

THE ROLE OF NEURONAL PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR
DELTA IN DIET-INDUCED OBESITY AND HYPOTHALAMIC INFLAMMATION

By

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This dissertation is dedicated to the memory of Professor Richard Fehn,
an inspiring teacher, scientist and mentor who instilled in me a lifelong love of research.

To my beautiful daughter Naomi and to my husband Paulo,
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CHAPTER I

INTRODUCTION

Obesity Epidemic

Obesity has increased at an alarming rate and now qualifies as a worldwide health epidemic (1-5). Currently, obesity affects 35.7% of adults and 20% of children in the United States of America (USA) (4, 6-8) and is an emerging disease in developing countries (1, 9-11). Obesity places a large burden on worldwide health care systems and economies. Many diseases associated with obesity have a serious impact on health, including diabetes, hypertension and coronary heart disease (12-20). These contribute to an increased risk of early mortality from stroke and heart attack (21-23). Chronic conditions like diabetes and hypertension require extensive medical intervention to manage. The direct medical costs in the USA associated with treating an increasingly obese patient population are estimated to be over \$160 billion per year (24, 25). Equally costly, but less well known, is the strain obesity places on our economy, costing an additional estimated \$140 billion per year due to lost productivity (26). In addition to these economic costs is the high toll that obesity takes on human life, contributing to an estimated 365,000 to 400,000 deaths per year in the USA (12, 18, 20).

Factors involved in obesity epidemic

In simple terms, obesity results when there is an imbalance between energy (food) intake and energy expenditure. J.V. Neel proposed a role for the environment to explain the obesity epidemic and its associated metabolic diseases over 50 years ago, and theorized that famine during human evolution favored genes that promote fat storage, but possession of this genotype may lead to greater susceptibility to obesity, when food is abundant and obtained with little effort (27). Certainly Neel's "thrifty genotype" hypothesis provides a plausible explanation for why

some individuals become obese in an obesogenic environment, as well as the high heritability of obesity observed in families. A number of loci with small effect sizes for body weight, diabetes and fat mass have been identified by genome wide association scans (28), but a genetic basis for obesity susceptibility remains elusive

Environment factors, including a sedentary lifestyle and consumption of high calorie, energy rich foods have been implicated in the obesity epidemic (10, 29). Epidemiological data show a positive correlation between obesity, measured by body mass index within populations, and dietary fat intake (30). The mechanisms involved in diet-induced obesity (DIO) have been studied extensively in rodent models for over 20 years (31). These models have shown that dietary lipid consumption induces pathophysiological changes in systems controlling energy balance within the central nervous system (CNS) (32).

Energy homeostasis

Coinciding with this alarming increase in the prevalence of obesity has been an exponential increase in our understanding of the systems involved in the regulation of energy homeostasis (33). Evidence indicates that body weight and adiposity can be tightly regulated physiologically through the coordinated action of distributed neurons and brain circuits, which regulate feeding and energy expenditure in response to changes in circulating hormones (34) and nutrients (35). A number of different signals are generated from the ingestion of food, and the metabolism and storage of nutrients (36). These signals control both long term and short term energy balance by regulating the size and frequency of meals (short term) as well as controlling total food intake and energy expenditure (long term) (37). In the model illustrated in Figure 1, energy homeostasis is controlled by an endocrine feedback loop in which adiposity signals, such as insulin and leptin, are involved in the long term regulation of energy homeostasis.

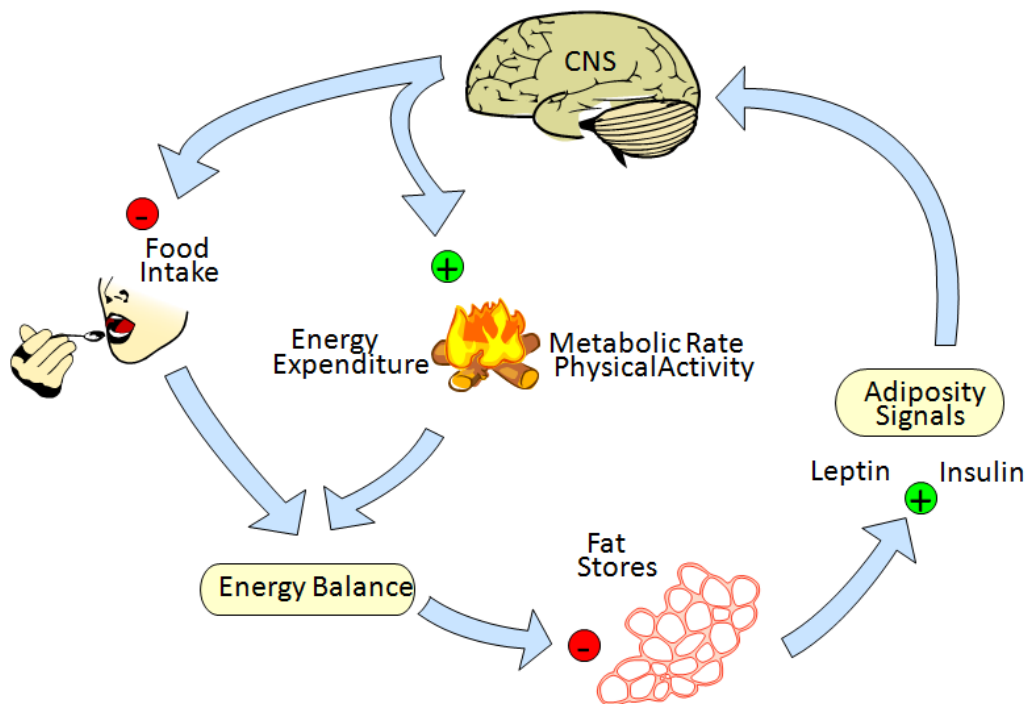


Figure 1. Central nervous system control of energy homeostasis.

Energy balance is regulated by adiposity signals, such as insulin and leptin, which circulate in proportion to fat mass and signal energy excess to the central nervous system (CNS). By activating their respective receptors on neuronal subsets found in the hypothalamus and elsewhere, these hormones increase catabolic while decreasing anabolic activity resulting in lower food intake and greater energy expenditure. Figure adapted from Schwartz et al. 2000. *Nature* 404, 661-671.

Adiposity signals

Adiposity negative feedback signaling in the CNS is a key mechanism involved in the regulation of energy balance. Several criteria define an adiposity negative feedback signal; it must be secreted in proportion to fat mass, it should be capable of interacting with neurons and cell types of brain areas that regulate energy balance, a deficiency should result in an obesity syndrome and administration of an adiposity negative feedback signal should affect feeding and/or energy expenditure.

Leptin

Leptin is an adipocyte-derived hormone that is secreted in proportion to energy stored as fat in adipose tissue (38) (Figure 1). Leptin was discovered through positional cloning experiments by J. Friedman's group in 1994 (39) as a result of efforts to identify a genetic defect causing the obesity syndrome in the obese (OB) mouse (40). Leptin enters the brain where it activates receptors expressed on specialized neurons in the hypothalamus that regulate food intake and energy expenditure (41). Reductions in leptin are sensed in the brain, and activate powerful adaptive responses that oppose excessive fat loss, suggesting these systems evolved to protect against starvation (42, 43). The conclusion that leptin acts as an important adiposity signal is supported by the profound phenotype that occurs in the absence of leptin signaling, in OB mice which have a mutation in the gene that encodes leptin. The phenotype of ob/ob mice, which includes profound hyperphagia and obesity (40) is normalized by leptin treatment (42, 44). The importance of leptin in feeding regulation is further evidenced by several reported cases of genetic leptin deficiency in humans resulting in extreme childhood obesity (45), which is also drastically improved with exogenous leptin therapy (46, 47). These examples demonstrate the importance of intact leptin signaling in the regulation of energy balance.

Insulin

The pancreatic β -cell hormone insulin is a well-established adiposity signal that acts in the brain (48). Like leptin, basal circulating levels of insulin are proportional to body fat stores (49-51). Insulin enters the brain through a receptor mediated saturable transport process (52). Insulin also reduces feeding and increases energy expenditure (Figure 1) by activating receptors expressed on neurons involved in energy balance in the hypothalamus (48). Intracerebroventricular (ICV) administration of insulin into the hypothalamus reduces food intake (53-55). Conversely, disruption of insulin signaling in the CNS, through specific genetic deletion of the insulin receptor (IR), causes hyperphagia and leads to obesity in mice (56).

Satiety signals

Satiety signals regulate short term feeding responses involving the size and frequency of meals by inducing feelings of satiation and satiety (57). Various chemo and mechano-receptors along the digestive track generate signals that are sent to the brain through vagal and taste afferents or through the circulation and provide information about the size and macronutrient composition (glucose, protein/amino acids, fatty acids) of a meal (58). Cholecystokinin (CCK) is one of the primary satiety signals involved in feeding regulation (57). CCK is released from duodenal mucosal cells in response to ingested protein and fat (59). The CCK signal is then transmitted by afferent fibers of the vagus nerve to the nucleus tractus solitarius (NTS) in the brain stem (60), which has projections to the hypothalamus where information is integrated into a response that results in meal termination (satiation) or suppression of hunger after meal (satiety). A number of other postprandial satiety signals are released from the gastrointestinal tract (GI) and have been shown to play important roles in feeding and appetite including glucagon-like-peptide (GLP)-1, amylin and peptide YY (reviewed in (57)). Ghrelin is another peptide hormone that is released from the GI tract and stimulates hunger and promotes feeding (61).

Reward circuitry: Mesolimbic dopamine system

Food reward promotes feeding and is a non-homoeostatic regulator of feeding and energy balance. Dietary fat and sugar intake engage the mesolimbic dopamine reward system, involving neurons in the ventral tegmental area (VTA) leading to dopamine and mu opioid release in the nucleus accumbens (62, 63), a region associated with drug addiction (64). Food restriction enhances the effects of drug reward (65-67). The reinforcing aspects of dopamine release on food reward are modulated by peripheral hormones and adiposity signals. Ghrelin signaling in VTA neurons increases dopamine levels in the nucleus accumbens (68) and promotes feeding (69). Leptin can inhibit feeding by modulating the activity of VTA neurons (70). Insulin is an important determinant of dopamine reuptake through regulation of dopamine transporter (DAT) translocation between intracellular vesicles and the plasma membrane. Evidence suggests that obesity is associated with defects in pre- and post-synaptic dopamine signaling and release (71-73) which may contribute to impaired feeding regulation in obesity (74). Obesity may also impair dopamine signaling through decreased expression of dopamine D2 receptors (72, 73, 75).

Central nervous system

The regulation of energy balance is complex and involves many brain regions and neurological systems (76). Of these, the hypothalamus serves as a key region involved in the regulation of energy balance (34). The arcuate nucleus is a region in the medialbasal hypothalamus that is located near the third cerebral ventricle and is in close contact with the median eminence, an area distinguished by an incomplete blood brain barrier (77). Therefore, neurons in the arcuate nucleus are positioned to sense changes in concentrations of circulating hormones and nutrients. Neuronal subsets found in the arcuate nucleus express receptors for insulin and leptin and are differentially regulated by these hormones (Figure 2) (Reviewed in (41)). These act as first order neurons that assimilate and integrate information about adipose

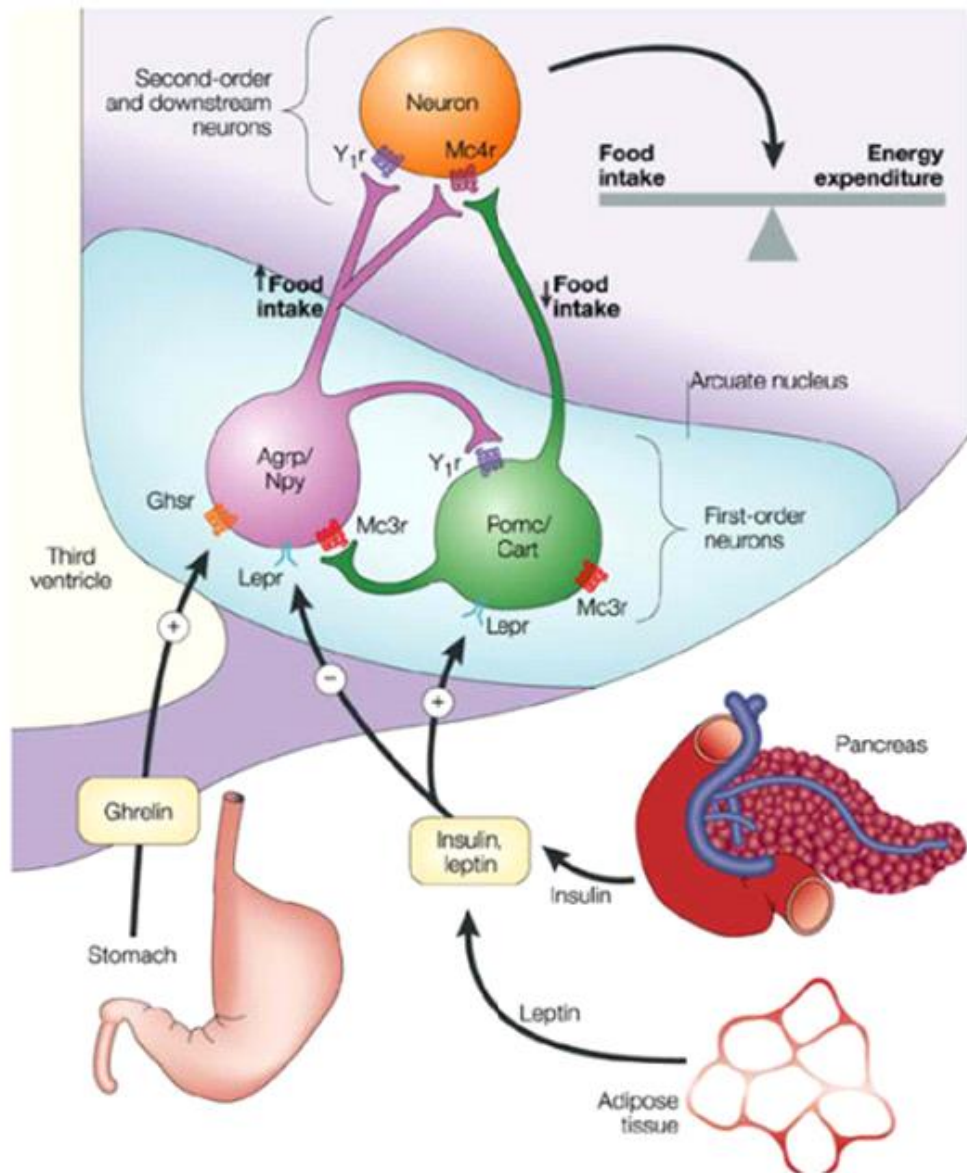


Figure 2. Hypothalamic neurons involved in energy balance regulation.

Two populations of neurons in the arcuate nucleus are known by the neuropeptides that they co-express, the neuropeptide Y (NPY) and agouti related protein (AgRP) orexigenic neurons as well as the proopiomelanocortin (POMC) and cocaine and amphetamine related transcript (CART) anorexigenic neurons. These co-expressing neurons are differentially regulated by circulating adiposity signals and satiety signals and differentially activate second order neurons that control food intake and energy expenditure (image from G.S. Barsh and M.W. Schwartz 2002. *Nature reviews. Genetics* 3, 589-600.

stores (long term energy availability), and orchestrate hormonal and autonomic responses via differential regulation of downstream neurons in the hypothalamus and other brain regions (37).

Anorexigenic POMC/CART neurons

The anorexigenic proopiomelanocortin (POMC) and cocaine and amphetamine related transcript (CART) (78, 79) co-expressing neurons are found in the arcuate nucleus and are activated during states of energy excess (Figure 2). Through the release of CART and the POMC cleavage product, α -melanocyte stimulating hormone (α -MSH), these neuropeptides activate CART and melanocortin receptors (MC3R/MC4R) receptors, respectively, expressed on second order target neurons to decrease food intake and increase energy expenditure (80). Genetic defects in multiple steps of melanocortin signaling result in obesity in rodents highlighting the importance of this system in energy balance (reviewed in (81)). A frameshift mutation in the gene encoding MC4R is responsible for the most common form of monogenetic obesity known to occur in humans (82)

Orexigenic NPY/AgRP neurons

The neuropeptide Y (NPY) and agouti related protein (AgRP) co-expressing neurons in the arcuate nucleus release orexigenic neuropeptides, NPY and AgRP that increase food intake and reduce energy expenditure (83, 84) (Figure 2). NPY is a neuropeptide that binds a variety of NPY receptor subtypes expressed on neurons throughout the brain (Y_{1-5}) (85, 86). Infusion of NPY into the third ventricle induces obesity in rodents (86). NPY is increased in the absence of leptin and genetic deletion of NPY attenuates obesity in ob/ob mice (87). AgRP is an endogenous MC3R/MC4R antagonist and blocks α -MSH signaling (88). In states of positive energy balance, insulin and leptin levels are elevated and inhibit NPY/AgRP neuronal firing and reduce gene expression of these neuropeptides (84, 89) thereby reducing orexigenic tone.

Second order neurons

NPY/AgRP and POMC/CART neurons project to many of the same second order neurons to regulate food intake and energy expenditure through differential modulation of these neuronal targets (37) (Figure 2). Neurons in the lateral hypothalamic area (LHA) express food regulatory neuropeptides, such as orexin, CART, neurotensin, α -MSH and histamine, and are involved in feeding regulation (90). Neurons in the paraventricular nucleus (PVN) regulate endocrine responses related to metabolism and stress via thyroid releasing hormone and corticotrophin releasing hormones (reviewed (90)). Activation of POMC neurons leads to increased energy expenditure via α -MSH, which activates receptors expressed on neurons in the brain stem to increase sympathetic outflow to brown fat, muscle, pancreas, and liver (91-94).

Hypothalamic insulin and leptin signaling

Leptin signaling

The effects of leptin are mediated through the long form of the leptin receptor, LEPR-B expressed on POMC/CART and NPY/AgRP neurons in the arcuate nucleus. LEPR-B is a type I cytokine receptor and activates a janus activating kinase 2 (JAK2) and signal transducer and activator of transcription-3 (STAT3) signaling cascade (44) (Figure 3). Leptin binding to the extracellular domain of LEPR-B induces a conformational change that induces JAK2 mediated tyrosine phosphorylation at three distinctive sites, Tyr1138, Tyr985 and Tyr1077 of the intracellular domain of LEPR-B. Phosphorylated-Tyr1138 recruits STAT3, where it is phosphorylated (pSTAT3) by JAK2, causing its nuclear translocation and transcriptional activation (reviewed (95)). In the nucleus, STAT3 promotes the expression of POMC and other gene targets including suppressor of cytokine signaling-3 (SOCS3), a STAT3

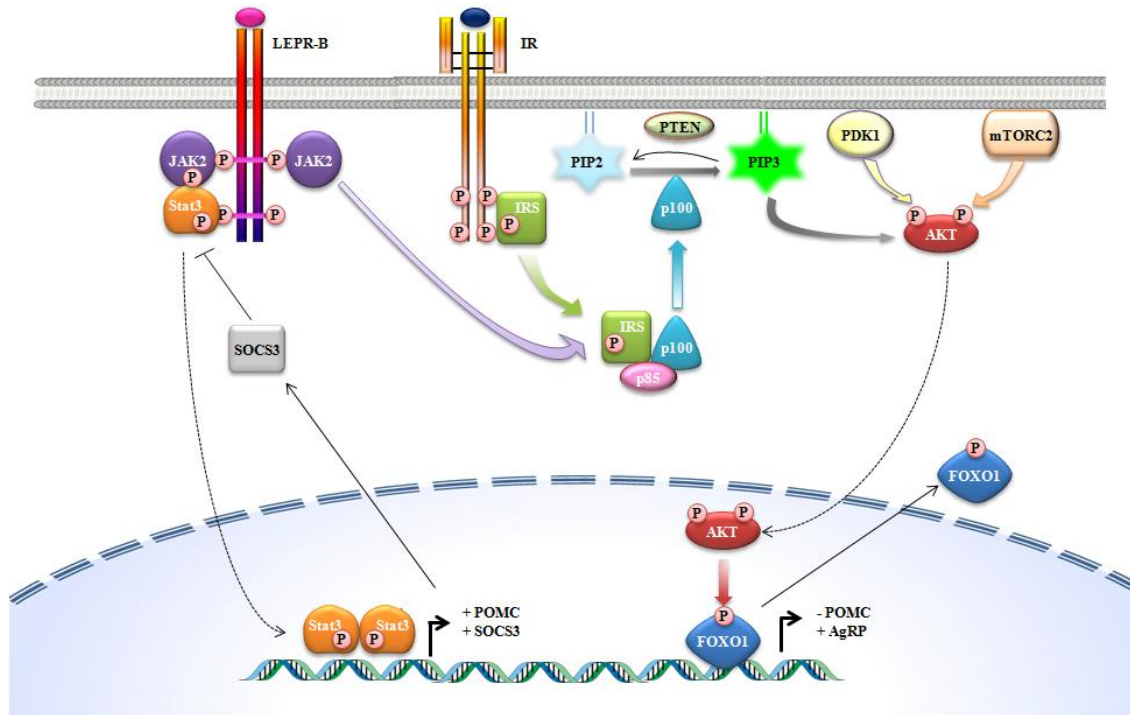


Figure 3 Insulin and leptin signaling in hypothalamic neurons.

Insulin binding to its receptors (IR) leads to receptor tyrosine auto-phosphorylation, which recruits insulin receptor substrate (IRS) to the IR, where it undergoes tyrosine phosphorylation leading to recruitment of the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K) and activation of and release of the catalytic subunit (p110) which phosphorylates membrane bound phosphatidylinositol (4,5) bisphosphate (PIP2) leading to the formation of phosphatidylinositol (3,4,5) trisphosphate (PIP3). Phosphatase and tensin homolog (*PTEN*) reverses the effects of PI3K, converting PIP3 to PIP2. PIP3 recruits protein kinase B/Akt to the membrane, where it is phosphorylated at two residues, by phosphoinositide dependent kinase 1 (PDK1) and mammalian target of rapamycin kinase complex (mTORC2) leading to its nuclear translocation. In the nucleus, Akt phosphorylates and promotes nuclear exclusion and inhibition of forkhead box protein O1 (FOXO1), resulting in regulation of neuropeptide gene expression. FOXO1 has differential effects in POMC and NPY/AgRP neurons. In POMC neurons, FOXO1 inhibits POMC expression. In these neurons, insulin signaling through PI3K promotes POMC expression via Akt mediated nuclear exclusion of FOXO1, which de-represses the POMC promoter. FOXO1 promotes AgRP expression in NPY/AgRP neurons. In these neurons, insulin signaling reduces AgRP expression by excluding FOXO1 from the nucleus. Leptin binding to the long form of the leptin receptor, LEPR-B, recruits and activates Janus kinase 2 (JAK2) that phosphorylates LEPR-B, creating a binding site for transducer and activator of transcription-3 (STAT3), where it is phosphorylated and activated by JAK2. pSTAT3 monomers form dimers and translocate to the nucleus where they act as transcription factors to promote expression of POMC and suppressor of cytokine signaling-3 (SOCS3), a STAT3 induced negative feedback regulator of LEPR-B activation in POMC neurons. Leptin signaling may also activate PI3K via JAK2 mediated phosphorylation and activation of IRS, resulting in decreased AgRP expression in NPY/AgRP neurons via FOXO1 inhibition. Ultimately, insulin dis-inhibits and leptin activates POMC expression while insulin deactivates and leptin inhibits AgRP expression. Figure adapted from Niswender and Schwartz. 2003. *Frontiers in neuroendocrinology* 24, 1-10.

induced negative feedback regulator of LEPR-B activation (96-98). Leptin signaling causes depolarization of POMC neurons (99) leading to neuropeptide release into the synaptic cleft near the axons of second order neurons, where CART and the POMC gene products α MSH activate their respective receptors ultimately reducing food intake and increasing energy expenditure. Leptin signaling has an opposite effect, hyperpolarizing anorexogenic NPY/AgRP neurons (78). The effects of leptin on POMC/CART neuron firing and gene expression are potentiated by insulin, which differentially regulates gene expression in POMC/CART and NPY/AgRP neuronal subtypes (Reviewed in (100)).

