprague dawley rats than in DIO rats. * $p<0.01$ when compared to KRH incubation

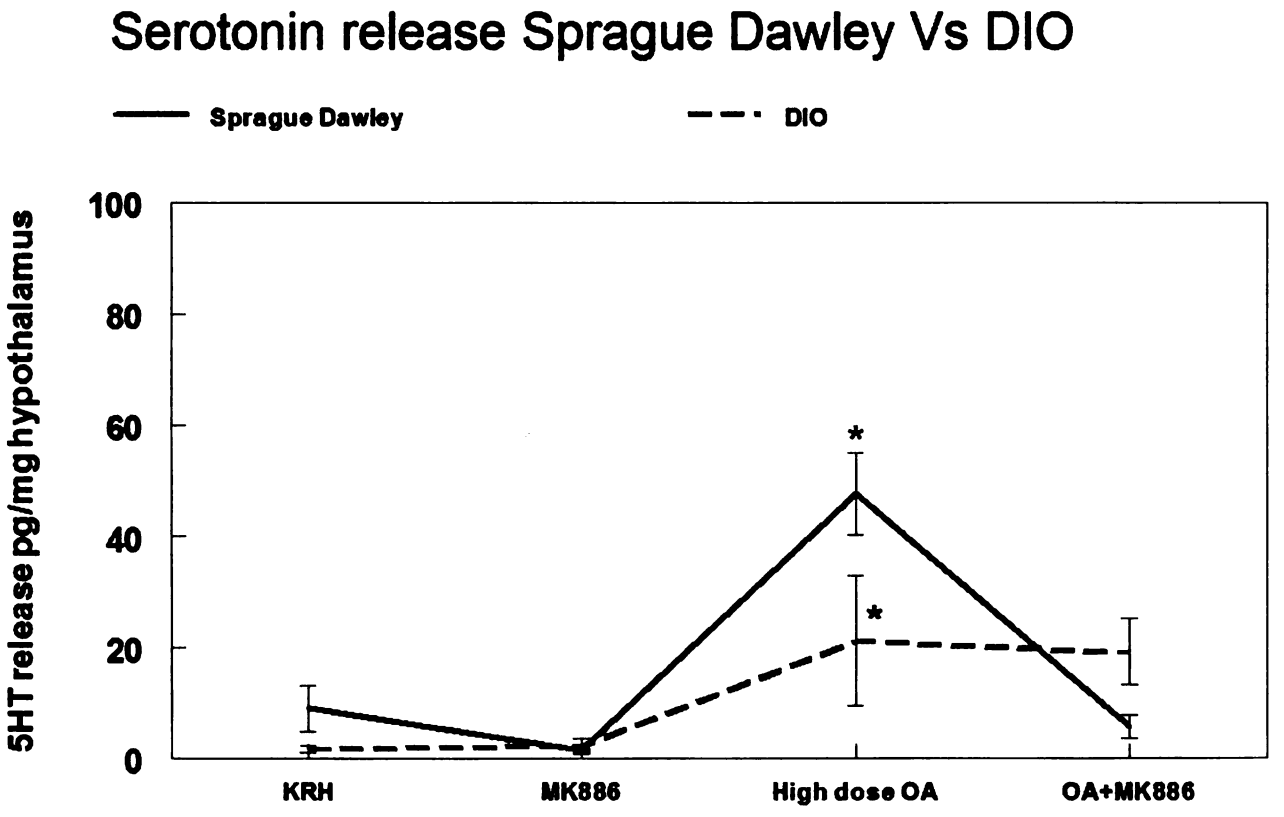

Fig 4-9. Comparison of the Serotonin response *in vitro* of hypothalami from Sprague Dawley and DIO rats when incubated with KRH, MK 886, high dose of oleic acid and OA+MK886. As shown, the 5HT release was greater in Sprague Dawley rats than in DIO rats. * $p<0.01$ when compared to KRH incubation.

IV. Discussion

Diet induced obese model of rats that we used showed significant changes in the pattern of neurotransmitter released when compared with Sprague Dawley rats. This difference also varied across the different monoamines that we measured namely norepinephrine, dopamine and serotonin.

High dose of oleic acid induced norepinephrine efflux which was significantly greater than control groups. However, this change was still not up to the extent of the norepinephrine release seen in the Sprague Dawley experiment. (Fig 4-7). Moreover, the dose response that was observed in Sprague Dawley rats was also absent in the DIO rats. Previous studies have shown that diet induced obese rats have impaired monoamine transmission in the hypothalamus [121]. Since monoamines act as important satiety signals, the obese animals probably do not attain a state of satiety like Sprague Dawley animals and hence tend to eat more leading to obesity. In our study we also show that this change is brought about by free fatty acids, particularly oleic acid. Although a number of lipid moieties including triglycerides and cholesterol circulate at high levels in the plasma of DIO animals, it is the free fatty acid that is most readily able to cross the blood brain barrier [63] and the hypothalamic centers regulating feed intake are one of the first regions receiving this metabolic information[33]. Although the fatty acids are readily available in DIO rats they may be unable to evoke a suitable satiety response thereby leading to weight gain. MK 886 blocked this effect in DIO rats also, showing the possible similarity between the two strains with respect to the mechanism of norepinephrine release.