Insulin signaling

Insulin binding to insulin receptors (IR) expressed on POMC/CART and NPY/AgRP neurons causes IR to undergo auto-phosphorylation at key tyrosine residues on the intracellular domain, which serve as recognition and docking sites to recruit cytoplasmic insulin receptor substrate proteins (IRSs) (Figure 3) (reviewed in (101)). IRSs are phosphorylated on tyrosine residues to facilitate recruitment through an interaction with the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K), a key mediator in arcuate neurons of the insulin-induced inhibition of feeding (53). The catalytic subunit of PI3K, p110, phosphorylates membrane bound phosphatidylinositol (4,5) bisphosphate (PIP₂) leading to the formation of phosphatidylinositol (3,4,5) trisphosphate (PIP₃), which recruits protein kinase B (Akt) to the membrane, where it is phosphorylated. Akt phosphorylation by phosphoinositide dependent kinase 1 (PDK1) at threonine 308 (102), and at serine 473 by the mammalian target of rapamycin kinase complex (mTORC2), induces nuclear translocation where it phosphorylates and promotes exclusion of transcription factor forkhead box protein O1 (FOXO1) (103) from the nucleus. In the un-phosphorylated state, FOXO1 binds to DNA and promotes AgRP expression in NPY/AgRP neurons and inhibits POMC expression in POMC/CART neurons (104). Therefore, insulin

reduces feeding, in part, by inhibiting AgRP expression and de-repressing POMC gene expression, via insulin induced phosphorylation and nuclear exclusion of FOXO1 (105).

Inhibition of adiposity negative feedback signaling

Negative regulation of insulin and leptin signaling occurs through several processes. SOCS3, which is upregulated by leptin signaling binds phosphorylated Tyr985 of LEPR-B and acts as a negative feedback regulator of STAT3 activation (96). SOCS3 is a negative regulator of insulin signaling, through ubiquitin mediated degradation of IRS1 and IRS2 (106). Consequently, chronic elevations in leptin can contribute to insulin and leptin resistance (98, 99). Phosphatase and tensin homolog (PTEN) reverses the effects of PI3K, converting PIP3 to PIP2 ultimately reducing the downstream effects of insulin and leptin signaling through this pathway (107) (Figure 3). A variety of protein tyrosine phosphatases (PTPs), enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins (108), can also act to reverse the effects of kinases involved in insulin and leptin signal transduction in hypothalamic neurons (109-112). Therefore, a balance between activation and deactivation of insulin and leptin signaling exists that allows for fine tuning and modulation of signal initiation, transduction and finally, squelching or conclusion of signal transduction. This allows for both acute regulation of neuronal activity, through effects on neuron firing, and chronic effects by modulating activity of the signaling molecules that regulate gene transcription.

Integrated regulation of energy balance

Reactive oxygen species

Nutrients, such as glucose and fatty acids, and their intermediate metabolites can act as signaling molecules to modulate short term, metabolic responses in multiple tissues, including the brain. Circulating free fatty acids (FFA) increase during prolonged fasting due to lower circulating insulin levels and increased lipolysis. FFAs are oxidized through mitochondrial β -

oxidation, which promotes production of reactive oxygen species (ROS) that are inherent byproducts of aerobic metabolism (113). At low concentrations, ROS can regulate insulin signaling through modulation of phosphatase activity (114, 115). Acutely, ROS serve as important nutrient signals and second messengers in hypothalamic neurons, where increased ROS simultaneously represses NPY while promoting POMC gene expression (116-118). Uncoupling protein 2 (UCP2) is a mitochondrial leak channel protein involved in hydrogen ion uncoupling across the mitochondria inner membrane. The regulatory effects of ROS to repress orexigenic neuronal activation and NPY neuropeptide expression are abrogated by mitochondrial uncoupling and reduction in ROS levels by UCP2 (116) through alterations in ATP production and neuronal firing.

Obesity: Impaired energy homeostasis

Caloric density of dietary fat

Dietary fat intake has been implicated in the obesity epidemic (30, 31, 119). Several possible explanations have been offered. The increased caloric density of dietary fat is thought to contribute to elevated weight gain (120). Laboratory rodents rapidly develop obesity when fed experimental high-fat (HF) diets that contain 45-60% of total calories derived from fat instead of a chow diet (31, 121, 122). HF food, gram for gram, is more calorically dense than chow, having 9 kilocalories (kcal) or 40% more kcal per gram than carbohydrates and protein which have 4 kcal per gram. Caloric intake tends to be elevated in ad libitum HF diet feeding paradigms, thus, inclusion of a pair-fed group, given access or restricted amounts of HF diet can help differentiate between the contributions of elevated caloric intake to DIO (123). A number of studies have reported detrimental metabolic effects of dietary fat that were independent of increases in total body weight, adiposity (124-128), and insulin resistance (129, 130). Therefore, while caloric intake is an important determinate of weight gain, dietary fat, among macronutrients, possesses unique obesogenic properties.

Hypothalamic insulin and leptin resistance

Consumption of a HF diet is associated with central insulin and leptin resistance such that elevations in insulin and leptin no longer reduce food intake or increase energy expenditure resulting in expansion of adipose tissue and obesity (*109, 131, 132*). In animals with normal levels of adiposity, leptin, either peripherally or directly into the third ventricle, leads to a modest but significant decrease in 24 hour food intake. DIO attenuates the effects of leptin action on feeding (*112, 133, 134*). Behavioral leptin resistance in DIO animals is accompanied by cellular leptin resistance, measured by reduced activation of downstream signaling targets of leptin receptor signaling, such as reduced levels of phosphorylated STAT3 and JAK2 (*132, 135, 136*). Elevated levels of SOCS3 have been associated with obesity and resistance to leptin. SOCS3 is a known negative regulator of leptin signaling and may also promote insulin resistance (*98, 99*).

Intracerebroventricular cannulation (ICV) is used to accurately assess behavioral and cellular insulin sensitivity in the CNS, due to the effects of exogenous peripheral insulin on glycemia. The effects of central insulin on feeding are attenuated or absent in DIO rodents (*105, 137, 138*). DIO rodents also exhibit cellular hypothalamic insulin resistance after ICV insulin treatment; with reduced phosphorylation of IR, IRS, PI3K, Akt, and increased nuclear localization of FOXO1 (*133, 137*).

Reduced energy expenditure

In addition to excess food intake, obesity may result from a reduction in energy expenditure (*139*). Energy expenditure is defined as the sum of resting energy expenditure plus the energy expended from the thermic effect of food and physical activity. Resting energy expenditure encompasses the energy utilized to support basic cellular and physiological processes including circulation, respiration thermoregulation, growth and reproduction. The majority of an organism's energy (~70%) is expended through these processes. When food is consumed, energy

is required for digestion, absorption and transport of nutrients, collectively termed the thermic effect of food which can account for 5-10% of total energy expenditure (140). Voluntary physical activity accounts for a small amount of all energy expenditure, although there is a large inter-individual range that is dependent upon levels of exercise. Reduced energy expenditure is a feature of several genetic mouse models of obesity, including ob/ob (141) and MC4R knockout mice (142), which are characterized by reduced oxygen consumption, an indirect measure of fuel utilization and metabolic rate.

Energy expenditure can be assessed by indirect calorimetry in which measurements of oxygen consumed (VO_2) and carbon dioxide produced (VCO_2) are measured and used to determine the respiratory exchange ratio (RER). RER is then used to determine the respiratory quotient (RQ) (VCO_2/VO_2), the value of which indicates the type of fuel being oxidized. Pure carbohydrate oxidation yields an RQ value of ~1.0 while the expected RQ for pure fat oxidation is ~0.7. Typically, mixed meals, composed of fat and carbohydrate yield an RQ value that is intermediate between 0.7-1.0. Heat production (kcal/hr) can be determined from RER and VO_2 and indicates the total energy expenditure of an organism in calories. While energy expenditure is an important determinant of energy homeostasis, fat mass can accumulate in the absence of large changes in body weight due to reciprocal changes in lean mass.

Fuel Partitioning

Energy expenditure in genetic and DIO rodent models of obesity can be difficult to interpret due to drastic differences in body weight and/or body composition (143). When food intake and energy expenditure are not altered, higher adiposity can also be due to fuel partitioning and preferential storage of energy in adipose tissue as fat. Fuel partitioning can occur through several mechanisms that include alterations in adipose and lean mass metabolism and function. In adipose tissue, fat mass can accumulate due to differences in the rate of uptake of fatty acids from circulating triglycerides in lipoproteins, increased rates of lipogenesis and storage of newly

synthesized fatty acids in triglycerides or decreased lipolysis whereby fat is retained in adipose tissue due to decreased release of stored fatty acids from triglycerides. The phenotype of mice with global genetic deletion of MC3R is an example of a fuel partitioning phenotype. These animals have similar food intake and energy expenditure but exhibit increased adiposity and reduced lean mass with higher RQ which suggests preferential glucose oxidation that promotes fat storage (*144, 145*). Fuel partitioning can also result from differences in the rates of protein synthesis and/or breakdown in lean tissue, which primarily occurs in skeletal muscle through myogenesis and proteolysis, respectively (*146, 147*). Skeletal muscle is a main site for fatty acid oxidation and glucose disposal; therefore reduced lean mass will have extensive metabolic implications related to glucose homeostasis and disposal as well as metabolic rate. Collectively, differences in fuel partitioning can lead to differences in body composition in the context of similar food intake and energy expenditure.

Mechanisms involved in diet-induced obesity

High-fat diet and hypothalamic inflammation

Dietary fat intake is associated with systemic and cellular low-grade inflammation, which may serve as a key step in the development of insulin resistance in peripheral tissues (*148-150*). Increasing evidence suggests that after consuming a diet rich in saturated fat, circulating saturated fatty acids (SFAs) are elevated, which contribute to hypothalamic insulin and leptin resistance by activating inflammatory signaling pathways that inhibit insulin and leptin signaling in the CNS (reviewed (*151*)). Manipulations that reduce inflammatory signaling in the hypothalamus can prevent obesity and improve glucose metabolism (*152*). Direct infusion of a proinflammatory cytokine, IL-4, into the hypothalamus to activate inflammatory signaling, increases weight gain during HF feeding (*153*). Collectively, the evidence supports a causal relationship between hypothalamic inflammation, CNS hormone resistance and DIO.

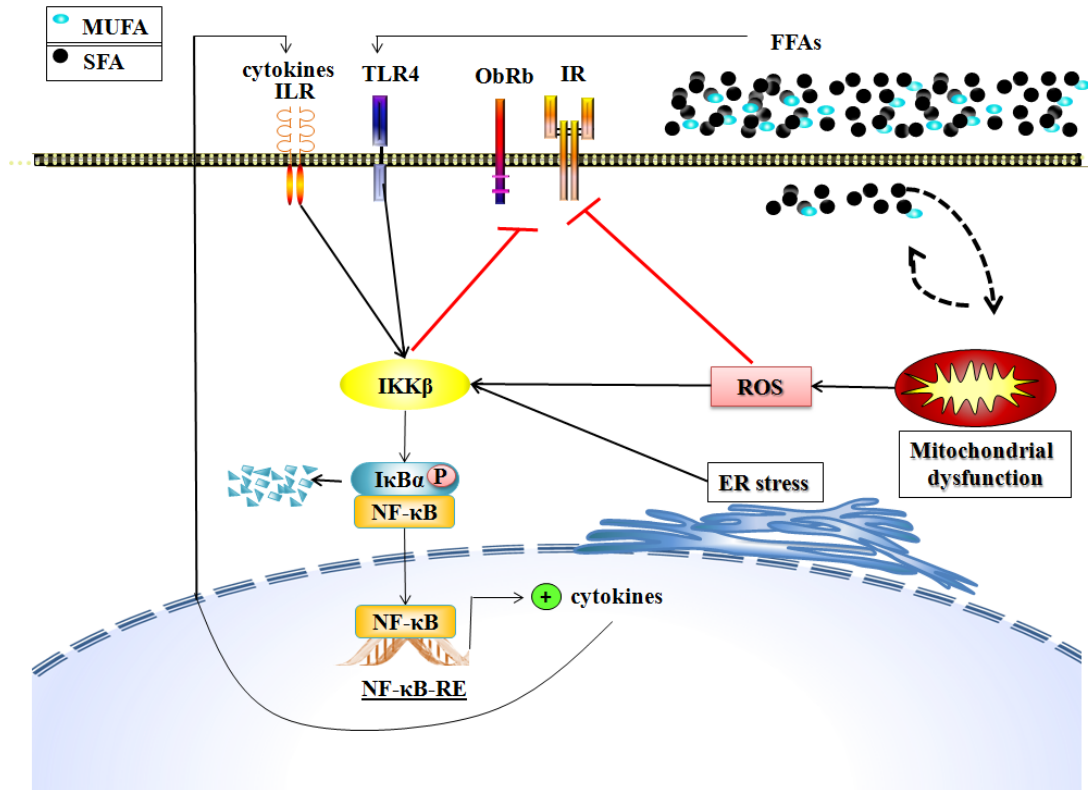


Figure 4. Upstream mediators of NF-κB activation.

During HF feeding, cells are flooded with FFA from plasma activate inflammatory pathways including NF-κB. NF-κB is normally retained in the cytoplasm by IκBα. ER stress, toll-like receptor 4 (TLR4) activation by SFAs, and ROS production activate IKKβ, which phosphorylates and promotes the degradation of cytoplasmic IκBα resulting in nuclear translocation of NF-κB (p50/p65) and transcriptional activation of proinflammatory NF-κB cytokine target genes. IKK can also contribute to insulin resistance through inhibitory serine phosphorylation of the insulin receptor substrate molecules and inhibition of insulin receptor (IR) initiated signal transduction.

Inflammation: nuclear factor-kappa B

DIO is commonly associated with activation of an inflammatory pathway involving the transcription factor, nuclear factor-kappa B (NF- κ B), its regulatory protein, inhibitor of NF- κ B alpha (I κ B α), and the upstream kinase, I κ B α kinase beta (IKK β) (133, 152). NF- κ B has been associated with hypothalamic inflammation in response to toll-like receptor 4 (TLR4) activation by saturated fatty acids (SFAs) (154, 155), endoplasmic reticulum (ER) stress (152), and long-chain fatty acyl CoA accumulation (133) (Figure 4). NF- κ B signaling involves activation of IKK β , which phosphorylates and promotes the degradation of cytoplasmic I κ B α , resulting in NF- κ B release and transcriptional activation of numerous NF- κ B target genes, such as proinflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor alpha (TNF α) and interleukin-1beta (IL-1 β) (156-159). These cytokines, produced locally within cells, can act in an autocrine manner to activate signal transduction cascades that contribute to oxidative stress and inflammatory signaling, which would further promote insulin resistance (149). IKK β -NF- κ B activation could also contribute to insulin resistance through IKK β mediated serine phosphorylation and degradation of IRS molecules (160).

Mitochondrial oxidative stress

Mitochondrial oxidative stress is linked to inflammation and insulin resistance in numerous peripheral tissues (161-163) and is a common feature of neurodegenerative disease (164-167). Nutrient excess and increased flux through the mitochondrial respiratory chain increases the mitochondrial membrane potential, a situation that promotes superoxide anion production (O₂⁻) through reverse electron transport at complex I and III of the electron transport chain (113) (Figure 4). ROS accumulation due to chronic oxidative stress can overwhelm the cellular antioxidant defenses resulting in oxidative damage to DNA, proteins and lipids (168-170). ROS also activate a variety of redox sensitive inflammatory kinases, including IKK β , which contribute to insulin resistance through inhibitory serine phosphorylation of IRS signaling

molecules and increased cytokine expression (171). Genetic deletion of antioxidant genes leads to increased inflammation and insulin resistance, while treatment with antioxidants or overexpression of antioxidant genes prevents oxidative stress, inflammation, insulin resistance and apoptosis (reviewed in (172)). Biomarkers of oxidative stress correlate with dietary saturated fat intake in diabetic humans (173) and evidence implicates mitochondrial dysfunction and oxidative stress in the pathogenesis of insulin resistance (162, 163, 174-178). However, the role oxidative stress might play within the hypothalamus during DIO is not known.

ER stress

The ER is the site of protein translation and folding, and defects in ER function are highly associated with obesity and insulin resistance (179). The higher demand on the ER to synthesize more proteins to respond to increased metabolic demands during overfeeding increases unfolded proteins in the ER inner membrane (180). These are sensed by ER stress sensors and unfolded protein response (UPR) transducers that halt protein translation until the defective proteins are cleared by ER chaperones (181). DIO models are associated with hypothalamic activation of the ER stress sensor protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activation of this ER stress response modulator in the hypothalamus by overnutrition or TNF α (182) also activates the IKK β -NF- κ B pathway (152).

Protective effects of monounsaturated fatty acids

Dietary fat is consumed predominantly as triglycerides, composed of a glycerol backbone and three fatty acid chains. Fatty acids have distinctive structures, vary by chain length and saturation as well as position of unsaturated bonds. Epidemiological evidence suggests diets rich in monounsaturated fatty acids (MUFAs) have beneficial effects including improved insulin sensitivity, weight loss and reduced cardiovascular disease risk.(183). In human trials, monounsaturated dietary fat is less obesogenic than saturated fat (184-186), and improves insulin

sensitivity in obese and diabetic populations (187-192). The health benefits are independent of caloric intake (189, 193). The insulin sensitizing effects of dietary MUFA along with polyunsaturated fatty acids (PUFAs) are hypothesized to be due to the ability to interact with several different cellular “*fatty acid sensors*” (194) that reduce inflammation and/or attenuate lipid accumulation (194, 195).

Peroxisome proliferator-activated receptors

Discovery

The peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors, are lipid ligand activated transcription factors belonging to the nuclear hormone receptor super family (196, 197). PPARs were discovered as a result of efforts to identify the molecular mediator of fibrates, a class of amphipathic carboxylic acids used in the treatment of hyperlipidemic and metabolic disorders since 1967 (reviewed in (198)). First discovered in the 1952 (199, 200), fibrates were termed peroxisome proliferators after early studies showed they increased the number and size of peroxisomes in livers of rodents (201), and were later found to act by increasing the expression of genes involved in peroxisomal fatty acid β -oxidation (202). Attempts to synthesize more potent fibrate molecules in the early 1980’s resulted in the discovery of thiazolidinediones (TZDs), a class of related compounds that potently reduced hyperglycemia, hyperinsulinemia and hypertriglyceridemia in rodents (203-205).

In 1990, Issemann and Green reported the discovery of a novel member of the nuclear hormone receptor super-family (197) that formed heterodimers with retinoid X receptor (RXR) and mediated the effects of fibrates and fatty acids on peroxisome proliferation and hepatic gene expression (206). In 1992, three related isoforms were identified in *Xenopus*, PPAR α , PPAR γ and PPAR β (later shown to be the same protein as PPAR δ) (196). The PPAR α isoform was confirmed as the molecular target of fibrates when genetic deletion ameliorated the effects of clofibrate and a related compound, Wy-14,643 in mice (207). PPAR γ was shown to be the predominant isoform

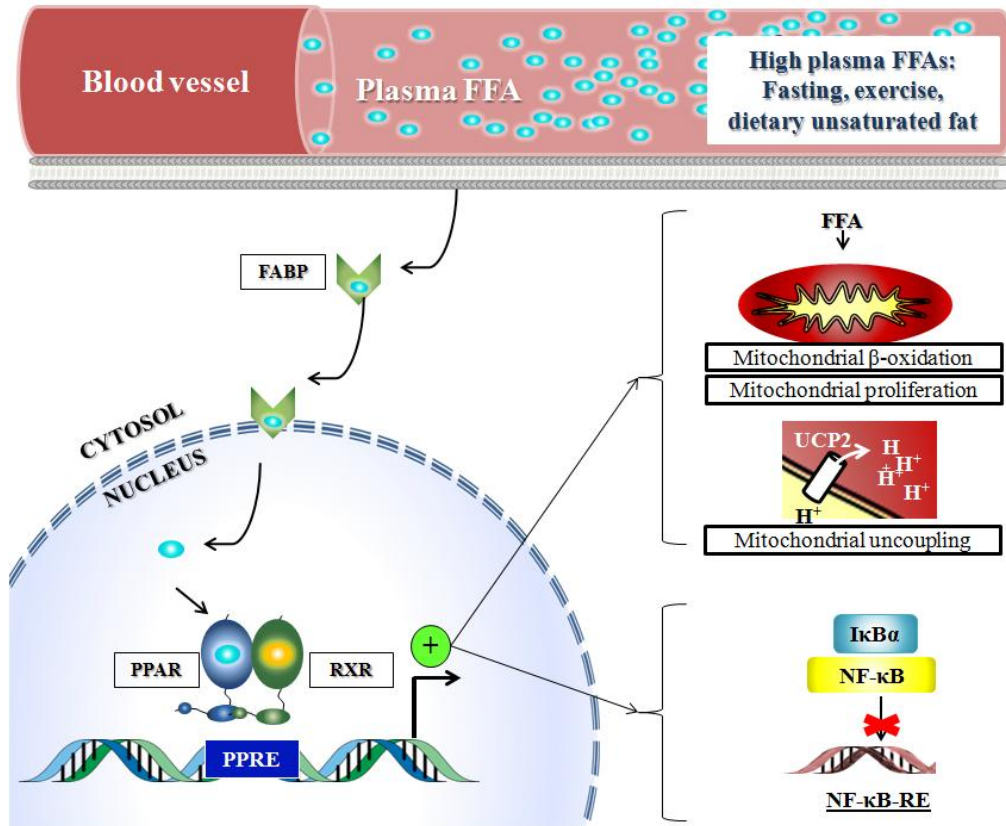


Figure 5. Peroxisome proliferator-activated receptors.

Circulating FFAs enter the cell and are transported to the nucleus by fatty acid binding protein (FABP). Once in the nucleus, FFAs are sensed by the peroxisome proliferator-activated receptors (PPARs) family of nuclear receptors. When bound to fatty acid ligands, these “fatty acid sensors” dimerize with their obligate heterodimer partner retinoid X receptor (RXR) and bind the promoter of target genes containing the consensus PPAR response element (PPRE) sequence, leading to transcriptional regulation of target genes.

in adipose (208) and was identified as the molecular target of TZD action the following year (209). The insulin sensitizing effects of TZDs were attributed to their ability to promote adipogenesis (210), thus reducing ectopic lipid accumulation and lipotoxicity in peripheral tissues such as liver and muscle (reviewed in (211)). PPAR δ exhibited low affinity for fibrates (212) and research into its function and target genes lagged behind the other isoforms prior to the introduction of a chemical agonist in 2000 and two potent, selective agonists in 2003 (213, 214).

PPAR biology

PPAR expression and function

Collectively, PPARs are thought to be activated by dietary and endogenous fatty acids and regulate the expression of a diverse collection of target genes involved in fatty acid metabolism, mitochondrial function and inflammation (Figure 5). PPAR α and PPAR γ are expressed in a tissue specific manner, which reflect their biological functions as regulators of hepatic and adipose lipid metabolism, respectively (215, 216). PPAR α is highly expressed in liver, where it regulates genes involved in fatty acid oxidation and lipoprotein metabolism (217-219), and is involved in the adaptive response to fasting (220) (Figure 6). PPAR γ is a master regulator of adipogenesis and adipose tissue function, and controls genes involved in fatty acid uptake and triglyceride storage (208, 211, 221) (Figure 6).

PPAR δ is ubiquitously expressed (212, 216) with the highest protein levels found in intestinal epithelium, liver, and keratinocytes (222) where it is involved in epithelial development, regulating lipid metabolism, and wound healing, respectively (223-226). In skeletal muscle, PPAR δ promotes the formation of oxidative, slow twitch fibers and is a key regulator of genes involved in fatty acid oxidation, mitochondrial biogenesis, electron transport and uncoupling (223, 227-231) (Figure 6). PPAR δ is a critical regulator of lipid metabolism and mitochondrial function in cardiomyocytes (232-234) and has similar effects on mitochondrial function and lipid metabolism in less oxidative cells, such as adipocytes (235) and pancreatic β -cells (236-238). Thus PPAR δ is positioned to be an important determinant of cellular and tissue lipid homeostasis, energy utilization and inflammation.

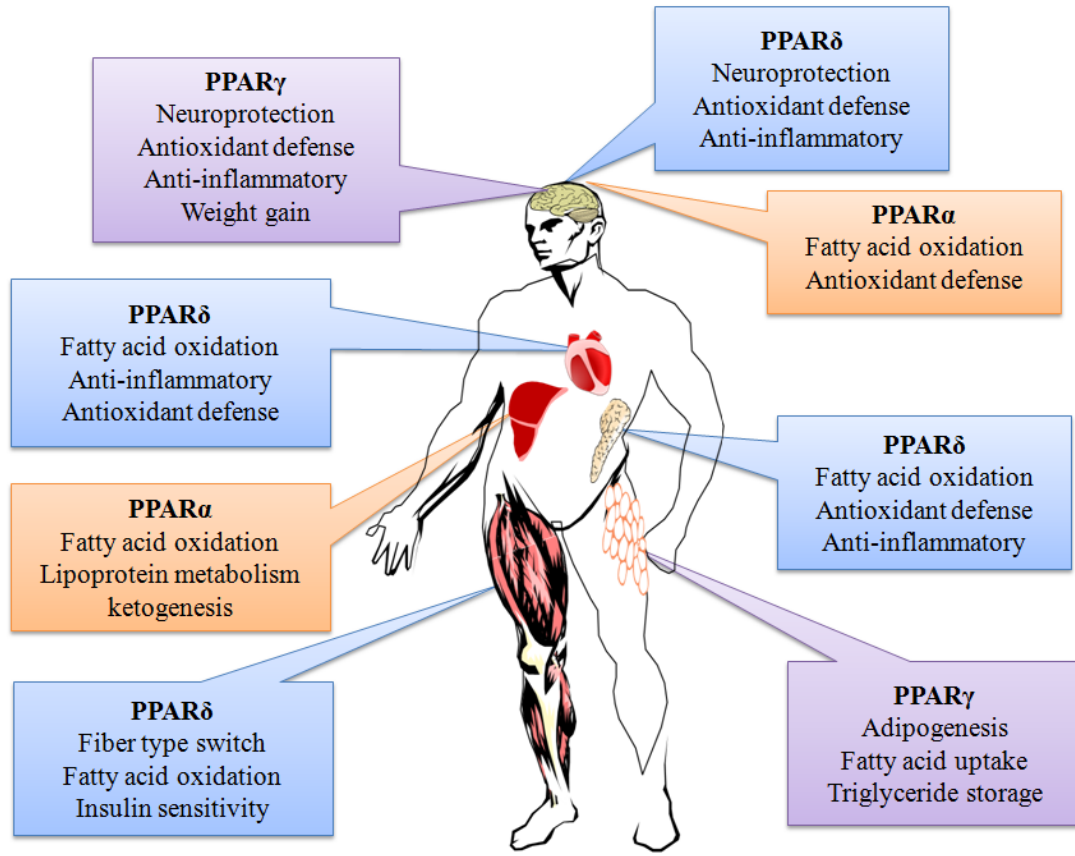


Figure 6. PPAR expression and function

Tissue expression and biological roles of PPAR isoforms are illustrated. In liver, PPAR α is involved in the adaptive response to fasting. PPAR γ is a master regulator of adipogenesis and adipose tissue function. PPAR δ is ubiquitously expressed and promotes fatty acid utilization as well as anti-inflammatory and antioxidant defense. All three isoforms are expressed in the CNS, where they are involved in neuroprotection.

Roles of PPAR isoforms in central energy homeostasis regulation

All three PPAR isoforms are expressed to different degrees in adult CNS (239). PPAR α and PPAR γ are expressed transiently during CNS development, while PPAR δ is expressed at high levels in embryonic and adult CNS (215). Very little is known regarding the role of centrally expressed PPAR isoforms in CNS lipid metabolism and energy balance regulation. PPAR γ is present in the medial basal hypothalamus, where it is co-expressed with NPY and POMC (117, 240). Deletion of a floxed allele of PPAR γ in neurons using Cre recombinase under the control of the Nex, a neuronal basic helix loop protein, promoter offers some protection against DIO (241).

Direct ICV administration of a chemical inhibitor of PPAR γ recapitulated the effects of neuronal deletion on body weight and also reduced food intake in rats (242). In line with this, ICV administration of a chemical agonist increased weight gain (242), raising the possibility that HF diet consumption pathologically activates neuronal PPAR γ facilitating obesity. PPAR α is expressed at extremely low levels in the CNS (215, 239); however, there is evidence that it regulates several of its canonical target genes involved in fatty acid oxidation as well as hypothalamic pituitary axis (HPA) axis function within the pituitary and hypothalamus in response to fasting (243-246). Activation of both PPAR α and PPAR γ in the CNS has also been shown to promote peripheral glucose utilization (241, 247). Although limited, this evidence suggests a critical role in the CNS for the PPAR family of receptors in regulating energy balance and peripheral glucose disposal as well as neuronal lipid metabolism.

Role of PPAR δ in the central nervous system

PPAR δ is the most prevalent PPAR isoform in the CNS and is expressed abundantly in neurons throughout the brain as well as some glial cells (239, 248). PPAR δ expression is particularly enriched in oligodendrocytes of corpus collosum, and agonists of PPAR δ promote oligodendrocyte differentiation and myelination in vitro (249-251). Higher levels of expression are found within neurons in the hippocampus, cortex and striatum, with the highest expression in the thalamus and hypothalamus (248, 252), where in situ hybridization and immunohistochemistry studies have shown it to be localized in neurons, cytosol and nucleus, with little expression in non-neuronal cells (239, 248).

Genetic deletion of the ligand binding domain (LBD) of PPAR δ , a region of the protein critical for transcriptional activation (253) leads to reduced brain size and myelination defects in corpus collosum of male mice (254), and altered CNS phospholipid composition in female mice (but not male) (255), providing some support for a role for PPAR δ in CNS lipid metabolism and myelin formation. Despite the fact that this potent transcriptional regulator is highly expressed in

the CNS, little is known of the transcriptional targets regulated by PPAR δ in the brain. More work is needed to understand the role of PPAR δ , the most highly expressed isoform, in the brain.

Neuroprotective effects of PPAR δ

Selective chemical modulators of PPAR α and PPAR γ , in particular, have potent anti-inflammatory and neuroprotective effects (256, 257). The first evidence that PPAR δ played an important protective role in the CNS came when PPAR δ LBD mutant mice showed increased susceptibility to ischemic injury, larger infarct size (258) and increased oxidative stress, the latter was accompanied by reduced expression of UCP2 (259). Studies conducted with highly selective PPAR δ agonists revealed them to possess extensive neuroprotective qualities. Agonists of PPAR δ attenuate brain inflammation, reduce demyelination in vitro (260) and reduce brain injury, in rodent models of neurodegenerative and neuroinflammatory diseases (261-263).

Molecular biology of PPAR δ

Structure

The mouse PPAR δ gene was cloned in 1994 (212), but had been first reported in 1992 as PPAR β in *Xenopus* (196) and as NUC1 in human cells (264). The mouse PPAR δ gene is contained in a 68,716 base pair (bp) region of DNA on chromosome 17 (Gene ID: 19015) containing 8 exons and encodes a 3,218 nucleotide (nt) mRNA transcript (NM_011145) that is translated to a 440 amino acid (aa) protein with a molecular weight of 49,584 kilodaltons (kD) (NP_035275). PPAR isoforms share a common structure with all nuclear hormone receptors by having 4 distinct domains; A/B, C, D, and E/F (Figure 7). While the A/B, activation function 1 (AF1), and E/F, LBD and activation function 2 (AF2), domains are poorly conserved regions, there is a high degree of sequence and structural homology in the C, DNA binding domain (DBD)

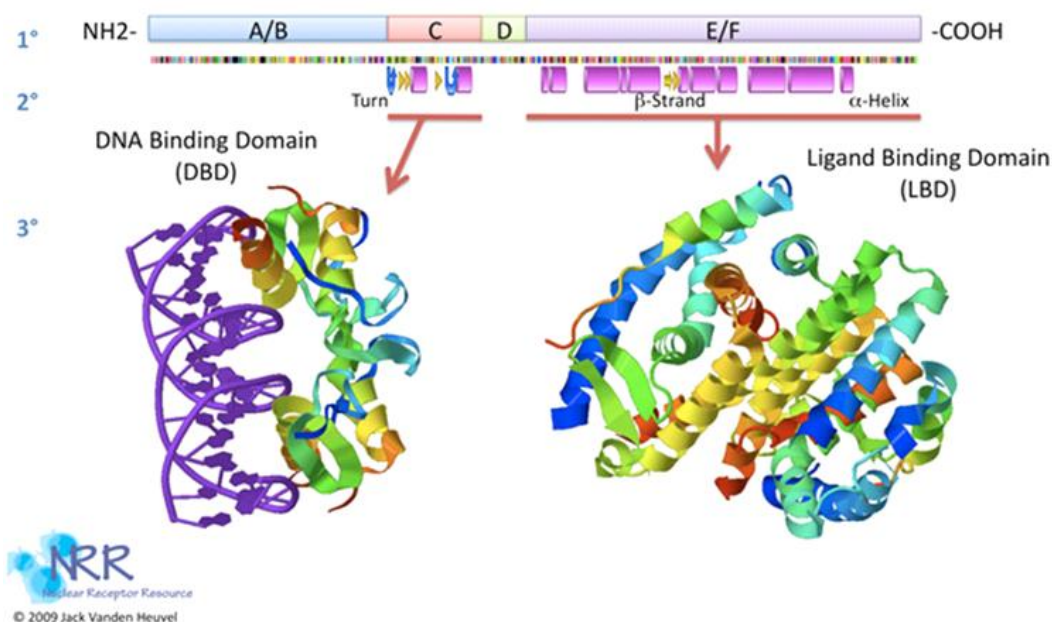


Figure 7. PPAR structural domains.

PPARs have a canonical nuclear hormone receptor structure with 4 distinct domains: N-terminal AF1 domain (A/B), DNA binding domain (DBD) (C), hinge region (D), and ligand binding domain (LBD) containing the C-terminal AF2 region (E/F). Image is from the Nuclear Receptor Resource Website .

and D, hinge region, domains across all isoforms. (265).

The highly variable AF1 region occupies the N-terminal end of the A/B domain (Figure 7) and is involved in ligand independent gene regulation and cofactor recruitment. Upon ligand binding, PPARs form heterodimers with RXRs that are activated by 9-*cis*-retinoic acid (Fig 7), and regulate gene expression through a direct interaction with PPAR response elements (PPREs) in target gene promoters that have a direct 1 (DR-1) repeat sequence (AGGNCA A AGGTCA) (206, 266) (Fig 9). The PPAR DBD possesses a canonical C₄ zinc finger motif, common to nuclear receptors. The α-helix motif in the proximal zinc finger of the P box region is involved in high affinity recognition of the PPRE core half site of the PPRE. The distal, D box region of the second zinc finger contains another α-helix that lies perpendicular to the P-box domain and mediates receptor dimerization (267, 268). Specificity of PPAR isoforms for target genes is detected when PPAR isoforms are co-expressed at similar levels in a single cell type (269), and

studies using chromatin immunoprecipitation sequencing and genome-wide analysis, show that PPARs have different affinities for specific genomic PPRE variants within target genes (269, 270). The PPAR LBD is flanked by the highly flexible D or hinge region and AF2, ligand-dependent activation domain, located near the C terminus (Figure 7) (271). The three PPAR subtypes have 60–70% amino acid sequence homology between their LBDs, resulting in a similar tertiary structure, composed of 13 α -helices and 4 β -sheet motifs, which fold to create a large (~1300Å), hydrophobic Y-shaped ligand binding pocket (272, 273).

Transcriptional regulation by PPAR δ

All of the PPAR isoforms induce target gene expression in response to ligand binding; however, PPAR δ is also a potent transcriptional repressor in the absence of ligands (274-276). The ability to repress or induce target gene expression is determined by ligand and co-regulator binding (277). PPAR δ represses basal transcription of PPRE containing target genes through a process termed ligand-independent repression (276). In the absence of ligand, the AF2 region assumes a three-turn α -helix structure that favors association with corepressor molecules, such as nuclear receptor corepressor (NCoR) and the related silencing mediator for retinoid and thyroid hormone receptor (SMRT) (278, 279) (Figure 8), which form large multi-protein complexes that include histone deacetylases (HDACs) and transducin beta-like protein-1 (TBL1) to actively repress gene expression (280-282). Agonist binding induces an allosteric shift in the C-terminal AF2 region, revealing a leucine zipper motif located in a conserved region of the LBD that serves as a docking site for RXR (283, 284). RXR hetero-dimerization releases corepressors in exchange for LXXLL motif containing coactivators, leading to formation of the “charge clamp”, a transcriptionally active form of the receptor (285-287) (Figure 8). In the charge clamp conformation, transcriptional coactivators such as steroid receptor coactivator-1 (SRC-1) and

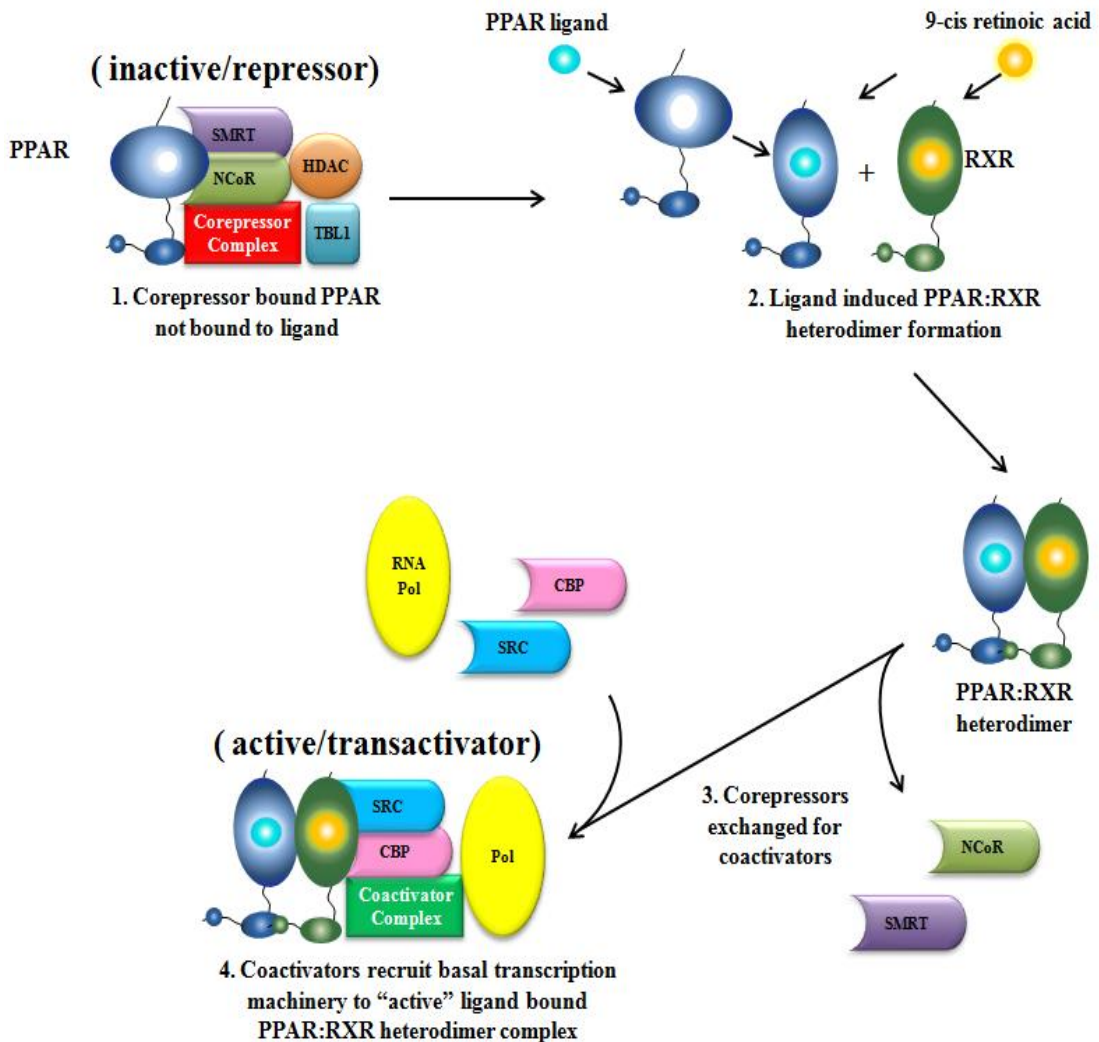
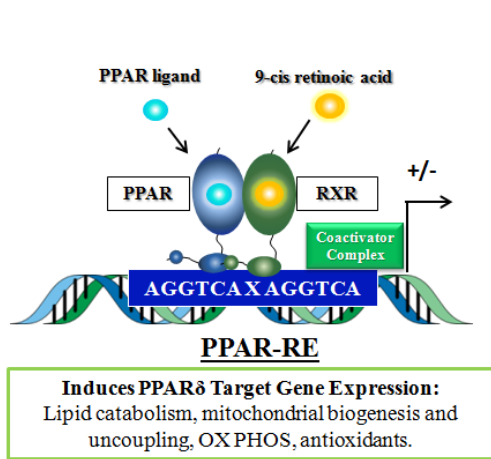


Figure 8. Steps involved in ligand-induced coregulator exchange.

1.) When not bound to ligands, PPAR δ associates with corepressors including nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) that form large multi-protein corepressor complexes containing histone deacetylase (HDAC) and transducin beta-like protein-1 (TBL1) to actively repress target gene expression. 2.) PPAR ligand binding induces dimerization with retinoid X receptor (RXR). 3.) PPAR:RXR heterodimer releases corepressors in exchange for coactivators, such as steroid receptor coactivator-1 (SRC-1) and CREB binding protein/p300 (CBP). 4.) CBP and SRC form a coactivator complex and recruit basal transcriptional machinery including RNA polymerase to the active, ligand bound PPAR:RXR heterodimer ("charge clamp" formation), leading to transcriptional activation and expression of target genes. Image adapted from Nolte et al. 1998 *Nature* 395, 137-143 and Perissi et al 2004. *Cell* 116, 511-526 .

Ligand-dependant Transactivation



Ligand-dependant Transrepression

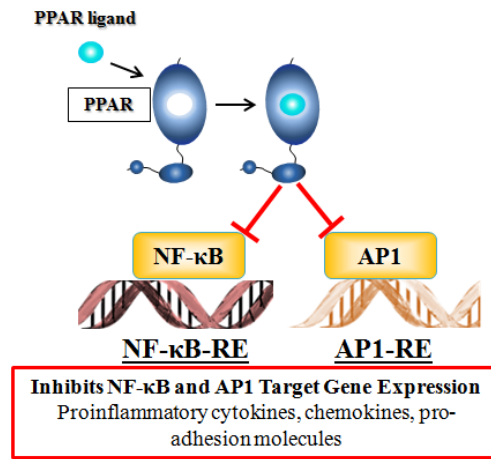


Figure 9. Ligand-dependant transcriptional regulation by PPAR δ :

Upon ligand binding, PPARs form a heterodimer complex with 9-*cis*-retinoic acid receptor (RXR), and regulate gene expression through a direct interaction with PPAR response elements (PPRE) in target gene promoters (Transactivation). PPARs reduce inflammation by preventing other transcription factors, such as NF- κ B and AP1, from binding to their DNA response elements (Transrepression). Image adapted from Coll et al. 2010. *PPAR research*.

CREB binding protein/p300 (CBP) bind and recruit basal transcriptional machinery to the target gene promoter. PPAR δ can also repress the expression of non-PPRE containing genes, by preventing other transcription factors from associating with their DNA response elements, a process termed ligand dependent trans-repression (Figure 9) (288, 289). Trans-repressed genes include inflammatory cytokines and other targets of NF- κ B as well as gene targets of the transcription factor, activator protein 1 (AP1) (290).

PPAR δ ligands

PPAR isoforms have different affinities for ligands due to differences in structure as well as charge of individual amino acids within the ligand binding pocket (291). PPAR ligands were first identified through the use of several different ligand screening assays which provided functional evidence that long-chain fatty acids (LCFA), prostaglandins, leukotrienes and eicosanoids were PPAR ligands (Table 1) (206, 292, 293). In one assay, ligands were screened for the ability to induce formation of the PPAR:RXR heterodimer, and activation of a consensus PPRE sequence fused to a luciferase reporter gene (292). Another functional assay was used to screen ligands for the ability to induce coactivator recruitment, which is a critical step for transcriptional activation (293). Crystallography and radioligand displacement helped consolidate findings from these two methods, as well as to provide quantitative affinities of fatty acids for PPAR LBDs (294). Data from these studies showed that LCFA can bind all PPAR isoforms with various affinities (292, 293, 295) (Table 1). PPAR α has a highly hydrophobic LBD and binds SFAs (C14-C16) with high affinity (1.1–5.4 μ M) in vitro (295) and in vivo (243). SFAs bind with slightly less affinity to the PPAR δ LBD (295), but do not activate expression of a PPAR δ reporter construct to any significant degree (292).

Table 1. PPAR δ ligands

Ligand	Comment	Reference
Oleic acid (C18:1)	MUFA, agonist (PPAR δ /PPAR α /PPAR γ)	(237, 293, 296, 297)
Linoleic acid (C18:2)	MUFA, agonist (PPAR δ /PPAR α /PPAR γ)	(292, 293, 295)
Linolenic acid (C18:3)	PUFA, agonist (PPAR δ /PPAR α /PPAR γ)	(293, 295, 298)
Arachidonic acid (C20:4)	PUFA, agonist (PPAR δ /PPAR α /PPAR γ)	(292, 293, 295)
Docosahexaenoic Acid (C22:6)	PUFA, agonist (PPAR δ /PPAR α)	(292, 293)
GW0742 and GW501516	Potent, selective chemical agonist (PPAR δ) 1000-fold selectivity	(214)
L165,041	Selective chemical agonist (PPAR δ), 100-fold selectivity	(292)
Palmitic acid (C16:0)	SFA, non-agonist ligand (PPAR δ) Agonist (PPAR α)	[47]
GSK0660	Highly selective antagonist (PPAR δ), 100-fold selectivity	(299)

Chemical ligands include L-165041, a potent PPAR δ agonist with 100-fold selectivity over other subtypes (300), and with 1000-fold greater selectivity, GW0742 and GW501516 (214, 301). GSK0660 is a potent selective inverse agonist with 100-fold selectivity over other isoforms (299). GW0742 and GSK0660 were utilized in cell culture and in vivo experiments described in this dissertation.

PPAR δ : Roles in obesity and metabolic disease

Genetic mutations of PPAR δ

Genetic mouse models have been used to study the function of PPAR δ in development, metabolism and disease in mice. Constitutive deletion of the N-terminal half of the DNA binding domain (221) led to over 90% embryonic lethality of knockout animals, whose death were due to

placental defects. Surviving knockout animals were smaller and had little brown or white adipose tissue on chow diet, but became profoundly obese when placed on a high fat diet (302). Deletion of the C terminal 60 amino acids, which encompasses part of the LBD and the AF2 region of the protein was not lethal, but mice exhibited abnormal growth, reduced adipose and skin defects (303, 304) with alterations in brain lipid metabolism.

To avoid the high rate of embryonic lethality of the global deletion mutant, a conditional “floxed” allele with lox-P sites flanking exon 4 (DBD encoding region) has been utilized to delete PPAR δ in a tissue specific manner (221). Deletion of PPAR δ in cardiomyocytes leads to reduced basal expression of fatty acid oxidative genes, cardiomyopathy and lipotoxicity (305). Inducible deletion of the same floxed allele in heart reduced the expression of genes involved in mitochondrial biogenesis and function, supporting a role for PPAR δ in mitochondrial function (234). The conditional allele has also been deleted in a number of tissues (reviewed in (306)), including skeletal muscle, where genetic deletion results in lipotoxicity, insulin resistance and obesity (307).

Effects of PPAR δ agonists on lipotoxicity and insulin resistance

Agonists of PPAR δ oppose HF diet-induced inflammation and insulin resistance in vivo, and reduce palmitate induced lipotoxicity and insulin resistance in vitro (Table 2). These protective effects of chemical agonists to prevent inflammation may be mediated by induction of several PPAR δ target genes involved in mitochondrial fatty acid oxidation and uncoupling (231, 234, 236, 238, 308). Agonist treatment is also associated with reductions in mRNA levels of the NF- κ B target genes, IL-6 and IL-1 β , whose expression are induced by palmitate exposure (231, 308), which improves insulin sensitivity (231, 307, 309-311). As a whole, these studies suggest that PPAR δ agonists should reduce inflammation and improve hypothalamic insulin sensitivity; and therefore, neuronal PPAR δ may be an attractive pharmacological target in the treatment of DIO.

Table 2. Effects of PPAR δ agonists on lipotoxicity and insulin resistance

	Effect	Comment	System	
Ischemia and GW0742	Increase cardiac fatty acid oxidation, prevent apoptosis and improve insulin sensitivity	Reduce expression: IL-6, MCP1, IL-8, and ICAM-1	Zucker fatty rat, heart	(309)
GW501516 and HFD or palmitate	Reduce inflammation and prevent hypertrophy	Reduce expression: TNF α , MCP-1 and IL-6	Mouse heart, cardiac cells	(308)
Palmitate and GW501516	Reduce inflammation, prevent DAG and PKC accumulation, and improve insulin sensitivity	Increase expression: CPT-1 and PDK4. Reduce expression: IL-6	Skeletal muscle cells	(231)
Palmitate and GW501516	Reduce inflammation (JNK)	Increase expression: Maf-A and GLUT2	Pancreatic β -cells	(312)
Palmitate and GW501516	Maintain mitochondrial function and prevent apoptosis	Increase expression: CPT-1, LCAD, PDK4 and UCP2	Pancreatic β -cells	(238)
Palmitate and GW501516	Maintain mitochondrial function	Increase expression: PGC1a, NRF-1, mtTFA and UCP2.	Pancreatic β -cells	(236)

Rationale and Hypothesis

Consumption of a HF diet has been shown to lead to inflammatory signaling in key neuronal subsets involved in the regulation of energy homeostasis (74, 133, 137, 313) resulting in behavioral and biochemical resistance to insulin, leptin and other regulatory hormones and nutrient signals in the CNS. MUFAs in the diet are beneficial and improve insulin sensitivity (187-192). Diets rich in MUFAs are also less obesogenic than those high in SFAs (184-186). The MUFA oleate has anti-inflammatory properties and can prevent palmitate induced insulin resistance in skeletal muscle cells (314-316). This suggested that substitution of SFAs with MUFAs in the diet may offer some protection against the development of DIO related hypothalamic inflammation as well.