However, with dopamine, the changes observed in Sprague Dawley rats were not seen in DIO rats. High dose of oleic acid completely failed to stimulate dopamine release from DIO rat hypothalamus. In a study involving mice, the opposite effect was noticed with high fat feeding[103]. In this study, high fat diet produced an increase in genes increasing dopamine availability in the hypothalamus. However, the changes observed in mice were in response to high fat diet, while we examined the acute effect of fatty acid alone in DIO rats that were still maintained on chow diet. These differences could have contributed to the contradictory results. Dopamine at the level of the hypothalamus has a less established role in feeding behavior as studies show that it can increase as well as decrease feed intake depending on the region involved[115]. Since dopamine reuptake inhibitors are also popular antiobesity drugs, it is safe to assume that the overall effect of dopamine is to promote satiety. In that view, it appears that free fatty acids fail to signal a state of satiety to the brain through the hypothalamic dopamine in DIO animals.

Serotonin showed an even more peculiar pattern in the DIO rat model. While the high dose of oleic acid continued to stimulate serotonin efflux from the DIO rat hypothalamus, this effect was not abolished by the addition of PPAR alpha antagonist. Although the release of serotonin was significantly higher when compared to control DIO rats, comparison with Sprague Dawley rats showed that this was still much lesser than that seen in the latter. The lack of response in serotonin release with respect to MK 886 induced PPAR antagonism was an unexpected finding. Diet induced obesity itself is associated with a number of

changes in the brain and specifically the hypothalamus of rats right from birth [131]. Changes in hypothalamic responses are seen in these animals even before they are exposed to high fat diet[132]. Hence it is not unlikely that the DIO animals recruit different mechanisms at the level of hypothalamus to regulate serotonin. However, more studies on the mechanisms of serotoninergic transmission in the hypothalamus are warranted before making any conclusions. Nevertheless, the difference in serotonin release between Sprague Dawley and DIO animals provides evidence that changes in serotoninergic transmission may be another difference between these two animals and possibly an explanation to why these animals are obese-prone.

Thus it appears that both norepinephrine and serotonin responses are blunted in DIO animals. This in turn opens the possibility that the signaling mechanisms leading to their release may be impaired. As MK 886 reversed the norepinephrine release induced by high dose of oleic acid, PPAR alpha may somehow be associated with noradrenergic neurotransmission in DIO rats. The same cannot be said with certainty in the case of serotonin. Understanding the exact link between fatty acids, PPAR alpha and monoamine release could provide valuable insights into this phenomenon. Any of the downstream molecules in this pathway could act as potential pharmacological targets in the treatment of obesity.

A number of new drugs are available to treat obesity and many of them act by increasing the monoamine neurotransmitter availability in the brain. However, since they are not region specific, they affect all the monoaminergic

systems of the brain leading to unfavorable results[115]. If the exact mechanisms of central nutrient sensing could be clearly delineated, drugs could be designed that target particular molecules which are probably most associated with the monoaminergic systems regulating feeding. This kind of specificity would be a great boon to those undergoing treatment for obesity.

Chapter 5

Summary and Conclusions

Summary and Conclusions

Oleic acid at a high dose comparable to that seen in the circulation of obese individuals was found to increase norepinephrine release from isolated rat hypothalamus maintained under *in vitro* conditions. Although this response was not significant with the medium and low doses, an increasing trend was noticed with higher doses. This effect was completely blocked by the PPAR alpha antagonist MK 886. Hence it is possible that free fatty acid sensing in the hypothalamus involves PPAR alpha. This could possibly lead to a number of molecular events culminating in the release of norepinephrine from the neuron. Thus fatty acids could act as a potential signal at the level of hypothalamus to induce satiety through the release of norepinephrine. The same response was also noticed in DIO rat hypothalami subject to similar conditions. However the release was much lower in DIO animals indicating that the satiety effect was less pronounced in DIO rats. This may be a contributing factor in the hyperphagia noticed in DIO rats and their subsequent tendency to gain weight.

Dopamine release was also increased following treatment of rat hypothalami with a high dose of oleic acid. This effect was again blocked with the addition of PPAR alpha antagonist. Hence in normal rats a high level of free fatty acids can induce satiety by stimulating dopamine secretion. However, in contrast to the noradrenergic response, dopamine stimulation was completely absent in DIO rats. Thus DIO rats fail to produce enough dopamine at the level of the hypothalamus to induce satiety. Although the effects of dopamine at the level of the hypothalamus in controlling feeding behavior is still subject to controversy[115],

this difference seen between Sprague Dawley and DIO could have an important relevance to the pathogenesis of diet induced obesity which may not be obvious from the information from the current study.

Serotonin release was also stimulated by high dose of oleic acid in Sprague Dawley rats and this effect was abolished by MK 886 in these animals. However, in DIO rats although serotonin release was stimulated by a high dose of oleic acid, PPAR alpha antagonism failed to reverse this effect. Hence it is possible that different mechanisms controlling serotonin release may be dominant in the DIO rat brain when compared with Sprague Dawley animals. The amount of serotonin released was still lower in DIO rats than Sprague Dawley rats. Hence, DIO animals still have reduced satiety recognition in response to free fatty acids.

Thus free fatty acids can act at the level of hypothalamus to modulate the release of various neurotransmitters involved in the regulation of feeding. Under physiological conditions fatty acids could signal a fed state and inhibit further feeding by stimulating the release of monoamines from nerve terminals in the hypothalamus. This may be crucial mechanism in the regulation of feed intake and body weight in a normal animal. Any disturbance in this intricate balance could contribute to abnormalities such as obesity and metabolic syndrome. Understanding the cellular mechanisms of feed intake regulation would help in developing pharmacological agents to specifically target the central regions regulating feed intake. Such specific pharmacological agents have the potential to be safer and more efficacious anti obesity drugs.

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