My dissertation work has focused on understanding how different types of dietary fats modulate the signaling events that lie upstream of inflammation and CNS hormone resistance. Chapter III explores how dietary fat composition influences the development of DIO related

hypothalamic inflammation and insulin resistance. The protective effects of MUFAs have been attributed to a diverse group of “fatty acid sensor” molecules that bind fatty acids. Of these, PPAR δ was a likely candidate based on its high expression in the CNS, its ability to regulate genes that increase fatty acid oxidation, mitochondrial biogenesis and induce mitochondrial uncoupling. Additionally, chemical agonists of PPAR δ are protective against palmitate induced inflammation and insulin resistance in peripheral tissues, have neuroprotective and anti-inflammatory effects and attenuate oxidative stress in the CNS. This evidence supported two complementary hypotheses, the first being that circulating or endogenous fatty acids act as ligands and activate neuronal PPAR δ to increase expression of its target genes in neurons. The literature also supports a second hypothesis, that PPAR δ is a protective MUFA sensor, which opposes lipotoxicity and supports insulin and leptin sensitivity in hypothalamic neurons, and provides protection from DIO (Figure 10). The results of experiments directly testing these hypotheses in an immortalized hypothalamic neuronal cell line, treated with different combinations of a SFA and MUFA, as well as selective chemical ligands of PPAR δ to directly probe the interaction between fatty acids and PPAR δ are described in chapter IV.

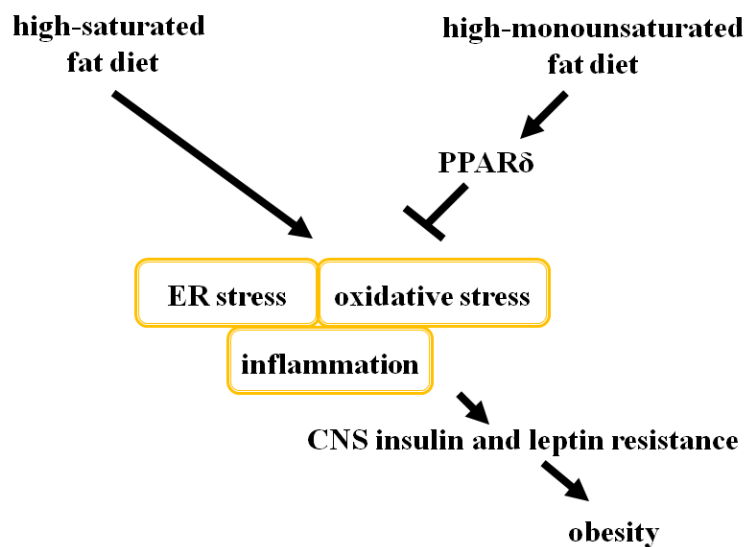


Figure 10. Hypothesis: PPAR δ is a protective monounsaturated fatty acid sensor in neurons.

Chapter V investigates the effects of PPAR δ deficiency on energy balance, DIO, hypothalamic inflammation and hypothalamic gene expression. A genetic deletion mouse model was used to test the hypothesis that loss of PPAR δ would cause CNS stress and inflammation, insulin and leptin resistance and increased susceptibility to obesity. A summary of the results from these studies is contained in chapter VI and placed in context of what is known about the PPAR family of receptors in the CNS, as they relate to energy balance, as well as suggested paths of future research.

CHAPTER II

MATERIALS AND METHODS

Experimental animals: rats

Eucaloric high-fat diets

Male, Long Evans rats (Harlan; Indianapolis, IN) were acclimated to a purified control diet with LF content (kcal%: fat 10%, carb 70%, protein 20%) (D12450B, Research Diets; New Brunswick, NJ) (Table 3). All animals were housed in a temperature (22 °C) and light (12 hour light/dark cycle) controlled room with free access to food and water except where indicated. Body weight and food intake were measured on a standard balance. Body composition was measured by nuclear magnetic resonance (NMR)/magnetic resonance imaging (MRI) in a Body Composition Analyzer (Echo MRI; Houston, TX). Rats were randomized into groups based on adiposity (fat mass/total body weight x 100) and housed in pairs to receive LF control diet or one of two micronutrient matched HF diets containing 45% of total calories as fat (45% fat, 35% carb, 20% protein) but different fatty acid compositions, one enriched in SFAs (formulated from lard: HF-SFA; Research Diets, D12451) and the other a custom formulated diet enriched in MUFAs (formulated from high-oleic safflower oil: HF-MUFA; Research Diets) (Table 3).

Table 3. Experimental high-fat diets

	D12450B	D12451	_____
Fatty acids	LF (10%)	HF-SFA (45%)	HF-MUFA (45%)
C14:0	0.8 (0.1)	0.8 (0.4)	0.1 (0.0)
C16:0	22.6 (2.3)	22.6 (10.2)	5.8 (2.6)
C18:0	12.9 (1.3)	12.9 (5.8)	2.0 (0.9)
Σ SFA	36.3 (3.6)	36.3 (16.3)	8.3 (3.8)
C16:1	3.5 (0.4)	3.5 (1.6)	-
C18:1 ω 9	41.3 (4.1)	41.3 (18.6)	71.4 (32.1)
Σ MUFA	45.3 (4.5)	45.3 (20.4)	71.7 (32.2)
C18:2 ω 6	14.9 (1.5)	14.9 (6.7)	19.1 (8.6)
C18:3 ω 3	1.9 (0.2)	1.9 (0.9)	1.0 (0.4)
C20:4 ω 6	1.6 (0.2)	1.6 (0.7)	-
C22:6 ω 3	-	-	-
Σ PUFA	18.5 (1.8)	18.5 (8.3)	20 (9.0)

ICV surgery and administration of GW0742

Male, Long-Evans rats weighing 300-350 g underwent third ventricle cannulation. Rats were placed in a Stoelting stereotaxic apparatus (Stoelting Co; Wood Dale, IL) and the head secured and prepared for sterile surgery. A 1 cm incision was made at the apex of the skull, and a small piece of bone (2 mm x 2 mm) was gently removed using a battery powered drill. A 22-gauge fused silica guide cannula (#C313G/FS; Plastics One; Roanoke, VA) fitted with a plastic internal cannula (#C313I/PK; Plastics One; Roanoke, VA) was inserted into the third ventricle using coordinates; anteroposterior, 0.2 mm; lateral, 1.5 mm; depth, 4.0 mm. The device was fixed to the skull with three nylon mounting screws and acrylic cement. Following a 7 day recovery period, rats were randomized to receive an ICV bolus of vehicle (in 2 μ L dimethyl sulfoxide (DMSO)) or the PPAR δ agonist GW0742 (4-[2-(3-fluoro-4-trifluoromethylphenyl)-4-methylthiazol-5-ylmethylsulfanyl]-2-methylphenoxy)-acetic acid; Cayman Chemical; Ann Arbor, Michigan) (50 μ g total in 2 μ L DMSO). After 24 hours, rats were anesthetized by CO₂ inhalation, and were euthanized by decapitation.

Effects of high-fat diet on hypothalamic oxidative stress

Male, Long Evans rats (Harlan; Indianapolis, IN) were housed individually and acclimated to IP injections (saline, 2 mL) for 7 days. A LF control diet (Table 3) was administered during the acclimation period. Rats were randomized into groups based on adiposity to receive LF control diet or HF diet (Table 3). Groups of LF and HF fed rats received the mitochondria targeted antioxidant MitoTEMPO (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride monohydrate) (Alexis; San Diego, CA) (1.5mg/kg/day, IP) or vehicle (saline). Fresh solutions of MitoTEMPO were prepared every two days in saline and stored at 4°C. After 7 days, rats were fasted for 4 hours then received heparin (1U/kg, IP), were anesthetized by CO₂ inhalation, and were euthanized by decapitation.

Experimental animals: Mice

Neuronal PPAR δ knockout model

Transgenic mice were obtained from Jackson Laboratories (The Jackson Laboratory; Bar Harbor, Maine) and were backcrossed to a C57Bl6 background for greater than 6 generations. Mice homozygous for a conditional allele of PPAR δ , containing Lox P sites flanking exon 4 of the PPAR δ gene (B6.129S4-PPAR δ tm1Rev/J) (302) (Figure 11) were mated with mice that express Cre recombinase under the control of the rat nestin promoter (317) (B6.Cg-Tg (Nes-cre)1Kln/J) (The Jackson Laboratory). These crosses resulted in heterozygous offspring (PPAR $\delta^{f/wt}$ Nestin-cre^{+/-}), which were mated together, or crossed to PPAR $\delta^{f/f}$ mice to produce neuronal PPAR δ knockout mice, referred to as KO (PPAR $\delta^{f/f}$ cre⁺) and “floxed” littermate (f/f) control mice (PPAR $\delta^{f/f}$ or PPAR $\delta^{f/wt}$).

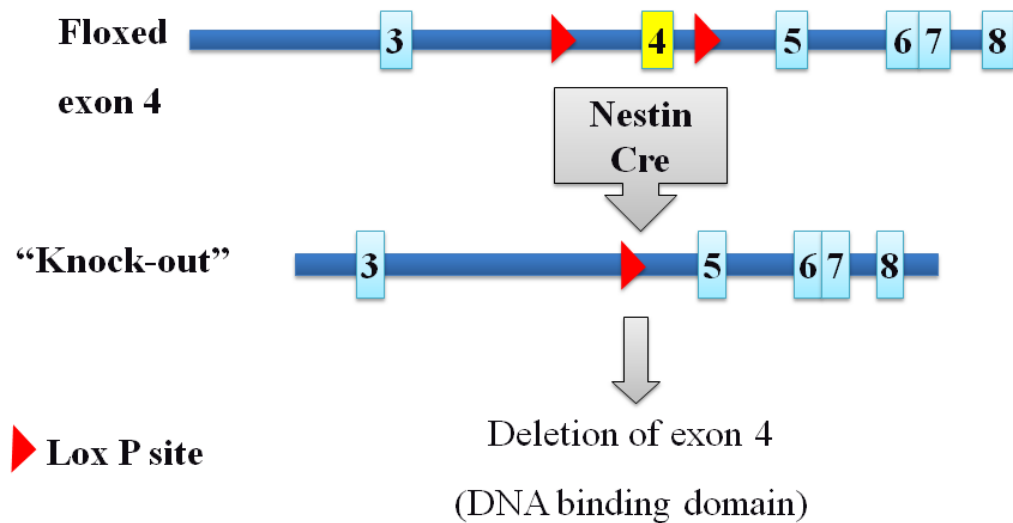


Figure 11. PPAR δ lox-P knock-out model.

CNS deletion of PPAR δ was achieved using mice that express a “floxed” PPAR δ allele containing Lox P recognition sites flanking exon 4, which encodes the amino terminal portion of the DNA binding domain of the protein (Figure 7). Nestin-Cre mediated recombination leads to excision of 160 bp region of DNA containing exon 4 region resulting in a mutated protein that lacks a 51 amino acid, ~5.24 kb region of the DNA binding and hinge region, and hence does not bind DNA.

Diet-induced obesity

Baseline body weight and body composition was assessed at 5 weeks of age. Mice were grouped by genotype and housed 3 to a cage. PPAR δ KO mice and their litter mate controls were studied on LF and HF diet (Table 3). Body weight and food intake were measured using a standard balance, and body composition was measured with the Minispec TD-NMR Body Composition Analyzer (Bruker Optics; Billerica, MA).

Indirect calorimetry

Energy expenditure (EE) was assessed by indirect calorimetry in 12-week-old chow fed mice and after 20 weeks on HF diet. Mice were housed individually in Oxymax cages (Columbus Instruments; Columbus, Ohio). VO₂ and VCO₂ (mL/hr) were measured after a 24 hour

acclimation period and calculated based on the input and output rates of O₂ consumption and CO₂ production. The RER ($RER = VCO_2 / VO_2$) and heat production ($kcal/hr = (3.815 + 1.232 \times RER) \times (VO_2)$) were calculated using software provided by the manufacturer (Columbus Instruments; Columbus, Ohio). EE data (kcal/hr) were normalized to body weight (g) and also lean mass (g) as measured by NMR on the day mice were placed in the chambers.

Glucose tolerance test

Glucose tolerance testing was performed in 12-week-old chow fed mice and after 20 weeks on HF diet. Mice were fasted for four hours prior to receiving an IP glucose bolus injection (D-glucose, 1 g/kg body weight in 200 μ L saline). Blood glucose was measured using a Freestyle handheld glucometer (Abbott Labs; Abbott Park, IL) in blood obtained from a small incision made at the tip of the tail with a sterile razor blade. Readings were made at baseline and at 5, 15, 30, 60, 90 and 120 minutes after glucose was administered.

Leptin sensitivity

To assess behavioral leptin sensitivity, individually housed, male, KO and f/f control mice were acclimated to IP injections (saline, 300 μ l) for 7 days. On the day of the study, food was withdrawn at 2 PM and mice were fasted for 4 hours. A known quantity of food was placed in the cage at 6 PM, at which time mice received an IP injection of vehicle (0.3 mL saline) or leptin (5 μ g/g body weight) (ProSpec; East Brunswick, NJ). Total caloric intake and food intake normalized to body weight were determined for the 24 hour period after leptin treatment. Cellular leptin sensitivity was assessed in a second group of mice treated similarly. Hypothalami were collected 30 minutes after leptin was injected and processed for immunological detection of STAT3 phosphorylation by Western blot analysis (see below).

Fasting-refeeding challenge

The effects of fasting on body weight, food intake and hypothalamic gene expression of NPY, POMC and UCP2 were determined in chow fed male, KO and f/f mice. To assess changes on body weight, body composition and food intake after prolonged fasting, mice were housed individually. On the day of the study, baseline body weight and body composition were measured and food was withdrawn at 10 AM. Mice were fasted for 24 hours and the measurements were repeated at the end of the fasting period. Food intake and weight gain were measured over a 24-hour refeeding period during which mice had free access to chow diet. Hypothalamic neuropeptide gene expression was determined in a second group of f/f and KO mice. Mice were given ad libitum access to chow diet, or fasted for 24 hours beginning at 10 AM. After 24 hours, both the fasted and fed groups of mice were anesthetized by isoflurane inhalation and euthanized by decapitation.

Hypothalamic-pituitary-adrenal axis function

HPA axis function was assessed in 12-week old, single-housed, chow-fed mice. Nadir blood samples (>100 μ L) were collected from the submandibular vein at 8 AM, and in a similar manner, peak samples were taken at 5 PM three days later. Stress induced corticosterone levels were measured after another 3 day recovery period. Mice were subjected to one minute of mild handling stress, returned to their home cages for 30 minutes, after which blood was collected via the submandibular vein. Only individual samples that were collected within one minute were included in the analysis.

Histology

Mice were anesthetized with sodium pentobarbital (60 mg/kg) and given heparin (1 U/kg). A vertical incision was made along the midline of the chest, and then mice were transcardially perfused with 250 ml of 4% formaldehyde in phosphate buffered saline (PBS; 0.1

M, pH 7.4). Brains were removed and post-fixed overnight in 4% formaldehyde. Coronal sections at 40 μm were made with a freezing microtome, sections were mounted on gelatin coated slides, and stained with Nissl stain (0.1% cresyl violet) (318). Epididymal white fat tissue samples were collected from chow fed mice and after 33 weeks of HFD exposure, fixed for 24 hours in 4% paraformaldehyde, then transferred to 70% ethanol before paraffin embedding and hematoxylin and eosin (H&E) staining (Vanderbilt Histology Core). Series of nonadjacent sections were processed for each staining protocol. Images were collected at 10x magnification using bright field light microscopy and qualitatively examined by an experimenter blinded to genotype.

Medial basal hypothalamus wedge dissection

Brains were rapidly removed and flash frozen under powdered dry ice and coronally sectioned at the optic chiasm and the rostral edge of the pituitary stalk. A wedge containing the arcuate nucleus, ventral and dorsal medial nucleus, and medial edge of the lateral hypothalamus were dissected with a razor blade, rapidly frozen and stored at $-80\text{ }^{\circ}\text{C}$.

Hypothalamic neuronal cell culture

Cell culture conditions

Clonal mouse hypothalamic mHypoE-N42 (319) neuronal cells were obtained from Cedarlane Laboratories Ltd. (Burlington, Ontario Canada). Cells cultured in 150 cm^2 flasks and maintained at $37\text{ }^{\circ}\text{C}$ with 5% CO_2 in low-glucose Dulbecco's Modified Eagle Medium (DMEM) growth media (D-glucose, 1 g/L; L-glutamine, 0.584 g/L; sodium bicarbonate, 3.7 g/L; and pyruvate, 110 mg/L) with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY). Experimental treatments were performed on cells plated in 100 mm culture plates.

Chemical reagents

Stock solutions (2 mM) of the PPAR δ agonist GW0742 (Cayman Chemical; Ann Arbor, MI) and the PPAR δ antagonist GSK0660 (3-[[[2-Methoxy-4-(phenylamino)phenyl]amino]sulfonyl]-2-thiophenecarboxylic acid methyl ester; Sigma Aldrich; St. Louis, MO) (299) were prepared in DMSO and stored at -20 °C. Cells were treated with vehicle (0.05% DMSO) or chemical PPAR δ ligands at final concentrations of 1 nM (GW0742) or 1 μ M (GW0742 or GSK0660) for 24 hours.

Fatty acid treatment in cell culture

Stock solutions (100 mM) of palmitate (C16:0) and oleate (C18:1) (Sigma Aldrich; St. Louis, MO) were prepared in 0.1 N NaOH (70 °C for 30 minutes). Concentrated stock solutions of fatty acids (FFA) were added to a solution of ultra-pure, fatty-acid-free, bovine serum albumin (10%, cell culture grade water) (Sigma Aldrich; St. Louis, MO), and left at room temperature for 30 minutes to allow FFA:BSA conjugation. Serum-free DMEM was added to give a final concentration of either FFA at 100 μ M with excess BSA (150 μ M) (final FFA:BSA ratio of 0.67:1). To determine if PPAR δ protects neurons from FFA induced lipotoxicity in DIO, the concentration of BSA was held at 100 μ M and mHypoE-N42 cells were treated with a mixture of oleate 100 μ M and palmitate 250 μ M (FFA:BSA ratio of 3.5:1) with or without the PPAR δ inhibitor GSK0660 (1 μ M).

Insulin signaling in cell culture

Following treatment with FFAs, cells were stimulated with recombinant human insulin (10 nM, 5 minutes) (Sigma Aldrich; St. Louis, MO). Media was rapidly removed, and cells were rinsed with ice cold PBS, harvested by scraping directly into protein extraction buffer and immediately frozen on dry ice.

Molecular and biochemical techniques

Genotyping of mice

Genotyping of mice for the “floxed” PPAR δ allele and Nes-Cre transgene was performed by PCR using DNA extracted from tail clippings (1-2 mm) by the Hot Shot method (320). Clippings were heated in an alkaline lysis reagent (100 μ L, 25 mM NaOH, 0.2 mM EDTA, pH 12) on a heat block (90 $^{\circ}$ C, 25 minutes) with constant shaking, after which an equal volume of neutralization reagent (40 mM Tris HCL, pH 5) was added. PCR was performed with 2 μ L of DNA using 5Prime PCR Supermix (5Prime; Gaithersburg, MD) (annealing temperature 65 $^{\circ}$ C, 40 cycles) and appropriate PCR primers for the floxed PPAR δ allele (common: 5' GAG CCG CCT CTC GCC ATC CTT TCA G 3' and wild type 5' GGC GTG GGG ATT TGC CTG CTT CA 3') as well as for the nestin cre transgene (5' GCG GTC TGG CAG TAA AAA CTA TC 5' and 5' GTG AAA CAG CAT TGC TGT CAC TT 3') (annealing temperature 57 $^{\circ}$ C, 40 cycles). The amplified cDNA was separated by electrophoresis on an agarose gel (nestin, 1%; agarose; PPAR δ , 2.5% agarose; 0.5 g/mL ethidium bromide (EtBr), 1X Tris-acetate (TAE) composed of 40 mM Tris (pH 7.6), 20 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)) followed by ultraviolet detection of bands stained with EtBr. Mice were genotyped based on the presence of expected PCR products for the PPAR δ allele; homozygous floxed mutant (400 bp), heterozygotes (359 bp and 400 bp) and wild type mice (359 bp). A single band (100 bp) indicated the presence of the Nestin-Cre transgene.

Measurements of plasma hormones and metabolites

Trunk blood was collected into EDTA treated 3 ml tubes and plasma was separated by centrifugation (20 min, 4 $^{\circ}$ C, 1,500 xg) then stored in 1.5 mL tubes at -80 $^{\circ}$ C. Plasma levels of insulin, leptin and corticosterone were quantified by radioimmunoassay by the Vanderbilt Diabetes Center Hormone Assay Core. Plasma triglycerides and free fatty acid levels were determined by enzymatic assay (Waco Chemicals; Richmond, VA).

Brain and plasma free fatty acid composition analysis

Lipids were extracted from whole brain or plasma using the method of Folch-Lees (321). The extracts were filtered, and lipids recovered in the chloroform phase. Individual lipid classes were separated by thin layer chromatography using Silica Gel 60 A plates (Sigma Aldrich; St. Louis, MO) developed in petroleum ether, ethyl ether, acetic acid (80:20:1), and visualized by rhodamine 6G. Diglycerides and triglycerides were scraped from the plates and methylated using BF₃/methanol as described by Morrison and Smith (322). The methylated fatty acids were extracted and analyzed by gas chromatography. Gas chromatographic analyses were carried out on an Agilent 7890A gas chromatograph (Agilent; Santa Clara CA) equipped with flame ionization detectors, a capillary column (SP2380, 0.25 mm x 30 m, 0.25 µm film, Supelco, Bellefonte, PA). Helium was used as a carrier gas. The oven temperature was programmed from 160 °C to 230 °C at 4 °C/min. Fatty acid methyl esters were identified by comparing the retention times to those of known standards. Inclusion of lipid standards with odd chain fatty acids permitted quantitation of the amount of lipid in the sample.

Western blot analysis

Frozen tissues or cultured cells were sonicated in cold TPER buffer (10 µL/mg tissue) with added protease and phosphatase inhibitors (Promega; Madison, WI) with a Q55 Sonicator (Misonix Sonicators; Newton, CT) (setting 3, <10 seconds). Protein levels were measured with the Micro BCA Protein Assay Kit (Thermo Scientific; Rockford, IL). Diluted protein samples (1 mg/mL) were mixed with 4X Sample Buffer and 20X Reducing Agent (Bio-Rad; Hercules, CA), heated to 85 °C for 10 min, and immediately loaded into a well on a gel (20 µg total protein per lane) along with a Kaleidoscope Precision Plus Protein Standard (Bio-Rad; Hercules, CA). Samples were subjected to denaturing electrophoresis on a 4-12% Tris Acetate gel with XT Tricine Running Buffer using the Bio-Rad XT Criterion System (Bio-Rad; Hercules, CA). Protein was transferred to 0.2 µm nitrocellulose or 0.45 µm polyvinylidene fluoride (PVDF) (Millipore;

Billerica, MA) membranes using the Criterion Blotter module, according to the manufacturer's instructions (Bio-Rad; Hercules, CA). Membranes were blocked in Starting Block T20 Blocking Buffer (Thermo Scientific, Rockford, IL) for 1 hour at room temperature and incubated in primary antibodies (diluted 1/1000) in blocking buffer at 4 °C with gentle rocking overnight. Blots were washed in TBS-T (Tris Buffered Saline; 150 mM NaCl, 20 mM Tris pH 7.5 with 0.1 (v/v) Tween 20 (Sigma Aldrich; St. Louis, MO) for (3 x 10 minutes) at room temperature, and incubated with species specific horse radish peroxidase-conjugated secondary antibody in 50/50 blocking buffer and TBS-T for 1 hour at room temperature and washed in TBS-T (3 x 10 minutes). Antibody detection was performed with ECL Enhanced Chemiluminescence Substrate Kit (Amersham Biosciences; Piscataway, NJ) and BioMax XAR scientific imaging film (Kodak; Rochester, NY). Band intensity from X-ray film detection was analyzed by densitometry using ImageJ software from the National Institutes of Health.

Primary antibodies used for immunological detection were: anti-p(Y705)Stat3 (#2687, Cell Signaling; Danvers, MA), anti-p(T980)PERK (#3179, Cell Signaling), anti-p(S473)Akt (#9271, Cell Signaling), anti phosphorylated p66 Src homology 2 domain containing transforming protein (p66shc) p(S36)p66shc (6E10, Santa Cruz; Santa Cruz, CA), anti-IkBa (#9242, Cell Signaling), anti-PERK (#3192, Cell Signaling), anti-STAT3 (#4904, Cell Signaling), anti- β -tubulin (#2146, Cell Signaling), heat shock cognate protein 70 (HSC70) (K-19, Santa Cruz) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#2118, Cell Signaling). Secondary antibodies used for immunoblotting included: horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000; W401B, Promega; Madison, WI) and bovine anti-goat IgG (1:7500; sc-2350 Santa Cruz; Santa Cruz, CA).

Real-time PCR

Total RNA was extracted from brain tissues using the RNAqueous kit (Ambion; Austin, TX) or from confluent, 100 mm cell culture plates using Trizol (Invitrogen; Grand Island, NY).

RNA was precipitated with an equal volume of lithium chloride (1:1 dilution, 7.5 M LiCl), mixed, stored at -20 °C overnight, and the pellet collected by centrifugation (13,000 xg, 20 minutes, 4 °C). The pellet was washed with 70% ethanol, allowed to dry, and after which, RNA was re-suspended in a small volume (~20µl) of ultra-pure molecular biology grade water. Sample RNA concentrations were quantified on a Nanodrop 1000 spectrophotometer (Nanodrop; Wilmington, DE). RNA integrity was determined using a total of 2 µg of RNA by electrophoresis on a agarose gel (1.5% agarose, 0.5 µg/mL EtBr, 1X TAE) followed by UV detection of 28S, 18S and 5S RNA stained with EtBr. A cDNA template was synthesized from equal amounts of each sample of RNA (1-2 µg) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Carlsbad, California). Target gene mRNA expression was analyzed with iQ SYBR green Supermix reagent (Bio-Rad; Hercules, CA) by real-time PCR (RT-PCR) on an iCycler PCR machine (Bio-Rad; Hercules, CA). Standard curves (six-point serial dilution) were generated using cDNA synthesized from XpressRef™ mouse universal total RNA (SuperArray Bioscience; Frederick, MD) and used to calibrate relative quantification of product in the exponential phase of the amplification curve in all experiments at 95-105% amplification efficiency. Target gene expression was normalized to endogenous expression of the internal control 60S ribosomal protein L13A (RPL13A). Real time primers were previously published or designed using Beacon Designer (Beacon Design Software; Palo Alto, CA) (Vanderbilt Molecular Cell Biology Resource Cores) (Table 4).

Detection of superoxide production in rat hypothalamus

Hypothalami were dissected into quarters in ice cold Krebs HEPES buffer (99mmol/L NaCl; 4.7mmol/L KCl; 1.2mmol/L MgSO₄; 1.0mmol/L KH₂PO₄; 1.9mmol/L CaCl₂; 25mmol/L NaHCO₃; 11.1mmol/L glucose; 20mmol/L NaHEPES). Mitochondria derived superoxide levels were determined by HPLC using the fluorescent probe MitoSOX (Excitation/Emission: 510/580 nm, Invitrogen; Carlsbad, CA). Samples were supplemented with 4 µM MitoSOX for 20 minutes

and then washed with Krebs/HEPES buffer. The cells were then harvest in methanol for extraction of 2-OH-Mito-E⁺ and Mito-E⁺. HPLC analysis of the superoxide specific product of MitoSOX (2-OH-Mito-E⁺) was performed by the Free Radicals in Medicine Core Laboratory.

Detection of intracellular H₂O₂ derived oxidant production

Intracellular ROS levels were measured using the membrane permeable H₂O₂ specific dye 2',7'-Dichlorofluorescein diacetate (DCFH-DA) (Invitrogen, Grand Island, NY). Cells were grown in the presence of GSK0660 (1μM), GW0742 (1μM), or vehicle (0.05% DMSO) for 24 hours. Fatty acids (oleate 100 μM, palmitate 250μM or both) were added during the last 4 hours. Cells were incubated with DCFH-DA (100 μmol/L) for 30 minutes, rinsed with PBS then florescence was recorded on the Infinite 200 PRO 96-well plate reader using excitation/emission wavelengths of 485nm/530nm (Tecan; Männedorf Switzerland). Intracellular levels of hydroxyperoxides were calculated as fluorescence intensity minus background fluorescence, normalized to the amount of total protein, as measured by the Micro BCA Protein Assay Kit (Thermo Scientific; Rockford, IL).

Statistical Analysis

Data are reported as the group mean ± SEM. Statistical analysis of differences was analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's multiple comparison test or two-way ANOVA (to assess diet x genotype interactions) followed by post hoc Bonferroni's multiple comparison test, using Prism version 5.0 for Windows (GraphPad; San Diego, CA). The student's t-test for non-paired values was performed when two groups were compared. A p value <0.05 was considered significant.

Table 4. Primers used to detect gene specific mRNA levels by RT-PCR

RT-PCR Primers		
Gene	Sense 5'-3'	Antisense 5'-3'
PPAR α	TGTCGGGATGTCACACAATGC	TTCAGGTCGTGTTACAGGTAAAG
PPAR δ	GGATTTTAGAGTGGGTGTTTTTTA	CACACCCGATTCCATGTTGAG
PPAR γ	TGGTTATTTGTAGGTTGGTTT	TTGATCGCACTTTGGTATTCTTGG
LPL	GGAGAGTTATTTAAGAAAGGTGG	AGTCAGGCCAGCTGAAGTAGGAGT
CPT1A	AACACCATCCACGCCATACTG	TCCCAGAAGACGAATAGGTTTGAG
UCP2	TAAAGGTTAAGGTGTAAGGAAAATTAAG	GCAATGGTCTTGTAGGCTTCG
GPAT	GGTTAAAGAATTAATTTTGT	ACAGAATGTCTTTGCGTCCA
SCD-1	GAGGGTAGTAAATTTGTTGGT	CCCAGTCGTACACGTCATTTT
ACC	TTTGTTGGTGTGGGATTTATAG	GTCATTACCATCTTCATTACCTCAATCTC
ACO	GGATTTTTTGTAGGAAGGAGA	CCACTCAAACAAGTTTTTCATACACA
FAS	TTAGTGATTGGAGTAGAGAGAAGTTG	ACAGAGGAGAAGGCCACAAA
DGAT2	AGGGGTTTTTTTTAAGGTTTTTTTTT	AATAGGTGGGAACCAGATCAGC
PDK4	GGGTTATAGGATGGATTGAG	CATGGAACTCCACCAAATCCA
NPY	GGATAGTTGGTAGTGTTAAAGATTT	AATCAGTGTCTCAGGGCTGGA
POMC	GAATTTTTTTTGGGGATTAGGG	GCGAGAGGTCGAGTTTGCA
TNF α	AGAGAGGTTGTTAGGAGTTTGGT	CTCCTCTGCTTGGTGGTTTG
IL-6	TTTTAAAGGAGTTTTTGAAAAG	GCATCATCGTTGTTTCATAC
IKB α	AGTTTGGTTAATAAGGGTGAGG	CAAAGTCACCAAGTGCTCCACGAT
IL-1 β	GGAAAATTTTTTAGGAGGTGATT	GATCCACACTCTCCAGCTGCA
CD36	AATTAGTAGAACCGGGCCAC	CCAACTCCCAGGTACAATCA
RPL13A	AGATGCACTATCCAAGA	AGTCTTTATTGGGTTTAC

CHAPTER III

DIFFERENTIAL EFFECTS BETWEEN HIGH-FAT DIETS ENRICHED IN MONOUNSATURATED AND SATURATED FATS AND A ROLE FOR NEURONAL PPAR δ

Introduction

Adoption of a Western-style diet, rich in calories and saturated fat, is associated with the increased prevalence of obesity that occurred over the last 40 years (4, 30, 323). Current hypotheses suggest dietary lipid consumption induces pathophysiological changes in systems controlling energy balance within the CNS (32). Neurons within the mediobasal hypothalamus regulate energy balance in response to circulating levels of nutrients and hormones (41, 324). Although the molecular mechanisms leading to impaired energy balance regulation are not completely understood, evidence from rodent models of DIO indicates that HF diets are associated with hypothalamic inflammation and neuronal resistance to insulin and leptin action (133, 137, 325).

Nutrient excess leads to elevated circulating FFA levels that promote lipid accumulation, oxidative stress and inflammation, collectively termed lipotoxicity (326, 327). The cytotoxic effects of SFAs involve several cellular organelles and metabolic processes including ER stress, mitochondrial dysfunction, and accumulation of proinflammatory bioactive lipids; all of which can promote insulin and leptin resistance by activating proinflammatory signaling through any one of several signaling pathways (182, 328-330). Our group and others have shown that DIO is associated with hypothalamic insulin and leptin resistance, and activation of an inflammatory pathway involving NF- κ B, I κ B α and IKK β (133, 152). This pathway has also been implicated in TLR4 activation by SFAs (154, 155) and oxidative stress (331). Once activated, IKK β phosphorylates and promotes the degradation of cytoplasmic I κ B α , resulting in NF- κ B release

and transcriptional activation of numerous NF- κ B target genes, such as proinflammatory cytokines IL-6, TNF α and IL-1 β (156-159). These cytokines, produced locally within cells, can act in an autocrine manner to activate signal transduction cascades that contribute to additional oxidative stress and inflammatory signaling to promote insulin resistance (149).

Substitution of SFAs with MUFAs in the diet is beneficial in improving manifestations of metabolic diseases (183, 188, 332). The insulin sensitizing effects of dietary MUFAs are mediated, in part, by their ability to reduce inflammation, in both the periphery as well as the CNS (191, 333, 334). The MUFA oleate can reverse lipotoxic effects associated with palmitate exposure in several cell types in vitro (314, 316, 335). When injected directly into the CNS, MUFAs and PUFAs attenuate hypothalamic inflammation and promote weight loss (333). Abundant evidence indicates that MUFAs can act as signaling molecules and ligands of a number of molecular mediators including PPARs (197, 292). PPARs can reduce inflammation by repressing the activity of proinflammatory transcription factors, such as NF- κ B and AP1 (289, 290). Of the three known isoforms (α , δ/β , γ), PPAR δ in particular is capable of regulating adaptive metabolic responses to changes in plasma FFA levels (336, 337). PPAR δ is expressed at high levels throughout the CNS and is enriched in areas known to be involved in energy homeostasis, such as mediobasal hypothalamus (248, 252). Moreover, agonists of PPAR δ have neuroprotective effects, reducing inflammatory responses to cytokines and oxidative stress in a variety of in vivo and in vitro neurological disease models (262, 263, 338).

Evidence that PPAR δ is activated by dietary MUFAs and has anti-inflammatory effects in peripheral tissues and the CNS, supported the hypothesis that dietary MUFAs activate PPAR δ in hypothalamic neurons and that PPAR δ is capable of opposing inflammation, cytokine expression and insulin resistance induced by excess SFAs in the diet. These experiments showed that chemical and dietary FFA ligands of PPAR δ regulate target gene expression and oppose inflammation in mediobasal hypothalamus.

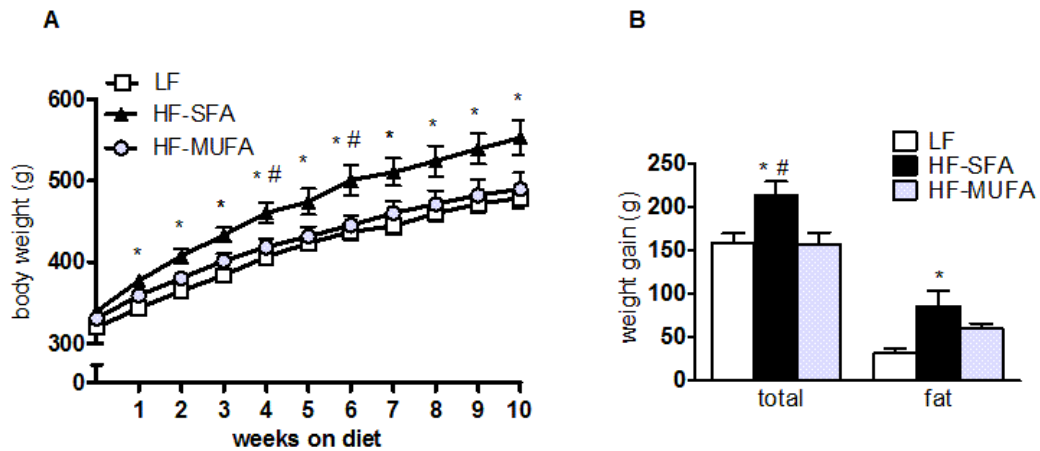


Figure 12. Effects of dietary fat composition on diet-induced obesity.

Long Evans rats were fed LF, HF-SFA or HF-MUFA diets for 10 weeks. A) Body weight and B) total amount body and fat mass gained over 10 weeks. Values represent group mean \pm SEM (n=5-6). Statistical significant is denoted as * ($p < 0.05$ vs. LF) and/or # ($p < 0.05$, HF-SFA vs. HF-MUFA).

Results

Differential effects of dietary monounsaturated and saturated fats on obesity

To determine the relative contributions of total dietary fat intake and dietary FFA composition to the development of obesity, male Long Evans rats were fed LF control diet or one of two eucaloric HF diets (Table 3) for 10 weeks. Rats consuming HF-SFA diet gained 1.3-fold more body weight and 2.8-fold more fat mass than rats fed LF control diet ad lib (Figure 12A,B). Rats that consumed the HF diet enriched in MUFAs gained a third less body weight than those fed a diet high in SFA (Fig 12A,B, Table 5).

Dietary fat composition is reflected in plasma and brain FFA

To begin to test the hypothesis that dietary MUFAs are less obesogenic via effects on hypothalamic inflammation, plasma and cerebral cortex FFA levels as well as their composition

Table 5 Metabolic parameters of Long Evans rats fed LF, HF-SFA or HF-MUFA diet for 10 weeks.

Weight gain, fat mass gain and food intake represent cumulative total for weeks 1-10. Plasma hormone or metabolite concentrations correspond to 4-hour fasted values. Values represent group mean±SEM (n=5-6). Statistical significance is designated as ^a p<0.05 vs. LF and/or ^b p<0.05 vs. HF; as measured by one-way ANOVA and post-hoc Tukey's multiple comparison test.

	LF	HF-SFA	HF-MUFA
body weight (g)	478.7±12.9	530.8±27.2 ^a	489.7±19.7
body weight gain (g)	159.1±11.1	200.2±18.5 ^a	157.3±13.9 ^b
fat mass (g)	64.8±8.1	113.0±19.6 ^a	93.9±6.8
fat mass gain (g)	32.2±5.5	86.3±17.7 ^a	60.2±5.8
adiposity (% fat)	13.4±1.4	20.5±2.6 ^a	18.9±0.9
food intake (kcal)	4,714±94	5,594±262 ^a	5,281±98 ^a
plasma leptin (ng/mL)	7.4±1.0	20.3±4.7 ^a	11.9±1.7
plasma insulin (ng/mL)	2.1±0.4	3.4±0.6	2.3±0.1
plasma glucose (mg/dL)	178.5±3.1	176.2±6.5	165.8±5.9
plasma TG (µg/mL)	2,065±292	1,141±147	2,256±386 ^b
plasma FFA (µg/mL)	74.5±14.6	67.4±5.3	80.1±13.8
brain FFA (µg/mg)	0.50±0.06	0.56±0.06	0.51±0.04

Table 6. Dietary fat composition is reflected in plasma and brain FFA

Percent of total fatty acids in rats fed LF, HF-SFA or HF-MUFA diet for 10 weeks are listed. Values represent group mean±SEM (n=5-6). Statistical significant is denoted as ^a ($p<0.05$ vs. LF) and/or ^b ($p<0.05$, HF-SFA vs. HF-MUFA).

Fatty acids	Plasma			Brain		
	LF	HF-SFA	HF-MUFA	LF	HF-SFA	HF-MUFA
C14:0	1.00±0.16	1.08±0.05	0.84±0.17	0.08±0.08	0.30±0.17	0.27±0.11
C16:0	26.61±0.40	24.21±0.42 ^a	18.79±0.50 ^{a,b}	26.67±0.72	23.03±1.18	20.74±1.76 ^a
C18:0	11.84±1.02	13.74±0.42	7.65±0.60 ^{a,b}	33.17±0.72	27.69±1.14 ^a	28.02±0.66 ^a
Σ SFA	39.45±1.30	39.03±0.63	27.26±0.79 ^{a,b}	59.92±1.25	51.01±1.04 ^a	49.47±1.97 ^a
C16:1	5.03±0.30	1.98±0.14 ^a	1.04±0.09 ^a	0.09±0.09	0.09±0.09	0.10±0.10
C18:1ω9	24.10±1.08	25.67±0.45	51.39±1.09 ^{a,b}	15.50±0.72	17.00±0.57	19.85±0.63 ^a
Σ MUFA	34.30±1.35	30.11±0.42 ^a	54.09±0.98 ^{a,b}	18.78±0.54	21.01±0.37	23.65±0.92 ^a
C18:2 ω6	18.60±0.72	21.42±0.38 ^a	14.56±0.37 ^{a,b}	0.64±0.29	2.34±0.26 ^a	1.67±0.16 ^{a,b}
C18:3 ω3	1.40±0.05	1.44±0.03	0.41±0.05	-	-	-
C20:4 ω6	5.40±0.67	6.97±0.42	3.60±0.13 ^b	18.04±0.60	19.98±1.05	20.69±1.12
C22:6 ω3	1.03±0.12	1.04±0.09	0.49±0.12 ^{a,b}	3.13±0.27	4.79±1.15	4.38±0.50
Σ PUFA	26.25±1.00	30.85±0.45 ^a	18.64±0.42 ^{a,b}	21.29±0.85	27.83±1.15 ^a	26.88±1.15 ^a

were measured, to determine if PPAR ligands consumed in the diet could be detected in plasma and brain tissue, and thus act as ligands in the brain (Table 6). Although total levels of FFAs were similar between diet groups, the MUFA oleate (18:1ω9) was enriched in plasma (2.1-fold > LF) and to some degree in brains (1.3-fold > LF) of rats that consumed the HF-MUFA diet (Table 6). Consistent with both LF and HF-SFA diets having the same fat source (lard), but different total fat content, the species of individual FFAs were similar in plasma from rats consuming HF-SFA and LF diets. Palmitate (16:0) and oleate (18:1) each consisted of ~25% of FFAs in plasma (Table 6). Consumption of the HF-MUFA diet resulted in a doubling of oleate concentration, to ~50% of total FFAs in plasma (Table 6). In addition to a small increase in the MUFA content in brain, HF-MUFA diet consumption increased the oleate to palmitate ratio from 0.6 with LF and 0.7 with HF-SFA to 1 with HF-MUFA diet (Table 6).

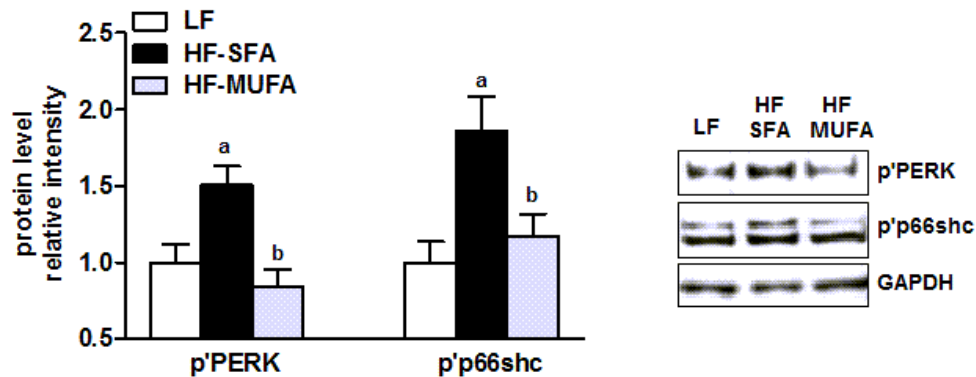


Figure 13. Effects of dietary fat composition on hypothalamic oxidative stress and ER stress.

Mediobasal hypothalamic total protein extracts from Long Evans rats fed LF, HF-SFA or HF-MUFA for 10 weeks were subjected to Western blot analysis and probed with antibodies directed against the phosphorylated forms of PERK (p'PERK) and p66shc (p'p66shc). Levels of GAPDH were determined and used as a loading control. Inset shows representative Western blots. Densitometry of blots yielded relative intensity protein levels, which are represented as dietary group mean \pm SEM (n=5-6). Statistical significance is designated as ^a (p<0.05 vs. LF) and/or ^b (p<0.05, HF-SFA vs. HF-MUFA), as measured by one-way ANOVA and post-hoc Tukey's multiple comparison test.

Effects of dietary fat composition on hypothalamic stress and inflammation

Protein markers of oxidative and ER stress in mediobasal hypothalamus were assessed by immunoblotting. After 10 weeks of consumption of HF-SFA diet, levels of an ER stress marker, phosphorylated PERK increased 1.5 \pm 0.1-fold relative to LF diet (Figure 13, p<0.05); additionally, an oxidative stress maker, phosphorylated p66shc increased 1.9 \pm 0.2-fold relative to LF diet (Figure 13, p<0.05). Levels of phosphorylated PERK and p66shc were similar between the HF-MUFA and LF-control groups, indicating that unlike HF-SFA diet, HF-MUFA diet for 10 weeks did not activate these ER and oxidant stress pathways in the hypothalamus.

Effects of dietary fat composition on biochemical markers of hypothalamic inflammation and PPAR δ activation

Rodent models of DIO exhibit increased hypothalamic cytokine expression after one week of HF diet exposure (313). To determine if dietary fat composition alters markers of hypothalamic inflammation, another cohort of rats were fed LF, HF-SFA or HF-MUFA diet for 7 days, euthanized the animals and prepared mediobasal hypothalamic RNA. mRNA levels of the cytokine IL-6, a NF- κ B target gene, as well as I κ B α , an inhibitor of NF- κ B (159, 339) were also determined. Relative to LF diet, HF-SFA diet consumption increased hypothalamic IL-6 gene expression 4.0 \pm 0.8-fold (Figure 14A, p <0.001), but did not significantly alter mRNA levels of I κ B α (Figure 14A). In contrast, the HF-MUFA diet did not increase IL-6 mRNA levels relative to those of LF diet, but did increase I κ B α mRNA levels 5.3 \pm 0.9-fold (Figure 14A, p <0.001). Induced expression of I κ B α would be predicted to attenuate NF- κ B signaling tone leading to reduced cytokine expression. Indeed, HF-MUFA diet led to 50% lower IL-6 mRNA expression relative to HF-SFA diet (Figure 14A).

PPAR δ is the most prevalent PPAR isoform in the CNS and is enriched in mediobasal hypothalamus (248). Hypothalamic PPAR δ expression was determined following 7 days of LF, HF-SFA or HF-MUFA diet exposure. PPAR δ mRNA was increased 1.9 \pm 0.2-fold only in animals fed the HF-MUFA diet (Fig. 14A, p <0.01). To determine if an increase in PPAR δ mRNA expression resulted in an increase in function, the mRNA expression level of a known PPAR δ target gene, uncoupling-protein 2 (UCP2) (340) was assessed. Indeed, the HF-MUFA diet increased hypothalamic UCP2 mRNA levels by 1.6 \pm 0.1-fold relative to LF control diet (Fig 14A, p <0.01). HF-SFA diet did not change UCP2 mRNA levels (Fig 14A), consistent with the possibility that HF-MUFA diet increased PPAR δ ligands and thereby transcriptional activity; whereas, PPAR δ ligands were not increased with HF-SFA diet consumption. To confirm that UCP2 is regulated by PPAR δ ligand activation in the hypothalamus, an additional group of rats were implanted with a cannula to allow access to the third ventricle. Hypothalamic neurons were

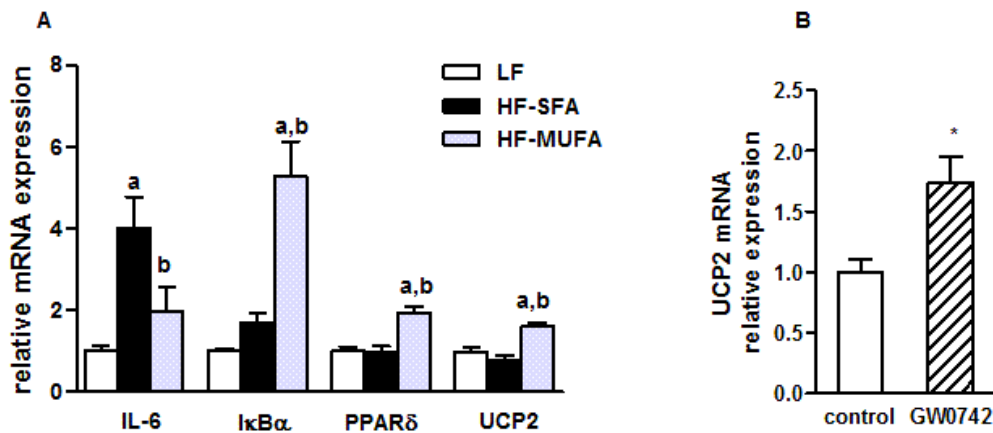


Figure 14. Effects of dietary fat composition on hypothalamic inflammation and PPAR δ activation.

Hypothalamic mRNA levels in Long Evans rats A) after one week of feeding LF, HF-SFA or HF-MUFA diet or B) 24 hours after ICV treatment with either vehicle or GW0742. Target gene mRNA levels were normalized to endogenous RPL13A levels, and then expressed relative to that of A) LF diet for IL-6, I κ B α , PPAR δ , and UCP2 or B) vehicle for UCP2. Values represent group mean \pm SEM (n=5-6). Statistical significance is designated as A) ^a (p<0.05 vs. LF) and/or ^b (p<0.05, HF-SFA vs. HF-MUFA), as measured by one-way ANOVA and post-hoc Tukey's multiple comparison test or B) * (p<0.05), as measured by student's t-test.

exposed to an ICV bolus of a specific PPAR δ agonist, GW0742 [36] (50 μ g in 2 μ L), and euthanized 24 hours later. Treatment with GW0742 significantly increased the mRNA level of UCP2 above vehicle treated by 1.7 \pm 0.2-fold (Fig 14B, p<0.05), confirming UCP2 in the hypothalamus as a specific PPAR δ ligand activated target gene.

Summary

Diets rich in SFAs are associated with increased risk of obesity, diabetes and heart disease (30, 31, 119, 123). Replacing dietary SFAs with MUFAs and PUFAs has been shown to promote insulin sensitivity, weight loss and cardiovascular health (188, 189, 193, 332). To identify possible mechanisms involved in the differential effects of SFAs and MUFAs this investigation focused on effects in the CNS; specifically hypothalamic neuronal inflammation and the possible role of PPAR δ . Long Evans rats were fed a LF control diet, HF diet enriched in

the MUFA oleate and low in the SFA palmitate or HF diet with levels of palmitate, nearly equivalent to oleate. The results of these studies suggested that different types of dietary fats have differential effects on weight and adipose gain and on hypothalamic markers of ER and oxidant stress, inflammation, and expression of PPAR δ as well as its target gene UCP2. These findings raise the possibility that PPAR δ in the CNS has a role in the adaptive response to MUFA diet consumption and may be an interesting target to modulate cellular inflammation and stress induced by diets enriched in SFAs.

To understand the influence of dietary fat composition on plasma and CNS FFAs, total and individual FFA levels in the FFA fraction of plasma and brains were determined in rats that consumed LF, HF-SFA or HF-MUFA diets for 10 weeks. Replacing the lard-based fat source of the HF diet with the oleate enriched safflower oil found in the MUFA diet led to a 24% increase in the relative amount of total MUFAs in plasma and reduced the relative amounts of SFAs by 12%. The HF-MUFA diet also increased oleate levels to a small degree in brain tissue. In a similar study, Citra and colleagues reported a modest change in plasma and hypothalamic FFA composition after feeding mice two high-fat diets with different FFA compositions for 8 weeks (333).

I also examined over the ten week experimental period several parameters involved in energy balance, such as body weight, fat mass gain and food intake. Both high-fat diets, HF-SFA and HF-MUFA, increased total caloric intake; yet only the high-fat lard based diet resulted in elevated body weight and increased fat mass gain. Given that dietary MUFAs, and not SFAs, are preferred substrates for fat oxidation (185, 341), energy expenditure may have increased with a MUFA diet based on the observation that animals fed a HF-MUFA diet gained less total body weight from a caloric intake nearly equivalent to HF-SFA fed animals, indicating reduced feed efficiency with HF-MUFA diet. Compared to HF-SFA diet, consumption of HF-MUFA diet resulted in reduced activation of protein markers of hypothalamic inflammation, ER and oxidative stress, and prevented an elevation of plasma leptin and insulin levels, which suggests

that MUFAs contribute less toward leptin and insulin resistance. From a comparison of various metabolic parameters of LF, HF-SFA and HF-MUFA diet fed rats (Table 5), the oleate enriched HF-MUFA diet group showed greater protection from the poor prognostic indicators associated with the group fed a HF-SFA diet, perhaps due to less inflammation.

In conclusion, in diets consisting of a high-fat content, dietary fatty acid composition is an important determinant in the physiological and molecular pathogenesis of DIO. In addition to providing calories to metabolism, some FFAs reduce cellular defense responses and promote lipotoxicity (SFA), whereas others are protective (MUFA). Therefore, activation of hypothalamic PPAR δ by dietary and/or endogenous fatty acids may serve as a protective mechanism to prevent neuronal inflammation via upregulation of UCP2 or other target genes ($\text{I}\kappa\text{B}\alpha$) in key brain regions (hypothalamus) involved in energy homeostasis. CNS PPAR δ gene expression is reduced in models of obesity and neurodegeneration (342, 343), raising the possibility that DIO is a state of acquired loss of PPAR δ function in hypothalamic neurons. My study sheds important new light upon plausible mechanisms by which a changing dietary environment may multifactorially enhance susceptibility to obesity, and raise the possibility that neuronal PPAR δ functions at a nexus involving gene transcription, inflammation, and sensitivity to leptin and insulin.

CHAPTER IV

HYPOTHALAMIC NEURONAL PPAR δ MEDIATES THE ANTI-INFLAMMATORY EFFECTS OF THE MONOUNSATURATED FATTY ACID OLEATE

Introduction

PPAR δ is the most highly expressed PPAR isoform throughout the CNS and is enriched in areas known to be involved in energy homeostasis, such as mediobasal hypothalamus. While PPARs in general have been relatively understudied in the brain, PPAR δ is perhaps the least understood. PPAR δ regulates genes involved in cellular adaptive metabolic responses to FFAs (236, 237, 336, 344), and has been implicated in protective responses to oxidative stress and inflammation in both peripheral and CNS tissues (231, 260, 308, 345-353). A number of recent publications have centered on hypothalamic inflammation and its potential role in obesity pathogenesis (133, 137, 313). Additionally, diets enriched in PPAR ligands are beneficial in improving symptoms associated with metabolic diseases (186, 187, 354-358). However, since the hypothalamus contains heterogeneous cell types, it is difficult to assign these effects to its action in specific neurons as well as to identify *in vivo*, with traditional biochemical techniques, the role of individual molecules, such as a SFAs or MUFAs, to regulate PPAR δ (359).

Historical methods used to study hypothalamic neuronal populations, such as stereotactic injection, electrical stimulation or lesioning, can destroy, activate or disrupt these neurons and produce erroneous results. Also, many *in vivo* approaches do not address the molecular and cellular events or provide specific information of the mechanisms involved in gene regulation and cellular signaling within a given cell type. Additionally, these techniques do not allow direct testing of an agent on specific hypothalamic neurons. Although rodent genetic models have been used to study the effects of altered gene expression on signaling pathways, these models are time and labor intensive to create and study. Established cell lines, derived from hypothalamic tumors

or immortalized from primary cultures, provide a simpler model that lacks much of the complexity and challenges associated with in vivo study. Immortalized cell lines provide a homogeneous, clonal population of neuronal cells that can be maintained in a controlled environment for the study of changes in gene expression and/or cell signaling in response to specific test agents.

For all these reasons, the differential effects of a MUFA, oleate, and that of a SFA, palmitate, were studied using a mouse clonal hypothalamic neuronal cell line, mHypoE-N42. This neuronal cell line allowed me to show that neuronal cells respond differently to MUFA and SFA FFAs and to explore more mechanistically the involvement of PPAR δ in this differential response. These experiments revealed that both chemical (GW0472) and common dietary (palmitate and oleate) FFA ligands of PPAR δ regulate target gene expression and oppose inflammation. Conversely, inhibition of PPAR δ with a chemical antagonist (GSK0660) in the presence of excess FFAs led to inflammation and insulin resistance.

Results

Regulation of PPAR δ target gene expression by chemical agonists/antagonists and fatty acids in clonal hypothalamic neurons

To investigate the role of PPAR δ in the molecular events involved in the differential effects of dietary SFAs and MUFAs on hypothalamic neuronal inflammation, in vitro experiments were performed using a clonal mouse hypothalamic neuronal cell line, mHypoE-N42 (319). Two highly specific chemical modulators of PPAR δ were used to manipulate PPAR δ functional activity, which was monitored via changes in target gene (UCP2) expression. Changes in PPAR δ and target gene mRNA levels were assessed by quantitative real-time PCR after a 24-hour exposure to the PPAR δ agonist GW0742 and/or the antagonist GSK0660 (214, 299). PPAR δ gene expression was not altered by any of the treatments (Figure 15A). Activation of PPAR δ with GW0742 increased UCP2 mRNA 4.1 \pm 0.2-fold above vehicle control levels (Figure 15A,

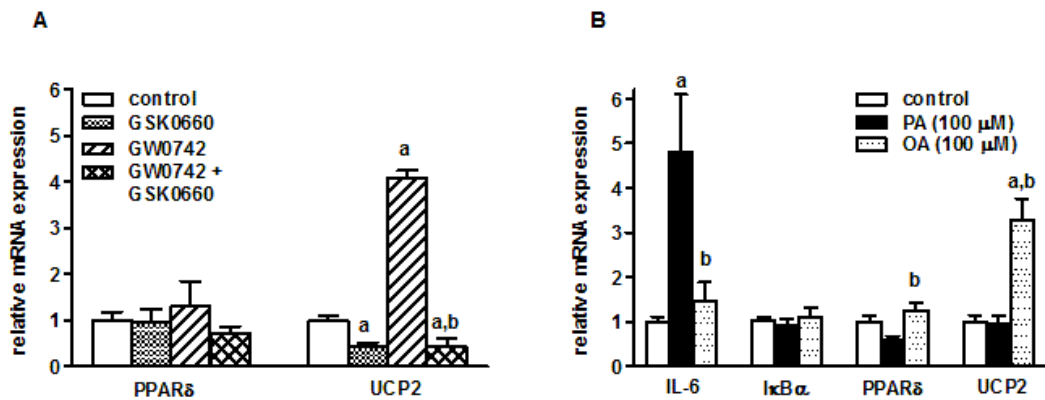


Figure 15. Effects of fatty acids and chemical ligands of PPAR δ on gene expression in clonal hypothalamic neurons.

A) PPAR δ and UCP2 mRNA levels in cells treated for 24 hours with vehicle (0.05% DMSO, control), the PPAR δ agonist GW0742 (1 nM) and/or the antagonist GSK0660 (1 μ M). B) IL-6, I κ B α , PPAR δ and UCP2 mRNA levels in cells exposed to vehicle (1% BSA, 0.05% DMSO; control), palmitate (PA, 100 μ M) or oleate (OA, 100 μ M) for 24 hours. Values represent group mean \pm SEM (n=4-5). Statistical significance is designated as ^a(p <0.05 vs. vehicle) and/or ^b(p <0.05 vs. PA), as measured by one-way ANOVA and post-hoc Tukey's multiple comparison test.

p <0.001). Conversely, treatment with a specific chemical antagonist of PPAR δ , GSK0660, reduced UCP2 mRNA to 42 \pm 9% of control levels (Figure 15A, p <0.05) and inhibited GW0742 induced expression of UCP2 to the level of GSK0660 alone (Figure 15A; GSK0660+GW0742 0.42 \pm 0.17 to GW0742 4.1 \pm 0.2, p <0.001).

I next utilized the mHypoE-N42 neuronal cell line to examine the effects of a common SFA and MUFA, palmitate (PA) and oleate (OA), respectively, on IL-6 and I κ B α mRNA levels as well as that of PPAR δ and its target gene, UCP2. Cells were exposed to low physiological concentrations of either oleate or palmitate bound to excess BSA (molar ratio of FFA to BSA 0.7:1) for 24 hours. Palmitate exposure of mHypoE-N42 cells increased IL-6 mRNA 4.8 \pm 1.3-fold above vehicle-treated control levels (Figure 15B, p <0.05), while oleate had no effect. In these neuronal cells, I κ B α mRNA levels were not altered by treatment with either fatty acid (Figure

15B). Compared to vehicle treatment, PPAR δ gene expression was not altered by oleate treatment but trended ($p < 0.1$) to be slightly reduced by palmitate (Figure 15B). Interestingly, relative to oleate treatment, PPAR δ gene expression was significantly lower with palmitate treatment (Figure 15B; PA 0.57 ± 0.11 to OA 1.2 ± 0.2 , $p < 0.05$). Finally, oleate induced UCP2 mRNA 3.3 ± 0.5 -fold above vehicle control levels (Fig 15B, $p < 0.001$), while palmitate had no effect on UCP2 gene expression relative to the vehicle control (Figure 15B).

Because cells are not physiologically exposed to a single fatty acid species (Table 65), mHypoE-N42 cells were exposed to a mixture of fatty acids to determine if increasing levels of oleate would increase expression of UCP2, a PPAR δ target gene in the presence of palmitate. When mHypoE-N42 cells were treated with either 100 or 250 μ M palmitate, UCP2 mRNA levels were similar to control levels (Figure 16A). Compared to cells treated with 100 μ M palmitate alone, the addition of 100 μ M oleate increased UCP2 levels 2.9-fold (Figure 16A; 100 μ M OA+100 μ M PA 1.35 ± 0.12 to 100 μ M PA 0.47 ± 0.02 , $p < 0.01$), while cells co-treated with 250 μ M oleate had 9.8-fold higher UCP2 mRNA levels relative to 100 μ M palmitate treated cells (Figure 16A; 250 μ M OA+100 μ M PA 4.58 ± 0.22 to 100 μ M PA 0.47 ± 0.02 , $p < 0.001$), suggesting a dose-dependent effect of oleate. Even in cells treated with a high dose (250 μ M) of palmitate, the presence of 100 μ M oleate increased UCP2 mRNA levels 2.8-fold (Fig 16A; 100 μ M OA+250 μ M PA 1.57 ± 0.08 to 250 μ M PA 0.56 ± 0.34 , $p < 0.05$), while 250 μ M oleate increased UCP2 mRNA levels by 5.2-fold (Fig 16A; 250 μ M OA+250 μ M PA 2.90 ± 0.46 to 250 μ M PA 0.56 ± 0.34 , $p < 0.001$).

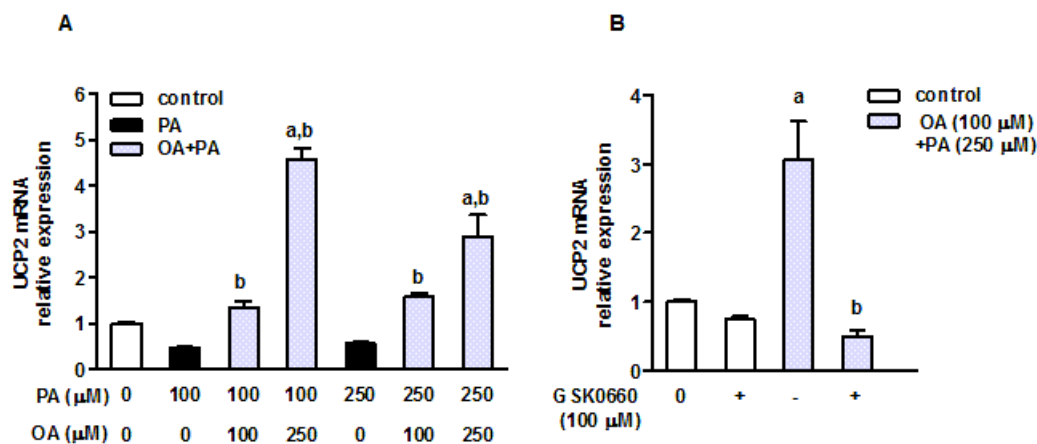


Figure 16. PPAR δ activation is required for oleate induced UCP2 mRNA expression in clonal hypothalamic neurons.

A) UCP2 mRNA levels in mHypoE-N42 cells treated with palmitate alone (PA) or co-treated with oleate (OA+PA) with constant BSA (100 μ M) at the indicated concentrations for 24 hours and expressed relative to vehicle (BSA) treated cells (control). B) UCP2 mRNA levels after co-treatment with OA+PA in the presence or absence of GSK0660 (1 μ M) or vehicle (0.05% DMSO; control). Values represent group mean \pm SEM (n=4-5). Statistical significance is designated as ^a (p<0.05 vs. vehicle) and/or ^b (p<0.05 vs. PA), as measured by one-way ANOVA and post-hoc Tukey's multiple comparison test.

The selective chemical PPAR δ inhibitor, GSK0660, was used to determine if PPAR δ activation is required for the effect of 100 μ M oleate to increase UCP2 mRNA levels in the presence of 250 μ M palmitate (Figure 16B). Treatment with oleate in combination with palmitate increased UCP2 mRNA levels by 3.1 \pm 0.6-fold compared to vehicle control treatment (Figure 16B; p<0.01), whereas inhibition of PPAR δ with the chemical antagonist GSK0660 reduced UCP2 mRNA levels to 16.1 \pm 0.2% of the levels of combined oleate and palmitate FFA treatment alone (Figure 16B; p<0.001). These data suggest that oleate increases PPAR δ functional activity to maintain or even induce target gene expression, as seen with UCP2.

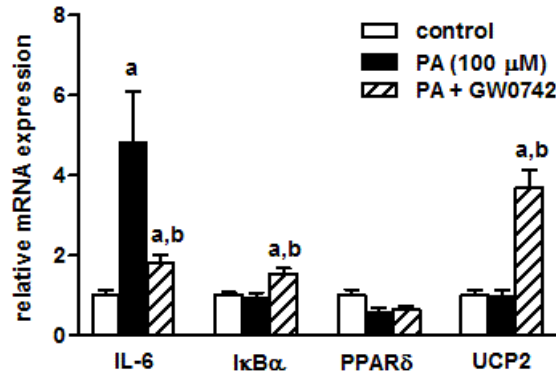


Figure 17. PPAR δ activation by GW0742 prevents palmitate induced inflammation in clonal hypothalamic neurons.

mHypoE-N42 cells were incubated for 24 hours with vehicle (1% BSA, 0.05% DMSO; control) or palmitate (PA, 100 μ M) in the presence or absence of GW0742 (1 μ M). Changes in IL-6, I κ B α , PPAR δ and UCP2 mRNA levels were assessed by quantitative real-time PCR and mRNA levels are expressed relative to that of control. Values represent group mean \pm SEM (n=4-5). Statistical significance is designated as ^a (p<0.05 vs. vehicle) and/or ^b (p<0.05 vs. PA), as measured by one-way ANOVA and post-hoc Tukey's multiple comparison test.

PPAR δ activation by GW0742 attenuates palmitate induced inflammation in clonal hypothalamic neurons

In the hypothalamic mHypoE-N42 cell line, IL-6 and I κ B α mRNA levels were measured as markers of pro-inflammatory and anti-inflammatory signaling, respectively (339, 360) in order to determine if ligand-specific activation of PPAR δ attenuates palmitate induced inflammation in neurons. In cells treated with palmitate, IL-6 mRNA levels increased 4.8 \pm 1.3-fold compared to vehicle-treated cells (Figure 17, p<0.05), whereas there was no change in I κ B α mRNA levels. Ligand-specific activation of PPAR δ with the chemical agonist GW0742 (1 μ M) almost completely attenuated (62% reduction) the palmitate-induced IL-6 mRNA levels (Figure 17; PA+GW0742 1.8 \pm 0.2 to PA 4.8 \pm 1.3, p<0.05). GW0742 treatment also resulted in a 1.7-fold increase in I κ B α mRNA levels in the presence of palmitate relative to palmitate alone (Figure 17;

PA+GW0742 1.5 ± 0.1 to PA 0.92 ± 0.14 , $p<0.05$). PPAR δ gene expression was not altered by any of the treatments (Figure 17). Similar to the effect of oleate, treatment of palmitate along with the PPAR δ agonist GW0742 increased UCP2 mRNA 3.7-fold above the level seen with palmitate alone (Figure 17; PA+GW074, 3.7 ± 0.4 to PA 0.97 ± 0.16 , $p<0.001$). These data suggest that PPAR δ activation has anti-inflammatory effects in the mHypoE-N42 hypothalamic neuronal cell line. Ligand activated PPAR δ mediates this effect by suppressing inflammatory cytokine gene expression, while promoting the expression of an "anti-inflammatory" NF- κ B regulatory gene, I κ B α .

Inhibition of PPAR δ with GSK0660 leads to inflammation and insulin resistance in clonal hypothalamic neurons exposed to excess fatty acids

The mHypoE-N42 cell line was next used to determine whether PPAR δ function was necessary to maintain the beneficial effects of the MUFA, oleate, in the presence of the SFA, palmitate. Cells were treated with a mixture of oleate (100 μ M) and palmitate (250 μ M). BSA was held at 100 μ M to yield an FFA to BSA ratio of 3.5:1 to examine how excess FFAs and loss of PPAR δ function might impact inflammation and insulin signaling in this hypothalamic neuronal cell line. Inhibition of PPAR δ with GSK0660 (1 μ M) in the presence of oleate and palmitate led to a loss of cytoplasmic I κ B α protein levels to $46\pm 8\%$ of the levels of combined oleate and palmitate treatment alone (Figure 18A, $p<0.01$). In the context of reduced I κ B α and presumed NF- κ B activation, treatment with GSK0660 increased IL-6 gene expression by 1.9 ± 0.1 -fold (Figure 18B, $p<0.05$). Likewise, expression of a second NF- κ B regulated proinflammatory cytokine gene, IL-1 β , was also increased 1.8 ± 0.4 -fold (Figure 18B, $p<0.05$).

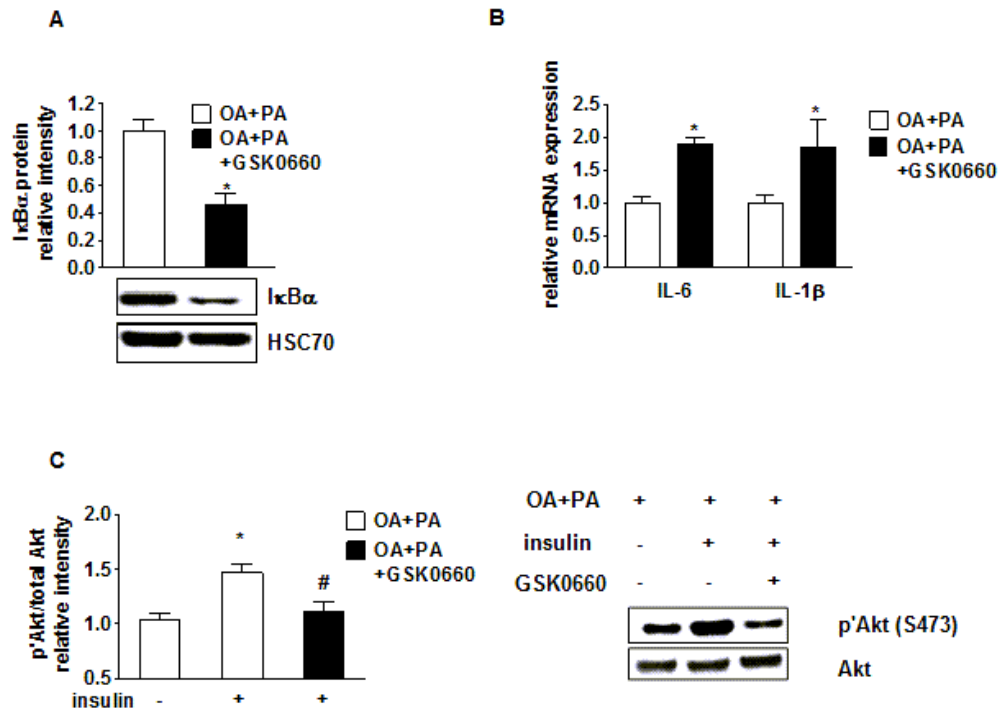


Figure 18. Inhibition of PPAR δ with GSK0660 leads to I κ B α degradation, inflammation, and insulin resistance in clonal hypothalamic neurons exposed to fatty acids.

mHypoE-N42 cells were incubated for 24 hours with a mixture of fatty acids conjugated to BSA (BSA, 100 μ M; oleate, 100 μ M; palmitate, 250 μ M; final FFA concentration 350 μ M) in the presence or absence of GSK0660 (1 μ M, 0.05% DMSO). A) Total protein extracts were subjected to Western blot analysis and immunological detection of cytoplasmic I κ B α protein was quantified by densitometry. Heat shock cognate protein 70 (HSC70) was used as a loading control and treatment levels are expressed relative to those in the absence of GSK0660. Insert of A shows a representative Western blot. B) Changes in mRNA levels of the cytokines, IL-6 and IL-1 β , with GSK0660 treatment were assessed by quantitative real-time PCR. Treatment levels are expressed relative to those in the absence of GSK0660. C) Insulin induced Akt phosphorylation was assessed in total protein extracts prepared from mHypoE-N42 cells stimulated with insulin (10 nM) for 5 minutes by immunological detection of phosphorylated and total Akt proteins using the indicated antibodies and expressed relative to total Akt levels. Treatment levels are expressed relative to those in the absence of insulin and GSK0660. Values represent group mean \pm SEM (n=4). Statistical significance is designated as * (p<0.05) by student's t-test; or in panel C, when measured by one-way ANOVA and post-hoc Tukey's multiple comparison test, * (p<0.05 vs. no insulin treatment) or # (p<0.05 vs. insulin treated, no GSK0660 treatment).

Rodent models of DIO are usually associated with increased inflammatory signaling accompanied with insulin resistance in mediobasal hypothalamic neuronal populations (133, 137). A chemical inhibitor of PPAR δ , GSK0660 was utilized to determine if loss of PPAR δ function also promotes insulin resistance in the hypothalamic neuronal mHypoE-N42 cell line. In cells treated with insulin in the presence of oleate and palmitate compared to this FFA mixture alone, insulin stimulation (10 nM, 5 minutes) increased phosphorylation of Akt at serine-473 by 1.5 \pm 0.1-fold (Figure 18C, p <0.01). Inhibition of PPAR δ with GSK0660 did not alter total levels of Akt, but prevented insulin-induced phosphorylation of Akt (Figure 18C; insulin with OA+PA+GSK0660 1.11 \pm 0.01 to insulin with OA+PA 1.47 \pm 0.08, p <0.05). Collectively, these results suggest that PPAR δ may sense and be activated by dietary MFUAs, like oleate, to oppose stress and inflammatory signaling, preventing hypothalamic neuronal insulin resistance.

Summary

High-fat diets are associated with inflammatory signaling in neuronal targets of insulin and leptin (133, 137), which may play a causative role in the development of central hormonal resistance and the pathogenesis of obesity. Conversely, the protective effects of omega-3 polyunsaturated fats on insulin sensitivity and inflammation have been appreciated for some time (361, 362). Diets rich in the MUFA oleate improve insulin sensitivity (187, 188, 191, 192, 332, 363) and promote weight loss (186, 341, 354). This led to the hypothesis that MUFAs activate PPAR δ in hypothalamic neurons resulting in increased expression of target genes that oppose inflammation, cytokine expression and insulin resistance induced by excess SFAs in the diet. To identify possible mechanisms involved in the differential metabolic effects of SFAs and MUFAs, the investigation was focused specifically on hypothalamic neuronal inflammation and insulin resistance, using the hypothalamic neuronal cell line, mHypoE-N42.

MUFAs and PUFAs activate PPAR δ with μ M affinity in vitro (364). However, cells are exposed to a heterogeneous mixture of both saturated and unsaturated fatty acids in vivo.

Therefore, the cell culture system provided an opportunity to test the effects of individual fatty acids on gene expression and inflammation at biologically relevant concentrations. Treatment of cells with the MUFA oleate increased UCP2 mRNA levels, while treatment with the SFA palmitate had no effect on UCP2 gene expression. The ability of oleate to restore and induce UCP2 gene expression in the presence of palmitate was dependent on both the concentration of each FFA as well as the ratio of MUFA:SFA with the largest response occurring at a MUFA to SFA ratio of 2.5:1, which closely matched plasma levels of oleate and palmitate after 10 weeks of HF-MUFA diet feeding (Table 6). Treatment with the PPAR δ antagonist GSK0660 abolished UCP2 expression induced by co-treatment with oleate and palmitate, indicating a specific role for PPAR δ in oleate induced UCP2 expression in hypothalamic neurons. Oleate induced UCP2 expression was also shown to be dependent upon PPAR δ in INS-1E cells, an insulin secreting cell line [61]. Additionally, mutant mice expressing a mutated form of PPAR δ that lacks the ligand-binding domain, a region necessary for fatty acid activation of gene transcription, cannot up-regulate UCP2 in response to fasting, and are more susceptible to neuronal oxidative stress and ischemic injury [65]. GSK0660 is a highly specific PPAR δ antagonist and only cross-reacts with other PPAR isoforms at an extremely high concentration (>10 μ M), which further supports a neuronal PPAR δ specific mechanism to increase UCP2 mRNA [53].

Dietary SFAs induce hypothalamic inflammation in rodents, but there is little evidence to support neurons and not glial cells as the primary source of increased inflammatory signaling and cytokine expression (313, 329). Similar to the effect after one week of SFA-rich HF diet feeding, palmitate increased IL-6 mRNA levels in mHypoE-N42 hypothalamic neuronal cells. Activation of PPAR δ with GW0742 attenuated the palmitate-induced gene expression of IL-6 as well as increased I κ B α mRNA levels 1.7-fold above palmitate treatment alone. These findings are consistent with extensive evidence that PPAR δ activation is protective against SFA induced lipotoxicity (Coll, Alvarez-Guardia et al. 2010; Jiang, Wan et al. 2010; Wan, Jiang et al. 2010; Wang, Liu et al. 2010; Alvarez-Guardia, Palomer et al. 2011. I κ B α sequesters NF- κ B in the

cytoplasm, preventing its translocation to the nucleus. The I κ B α promoter contains several confirmed PPREs and I κ B α mRNA expression and protein levels are increased in response to treatment with PPAR chemical agonists (339). PPARs are known to prevent inflammation through ligand dependent trans-repression of the p50/p65 subunits of NF- κ B, and with AP1, effectively blocks association of these transcription factors with their DNA response elements (231, 308). Although my studies do not differentiate by which mechanism PPAR δ activation reduces inflammation, they clearly implicate the involvement of PPAR δ .

Persistent exposure to elevated FFA levels during obesity induces lipotoxicity and insulin resistance (326, 365). The clonal neuronal cells were next treated with a mixture of oleate and palmitate to better recapitulate physiological FFAs, since neurons are never exposed to a single FFA species. This experiment tested the hypothesis that PPAR δ mediates the action of the MUFA oleate in mHypoE-N42 hypothalamic neuronal cells, by protecting these cells from the effects of SFA-induced lipotoxicity and thereby maintaining insulin responsiveness. Insulin signaling was maintained despite the presence of high levels of FFAs in the culture media as long as the MUFA oleate was present with the SFA palmitate. Specific inhibition of PPAR δ with GSK0660 in the presence of oleate and palmitate prevented insulin induced phosphorylation of Akt. Inhibition of PPAR δ action with GSK0660 in the presence of oleate and palmitate also led to a 50% reduction in I κ B α protein and increased mRNA levels of the proinflammatory cytokines IL-6 and IL-1 β . Loss of PPAR δ function leads to biochemical events that resemble those occurring in rodent models of DIO, which exhibit reduced hypothalamic levels of cytoplasmic I κ B α , increased cytokine expression and insulin resistance (133, 137, 313). PPAR δ gene expression is reduced in several disease models, including diabetes, aging, obesity and inflammatory disease (342, 343); therefore, these findings raise the possibility that DIO is a state of acquired loss of PPAR δ function.

In conclusion, in diets consisting of a high-fat content, dietary fatty acid composition is an important determinate in the physiological and molecular pathogenesis of DIO. In addition to

providing calories to metabolism, some FFAs reduce cellular defense responses and promote lipotoxicity (SFA), whereas others are protective (MUFA). My investigation and findings support a role for PPAR δ as a diet responsive fatty acid “sensor” that may mediate the protective effects of unsaturated fats from DIO associated inflammation and insulin resistance in hypothalamic neurons

CHAPTER V

CNS DELETION OF PPAR δ LEADS TO INCREASED SUCEPTABILITY TO DIET-INDUCED OBESITY

Introduction

All three PPAR isoforms are expressed to different degrees in the CNS (239). Recent evidence suggests that CNS activation of PPAR α and/or PPAR γ may contribute to weight gain and obesity (242, 366). Deletion of PPAR γ in neurons (366), or chemical inhibition of PPAR γ in the hypothalamus (242), protects against the development of DIO . Activation of this receptor with HF feeding or a chemical agonist increases weight gain (242), raising the possibility that HFD consumption activates neuronal PPAR γ , which may be a pathogenic mechanism associated with obesity. PPAR δ , the most highly expressed isoform throughout the CNS, is enriched in areas known to be involved in energy homeostasis, such as mediobasal hypothalamus (239, 248, 252). Accumulating evidence supports a role for CNS PPAR δ activation in preventing oxidative stress and inflammation in several neurodegenerative models (263, 338). Although limited, these reports support a key role for PPARs in central energy homeostasis regulation.

Evidence from various rodent models suggests that hypothalamic lipid accumulation and low-grade inflammation are associated with obesity (133, 313). Agonists of PPAR δ oppose HF diet induced inflammation and insulin resistance in peripheral tissues, in part through transcriptional activation of target genes whose encoded proteins are involved in mitochondrial fatty acid oxidation, proliferation and uncoupling (231, 234, 236, 238, 308). In the CNS, PPAR δ has been implicated in reducing neuronal inflammation via down regulation of NF- κ B signaling as well as neuroprotection from inflammation and excitotoxicity (reviewed in (323)). Despite the

fact that this potent transcriptional regulator is highly expressed in the CNS, little is known of the transcriptional targets regulated by PPAR δ in the brain.

UCP2 is a validated target gene of PPAR δ and global PPAR δ KO mice fail to express UCP2 in the CNS in response to fasting. Extensive data implicate UCP2 in neuroprotection, reduction of CNS oxidative stress and neuroinflammation. Genetic deletion of UCP2 in mice also results in chronic basal NF- κ B activation (367), which suggests an important role protecting against basal metabolic stress. Therefore, this gene can serve as a marker of PPAR δ activation.

In a hypothalamic neuronal cell line, PPAR δ was found to regulate UCP2 gene expression in response to a chemical agonist and FFAs commonly encountered in the diet. Conversely, exposure to the antagonist GSK0660 reduced basal expression of UCP2 and blocked oleate induced UCP2 expression. This suggested that activation of hypothalamic PPAR δ by dietary FFAs may serve as a protective mechanism to prevent neuronal inflammation via upregulation of UCP2 or other target genes expressed in this brain region. Inhibition of PPAR δ with the chemical antagonist GSK0660, led to biochemical events that resembled those occurring in rodent models of DIO, including reduced hypothalamic levels of cytoplasmic I κ B α , increased cytokine expression and insulin resistance (137, 313). PPAR δ gene expression is reduced in several disease models, including diabetes, aging, obesity and inflammatory disease [74-77]; therefore, my findings raise the possibility that DIO is a state of acquired loss of PPAR δ function.

Results

CNS PPAR δ knockdown and brain morphology

PPAR δ was deleted from neurons using cre-lox technology (368). Mice expressing Cre recombinase under the control of the rat Nestin promoter (Nestin Cre^{+/+}) (369) were crossed with mice engineered with Lox-P recognition sites flanking exon 4 of the PPAR δ gene (f/f; PPAR δ ^{f/f}) (Figure 11) to obtain double transgenic, heterozygous (Het; PPAR δ ^{f/wt} Nestin Cre^{+/-}) mice which were crossed to obtain CNS PPAR δ knockout (KO; PPAR δ ^{f/f} Nestin Cre⁺) mice.

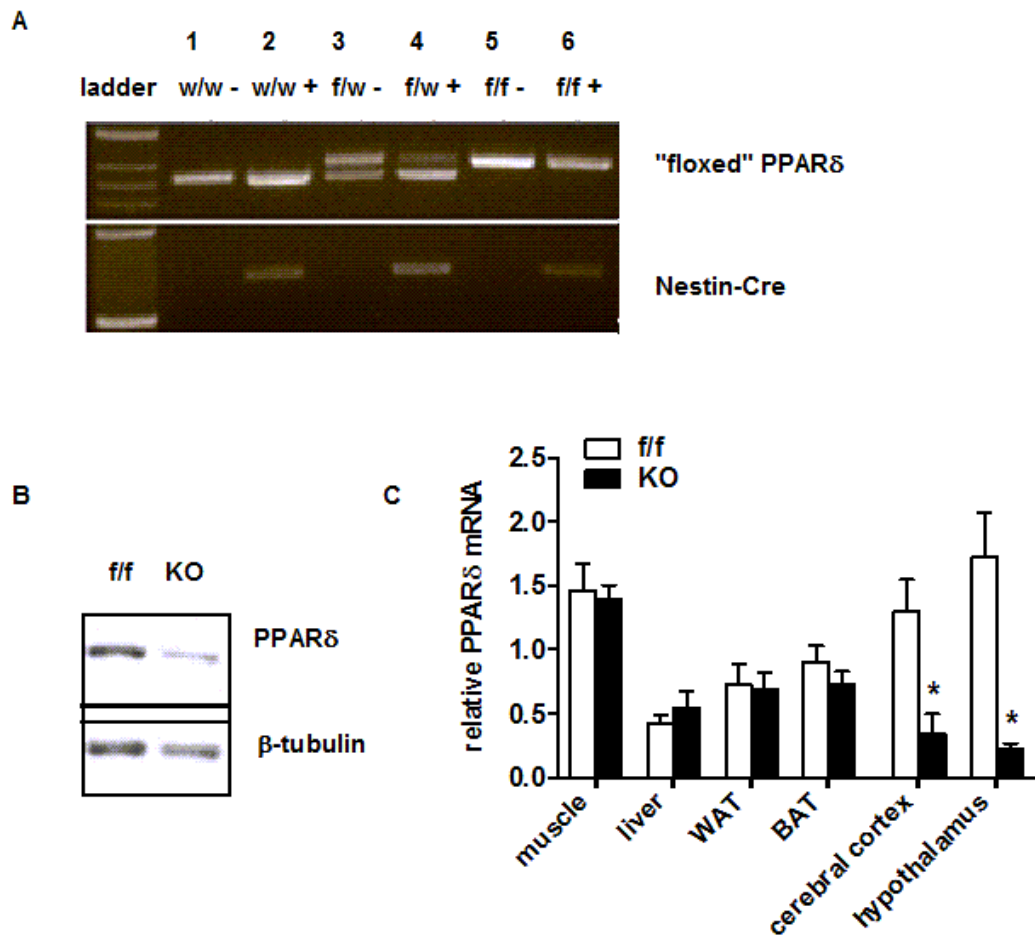


Figure 19. Validation of CNS PPAR δ lox-P knock-out mouse model.

A) UV detected PCR products, stained with EtBr, used to illustrate genotyping are shown. Genotypes were confirmed through PCR of hypothalamic DNA using primers for wild type (wt) and floxed (f/f) alleles of PPAR δ as well as Nes promoter-Cre recombinase transgene (+). Double heterozygous crosses yielded 6 genotypes (Lane 1, PPAR $\delta^{wt/wt}$ Nestin-Cre-; lane 2, PPAR $\delta^{wt/wt}$ Nestin-cre+; lane 3, PPAR $\delta^{fl/wt}$ Nestin-cre-; lane 4 PPAR $\delta^{fl/wt}$ Nestin-cre+ (Het); lane 5, PPAR $\delta^{fl/fl}$ Nestin-cre- and lane 6, PPAR $\delta^{fl/fl}$ Nestin-cre+ (KO)). B) Representative Western blot of PPAR δ levels in total cellular protein extracts from mediobasal hypothalamus of f/f and KO mice. β -tubulin was used as a loading control. C) Quantification of tissue PPAR δ mRNA expression in f/f and KO mice (muscle, liver, white adipose tissue (WAT), brown adipose tissue (BAT), cerebral cortex and hypothalamus). PPAR δ mRNA levels were measured by RT-PCR and normalized to endogenous levels of the housekeeping gene RPL13A (n=4-8). Values represent the mean \pm SEM of each genotype group. Statistical significance is designated as *(p<0.05 vs. f/f control group), as determined by two tailed student's t test.

Global deletion of exon 4 of the PPAR δ gene leads to embryonic lethality in mice (302). The effect of CNS PPAR δ deletion on embryonic viability and neonate survivability was assessed through a Chi square analysis of genotypes with a comparison to expected Mendelian ratios of offspring. This analysis of genotypes indicated that neuronal ablation of PPAR δ was not lethal ($p < 0.001$). PPAR δ Het and KO mice were fertile and had no apparent developmental abnormalities compared to their floxed (PPAR $\delta^{f/f}$) littermate control mice.

Genotypes were confirmed through PCR of hypothalamic DNA using primers for wild type and floxed alleles of PPAR δ as well as the Nestin-Cre transgene. The PCR products were visualized by UV detection of DNA bands stained with EtBr (Figure 19A). Mice with the wild type allele for PPAR δ were identified by a single band at 359 bp, mice homozygous for the floxed allele were identified by a single band at 400 bp and heterozygotes by the presence of both the wild type and floxed alleles (Figure 19A, top panel). A single 100 bp band indicated the presence of the Nestin-Cre transgene (Figure 19A, bottom panel). Hypothalamic PPAR δ protein levels, as determined by the presence of an immunoreactive species, were qualitatively reduced in KO compared to control mice, as assessed through Western blot analysis of total hypothalamic protein extracts (Figure 19B).

PPAR δ is ubiquitously expressed and is an important regulator of lipid metabolism in a number of peripheral tissues, including muscle (223, 227-231), liver (336, 370-374), brown adipose tissue (BAT) (375) and white adipose tissue (WAT)(235). Quantitative RT-PCR of PPAR δ mRNA confirmed that peripheral levels were not different between KO and control mice (Figure 19C). Neuronal deletion led to a $74 \pm 6\%$ reduction in cortex and an $88 \pm 1\%$ reduction in hypothalamus of PPAR δ mRNA of KO mice relative to f/f control mice (Figure 19C, $p < 0.001$).

Global genetic deletion of the LBD of PPAR δ leads to reduced brain size and myelination defects (254). Histological analysis was performed to determine if the CNS is grossly altered by neuronal deletion of the DBD of PPAR δ . Nissl stained coronal sections (Figure. 20) at the level

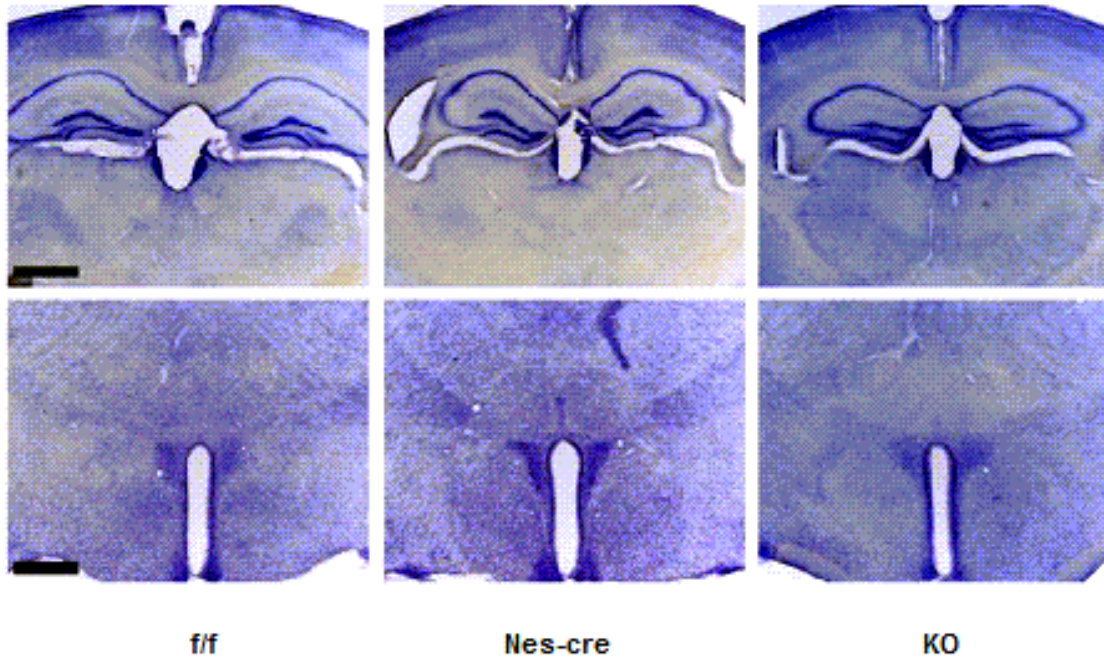


Figure 20. Brain morphology in CNS PPAR δ KO mice.

Photomicrographs of Nissl staining of brains from PPAR δ KO and control, PPAR $\delta^{f/f}$ (*f/f*) and Nestin-Cre, mice are illustrated. Representative sections shown at the level of the hippocampus (top) and hypothalamus (bottom). Scale bar = 500 μ m.

of the hippocampus (top panel) and hypothalamus (bottom panel) revealed no obvious differences between KO mice and control (*f/f*) or unfloxed negative controls (Nes-Cre, Figure 20), indicating no malformations in the structure of these or any other forebrain nuclei. Additionally, brain weight was not different between *f/f* and KO mice (*f/f*, 0.46 ± 0.01 g vs. KO, 0.47 ± 0.01 g, $p=0.82$), consistent with normal CNS morphology. PPAR δ KO mice were fertile and had no apparent developmental abnormalities compared to *f/f* control. I proceeded to use this animal model to study the effects of loss of neuronal PPAR δ function on energy homeostasis.

Body weight and composition of CNS PPAR δ KO mice fed chow diet

I hypothesized that PPAR δ regulates gene expression in response to endogenous lipids, and with its deletion, an obese phenotype on a chow diet would ensue.

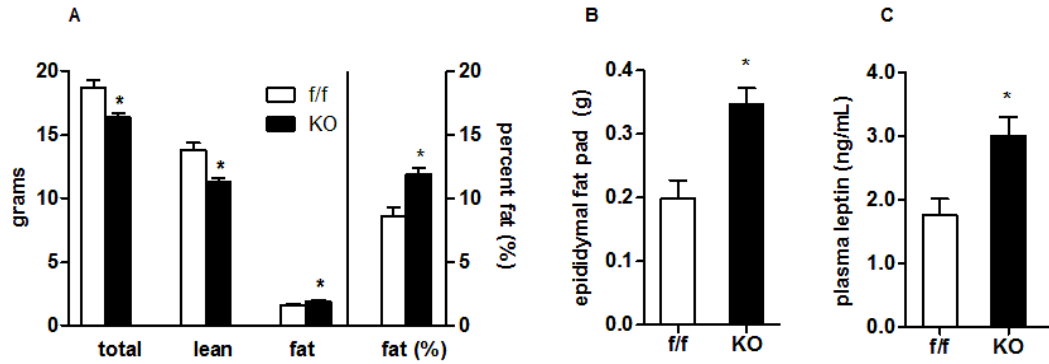


Figure 21. Effects of CNS PPAR δ deletion on body weight and adiposity.

A) Total body weight (total), lean and fat mass (body composition) and percentage fat (adiposity) was measured in 5-week-old, chow-fed f/f and KO mice. B) Epididymal fat pad mass and C) plasma leptin levels in aged-matched, chow-fed f/f and KO mice are shown. Values represent the mean \pm SEM. Statistical significance is denoted by * ($p < 0.05$, vs. f/f controls), as determined by two-tailed student's t test.

Body weight and body composition were assessed in 5-week-old f/f and KO mice fed a chow diet to determine if genetic loss of neuronal PPAR δ alters the basal phenotype of mice. Interestingly, body weight (BW) was reduced by 13 \pm 1% in KO mice (Figure 21A, $p < 0.001$), a difference that was largely due to a 12 \pm 1% reduction in lean body mass (Figure 21A, $p < 0.001$). Despite lower total BW, fat mass was slightly but significantly increased by 16 \pm 1% (Figure 21A, $p < 0.05$). Adiposity, which indicates the percentage body fat (fat mass/BW \times 100), was increased by 138 \pm 1% in KO mice (Figure 21A, $p < 0.01$). Elevated adiposity was due to increased fat storage in adipose, as seen by a near doubling of epididymal adipose tissue in 12-week-old PPAR δ KO mice (Figure 21B) and higher circulating basal leptin levels (Figure 21C).

CNS PPAR δ deletion leads to altered body composition and leptin insensitivity

Leptin acts as an adiposity negative feedback signal, controlling fat mass (34, 376) through the coordinated regulation of food intake and energy expenditure. Resistance to the

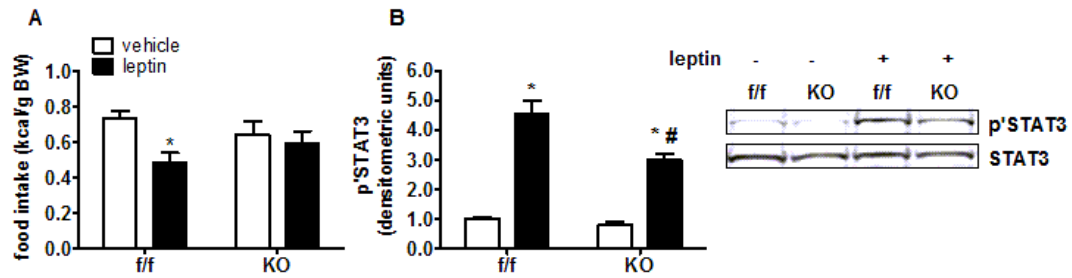


Figure 22. Effect of CNS PPAR δ deletion on hypothalamic leptin sensitivity.

A) Food intake in chow-fed f/f and KO mice were measured after receiving a bolus injection of leptin (5 mg/kg BW, IP) or vehicle (saline) at the onset of the dark period. Mice were housed individually and food intake was measured over 24 hours (n=10-12). Values represent the mean \pm SEM. (B) Hypothalamic total protein extracts from f/f and KO mice treated with leptin (5 mg/kg BW, IP) or vehicle for 30 minutes were used for Western blot analysis of STAT3 phosphorylation (Y705). Total STAT3 levels were determined and used as a loading control. Densitometry of blots yielded relative intensity levels, which are expressed as an activation index (pSTAT3/total STAT3) and represented as the group mean \pm SEM (n=4-6) relative to the f/f vehicle group. Statistical significance is denoted by * ($p < 0.05$, leptin (black bars) vs. vehicle (white bars) treated groups within each mouse genotype) and/or # ($p < 0.05$, KO vs. f/f mice treated with leptin), as determined by two-way ANOVA and Bonferroni post test.

behavioral and biochemical effects of leptin is a hallmark of obesity (136). Leptin treatment (5mg/kg BW, IP) reduced 24-hour caloric intake (kcal/g BW) by 24% in f/f mice (Figure 22A, $p < 0.05$) compared to vehicle, but failed to reduce food intake in KO mice (Figure 22A). STAT3 is a direct molecular target of leptin receptor activation (44) and its phosphorylation state can be used as a biochemical marker of leptin sensitivity (95). Leptin treatment (5mg/kg BW, IP) increased phosphorylation of STAT3 (Y705) by 4.6 ± 0.5 -fold in hypothalami of f/f mice, while KO mice exhibited a significantly reduced response to leptin treatment, which increased phosphorylation by only 3.7 ± 0.3 -fold in the KO group (Figure 22B, $p < 0.05$).

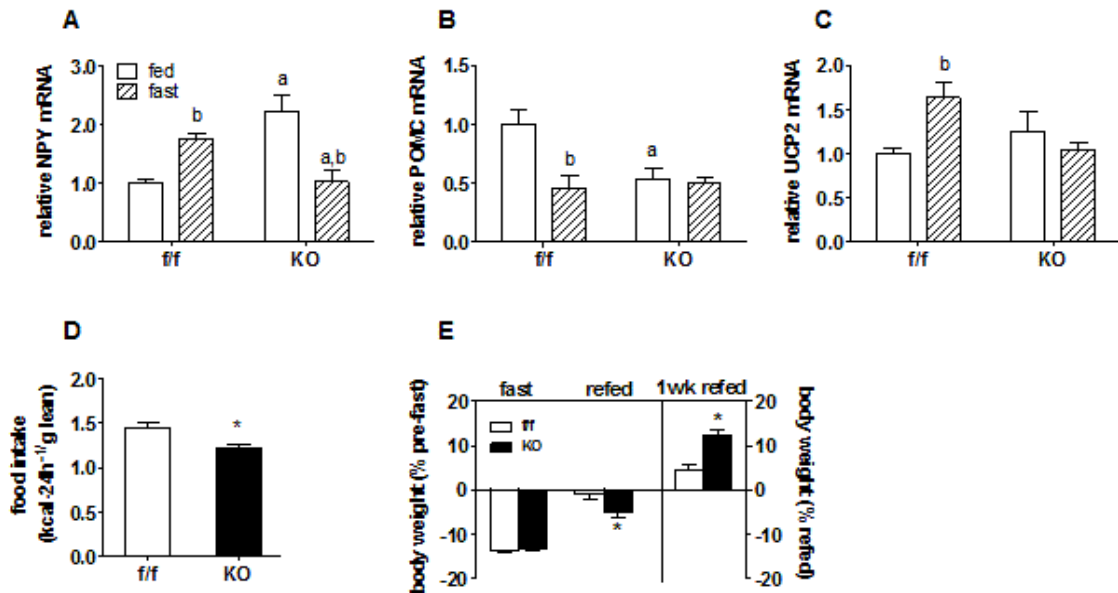


Figure 23. Effects of CNS PPAR δ deletion on neuropeptide expression and the refeeding response after a prolonged fast.

A-C) Fasting induced changes in NPY (A), POMC (B) and UCP2 (C) hypothalamic mRNA levels of f/f and KO mice fed chow diet ad libitum (fed) or fasted for 24 hours (fast). Target gene mRNA levels were normalized to RPL13A mRNA levels and shown as the group mean \pm SEM, expressed relative to the fed, f/f control. D) Food intake (chow diet) during a 24-hour refeeding period was measured after fasting in a separate cohort of individually housed mice (n=6-7). Graph shows food intake normalized to basal, pre-fast lean mass (kcal/g lean mass). E) Percent changes in body weight after fasting, refeeding for 24 hours or 1 week. In A-C, statistical significance is designated by ^a (p<0.05, f/f vs. KO, same treatment) and/or ^b (p<0.05, fed vs. fast, same genotype), as determined by two-way ANOVA and Bonferroni post test, or in D and E by * (p<0.05, vs.f/f), when determined by two-tailed student's t test.

Altered neuropeptide gene expression and compensatory refeeding after prolonged fasting in CNS PPAR δ KO mice.

NPY is a powerful orexigenic neuropeptide and activator of food intake that is potently induced by fasting and in the absence of leptin signaling (83, 84, 86, 377). In this situation, simultaneous inhibition of POMC neurons (and down regulation of anorexigenic POMC gene expression) facilitates subsequent hyperphagia and weight regain (89). Consistent with leptin resistance in KO mice (Figure 22), hypothalamic NPY mRNA levels were elevated (Figure 23A)

and POMC gene expression was reduced in the fed state (Figure 23B). Fasting elicited the appropriate neuropeptide gene expression pattern, increasing NPY mRNA levels by 1.75-fold (Figure 23C) and decreasing POMC gene expression by half (Figure 23D), in hypothalami of *f/f* mice. Fasting in KO mice, paradoxically, decreased NPY expression (Figure 23A) but did not further reduce POMC gene expression relative to fed KO mice (Figure 23B).

UCP2 mRNA levels are induced by fasting in hypothalamic NPY and POMC neurons, where it has been implicated in hypothalamic nutrient sensing, hormone signaling, neuronal firing and neuropeptide gene expression (induction of NPY and repression of POMC) (*116*). My previous experiments (Chapter 3, Figure 16) confirmed that UCP2 is a target gene of PPAR δ in a clonal hypothalamic neuronal cell line. KO mice should therefore exhibit an abnormal transcriptional response to physiological elevations in lipid, that could be detected at the level of UCP2 gene expression. Consistent with this, UCP2 gene expression was increased 1.6-fold by fasting in hypothalami of *f/f* mice but was unchanged in fasted KO mice (Figure 23C), given the UCP2 requirement for NPY induction (*116*) and that UCP2 is a gene target of PPAR δ , this suggests a potential molecular mechanism for disruption of neuropeptide gene expression in CNS PPAR δ KO mice.

To understand the functional implications of altered neuropeptide expression in KO mice, food intake was determined in a second group of individually housed mice following a 24 hour fast. Consistent with blunted fasting induced UCP2 and NPY expression, KO mice consumed significantly fewer calories (normalized to lean mass, kcal/g lean mass) after fasting (Figure 23D), resulting in attenuated weight regain after 24 hours of refeeding (Figure 23E). Interestingly, KO mice gained significantly more weight than *f/f* mice in the 8 days following the fasting challenge (Figure 23E), consistent with the reduced POMC gene expression in fed KO mice, suggesting that stress-induced weight gain may be exaggerated in the long term. Together with impaired leptin sensitivity, these data raise the possibility that loss of PPAR δ function in neurons impairs both anorexogenic and orexigenic tone.

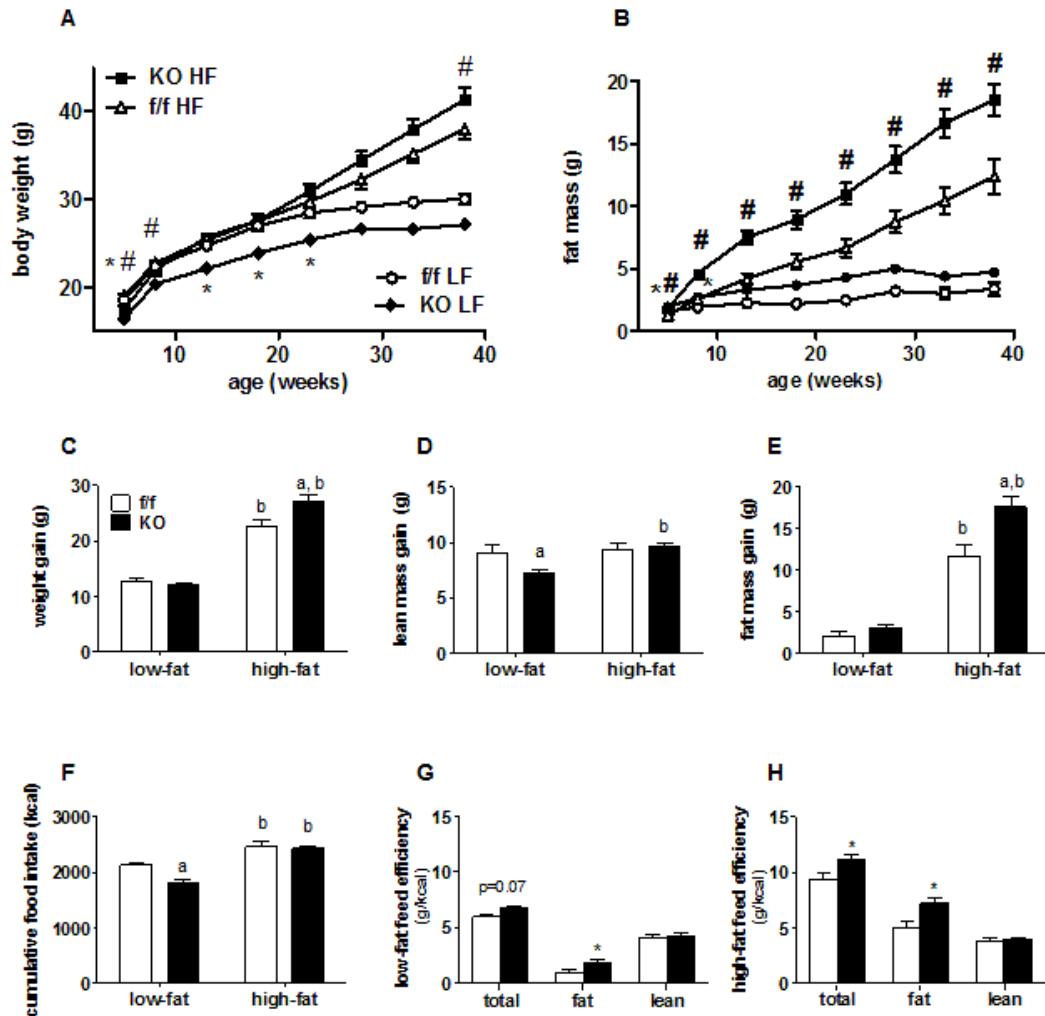


Figure 24. Effects of CNS PPAR δ deletion on susceptibility to diet induced obesity.

Growth curves of f/f and KO mice fed LF or HF diet for 33 weeks (age 5-38 weeks) are shown for body weight (A) and (B) fat mass, measured at the indicated ages. Total weight gain over the experimental period for body weight (C), lean (D), and fat mass (E) is shown for f/f and KO mice. Cumulative food intake as kcal (F) and feed efficiency of f/f and KO mice fed LF (G) or HF (H) diet (weight gained (either BW, fat or lean mass)/cumulative kcal). Values represent the mean \pm SEM. Statistical significance in A-B is denoted by * (LF f/f vs. LF KO) and # (HF f/f vs. HF KO) or in C-E as ^a ($p < 0.05$, f/f vs. KO, same diet) and/or ^b ($p < 0.05$, LF vs. HF, same genotype), when determined by one-way ANOVA and Bonferroni post test, or in G and H, by * ($p < 0.05$ vs. f/f controls), when determined by two-tailed student's t test.

CNS PPAR δ deletion leads to increased susceptibility to diet-induced obesity

Given the known role of PPAR δ in the regulation of genes that reduce oxidative stress and promote lipid oxidation (223) and its recognized anti-inflammatory effects in the CNS (323), Based on this evidence, PPAR δ could act as an important molecular determinant of susceptibility to environmentally induced obesity. To test this, at 5 weeks of age, mice were placed on a diet with HF content (45% kcal as fat) or a micronutrient matched control diet with LF content (10% kcal as fat).

Although smaller at weaning, KO mice have normal growth and gain a similar amount of weight as f/f mice over 33 weeks of LF diet feeding (Figure 24A, C). Exposure to HF diet led to significant weight gain in both genotypes. However, KO mice were more susceptible to DIO, becoming significantly heavier than HF fed f/f control mice after 33 weeks (Figure 24A). At the end of the 33-week experimental period, HF fed KO mice had gained 16% (~5g, Figure 24C) more body weight and were 6% heavier than f/f mice fed the same HF diet (Figure 24A), revealing a role for neuronal PPAR δ expression in the determination of body weight gain and DIO susceptibility.

Differences in body composition can have profound effects on the metabolic implications of weight gain. On LF diet, KO mice have reduced lean mass gain (Figure 24D), whereas HF feeding lead to identical lean mass gain. At baseline, KO mice have slightly more fat mass (Figure 24B), which is maintained over the course of 33 weeks of LF diet feeding (Figure 24E). The effect of HF diet consumption to induce obesity was augmented in KO mice, which had 6 grams or 33% more fat mass than f/f control fed HF diet (Figure 24B, EF). These data show that increased body weight gain in KO mice on HF diet was due to a profound accumulation of fat mass and reveals a significant interaction between CNS PPAR δ function and dietary fat exposure.

Absolute food intake was reduced in KO mice fed LF diet, whereas no differences in cumulative (33 weeks) food intake were observed between the genotypes on HF diet (Figure 24F). Feed efficiency (calories consumed to gain 1 gram of mass) was elevated for total body

Table . Effect of CNS PPAR δ deletion on energy expenditure

Energy expenditure (EE) and respiratory exchange ratio (RER) were measured over 24 hours by indirect calorimetry in individually housed f/f and KO mice after 20 weeks on HF diet (n=4). Values for 24 hours, daily, EE (kcal) and food intake were also normalized to body weight and lean mass. Values represent mean \pm SEM of each group and statistical significance is indicated by * (p<0.05), for comparisons between KO vs. f/f, same diet, using a Student's t test.

	f/f	KO
BW (g)	30.10 \pm 1.44	30.90 \pm 1.61
lean mass (g)	19.05 \pm 0.37	16.95 \pm 0.42*
Daily EE (kcal)	11.11 \pm 0.18	11.01 \pm 0.27
Daily EE (kcal/g BW)	0.38 \pm 0.01	0.35 \pm 0.01
Daily EE (kcal/g lean mass)	0.58 \pm 0.01	0.65 \pm 0.01*
RER dark period	0.86 \pm 0.01	0.82 \pm 0.02
Daily FI (kcal)	10.78 \pm 0.24	10.43 \pm 0.62
Daily FI (kcal/g BW)	0.37 \pm 0.01	0.37 \pm 0.01
Daily FI (kcal/g lean mass)	0.58 \pm 0.02	0.64 \pm 0.02*

weight gain on HF diet, and for fat mass gain on both LF and HF diet in KO mice, relative to controls (Figure 24F, G). Interestingly, feed efficiency of KO mice for lean mass gain was not different from control mice on either diet (Figure 24G, H). Together, these data suggest that KO mice are more efficient at storing dietary fat as adipose.

To determine if reduced energy expenditure contributes to increased fat mass gain in KO mice, energy expenditure (EE) was measured by indirect calorimetry. Interestingly, after 20 weeks of HF diet exposure, daily EE (kcal) and EE normalized to total body weight (kcal/g BW) were not different in KO mice, relative to f/f mice (Table 6). At this time point, KO animals had similar total body weight but reduced lean mass (Table 6). When normalized per gram lean mass (kcal/g lean mass), KO mice exhibit a slight but significant elevation in EE over 24 hours relative to f/f mice (Table 6). During the measurement period, both daily food intake (kcal) and daily food intake normalized to body weight (kcal/g BW) were similar in KO and f/f controls. When daily

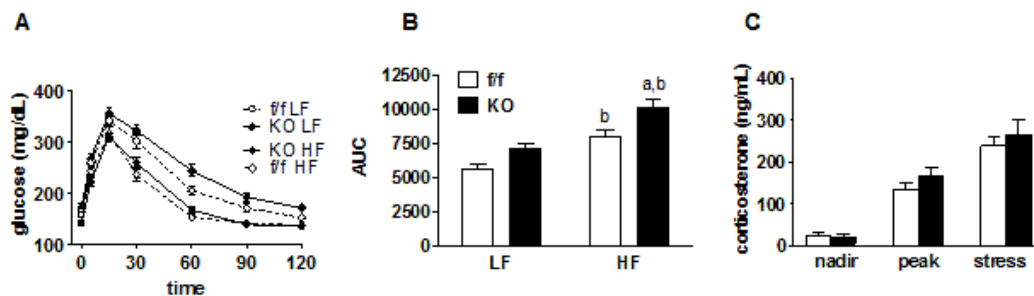


Figure 25. Effects of CNS PPAR δ deletion on glucose tolerance and corticosterone response.

A) Glucose tolerance tests are illustrated, showing the changes in blood glucose levels after a glucose injection (1 g/kg BW, IP), for f/f and KO mice fed either a LF or HF diet for 20 weeks (n=8-10). Mice were fasted for 4 hours and blood glucose levels were measured via tail blood at the indicated time points post glucose injection. B) Quantitation of area under the glucose curve (AUC) from glucose tolerance testing is shown for LF and HF fed f/f and KO mice. C) Plasma corticosterone levels in individually housed, chow fed, f/f and KO mice are illustrated. Blood was collected at nadir (8am), peak (5pm) or 30 minutes after a mild, restraint stress. Values represent the mean \pm SEM. Statistical significance is denoted in panel B as ^a (p<0.05, f/f vs. KO, same diet) and/or ^b (p<0.05, LF vs. HF, same genotype), as determined by two-way ANOVA and Bonferroni post test.

food intake was normalized to lean mass (kcal/g lean mass), KO mice exhibited increased intake (Table 6).

Consistent with elevated fat mass (Figure 24), KO mice had significantly higher plasma leptin levels after 33 weeks on LF diet and displayed a profound increase after HF feeding (Table 7). Insulin was also elevated in KO mice but only on HF diet (Table 7), which was accompanied by a greater glucose intolerance in KO mice fed HF diet for 20 weeks, compared to HF fed f/f control mice (Figure 25A, B).

Table 7. Effects of CNS PPAR δ deletion on circulating concentrations of hormones and lipids

Values represent mean \pm SEM of each group (n=9-11) and statistical significance is indicated by.^a ($p < 0.05$, f/f vs. KO, same die) and/or ,^b ($p < 0.05$, LF vs. HF diet, same genotype), as determined by two-way ANOVA followed by Bonferroni post test.

	LF (10% kcal fat)		HF (45% kcal fat)	
	f/f	KO	f/f	KO
insulin (ng/mL)	0.61 \pm 0.08	1.00 \pm 0.15	1.26 \pm 0.26	2.21 \pm 0.20 ^{a,b}
leptin (ng/mL)	5.7 \pm 1.0	18.1 \pm 2.3 ^a	64.7 \pm 21.4 ^b	129.3 \pm 11.4 ^{a,b}
FFA (mM)	0.66 \pm 0.08	0.63 \pm 0.06	0.82 \pm 0.08	0.73 \pm 0.03
TG (mg/dL)	38.1 \pm 3.0	53.1 \pm 5.2 ^a	63.6 \pm 5.5 ^b	46.5 \pm 4.2

Effects of CNS PPAR δ deletion on glucose tolerance and corticosterone response

PPAR δ is expressed throughout the CNS and is enriched in a number of other brain regions (248). To determine if neuronal loss of PPAR δ resulted in dysregulation of the HPA axis, diurnal and stress induced levels of the hormone corticosterone, which is associated with elevated adiposity and insulin resistance, were measured. Diurnal plasma concentrations (nadir and peak), and stress induced plasma corticosterone levels, did not differ between KO and f/f mice (Figure 25C), ruling out gross abnormalities in the HPA axis as a cause for elevated fat mass gain.

Effects of CNS PPAR δ deletion on brain lipid content and composition and hypothalamic gene expression of lipid metabolism genes

Hypothalamic lipid accumulation/lipotoxicity is implicated in obesity (133). On LF diet, total levels of brain lipids (free fatty acid (FFA), diglyceride (DG), triglyceride (TG)) were not different between the genotypes (Figure 26A). Exposure to HF diet increased brain FFA content by 1.5-fold in f/f mice but did not alter brain FFA levels in KO mice, which had significantly lower levels of FFAs, relative to the HF fed f/f control mice (Figure 26A). HF diet also led to a

significant reduction in total TG levels in brains of KO mice, relative to the level of the LF fed KO group (Figure 26A).

To determine if a transcriptional response was involved in the observed alterations in CNS lipid content, hypothalamic mRNA levels of several proteins involved in FFA uptake, lipoprotein lipase (LPL) and cluster of differentiation 36 (CD36), as well as triglyceride

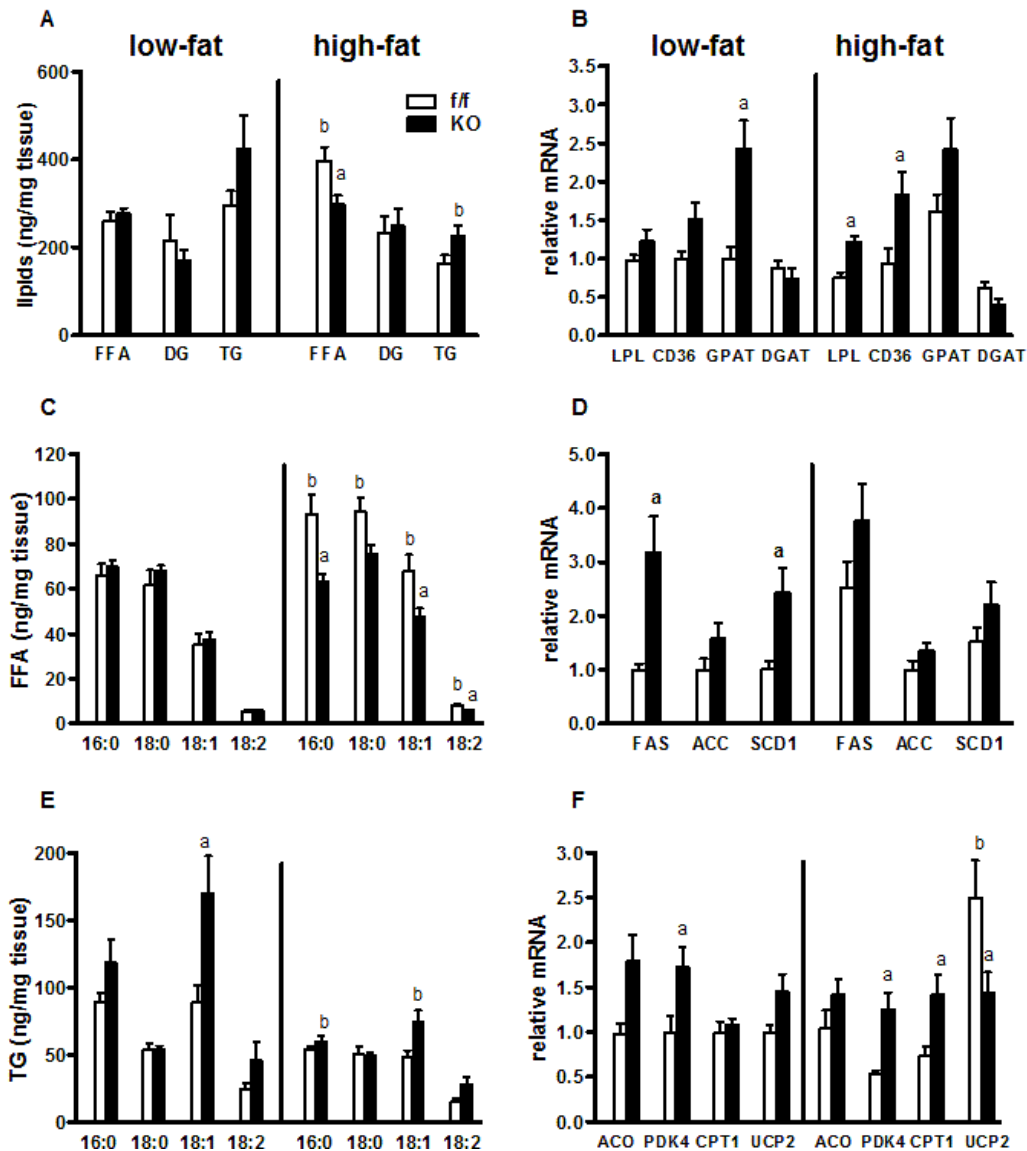


Figure 26. Effects of CNS PPAR δ deletion on brain lipid content and composition and hypothalamic gene expression of lipid metabolism genes.

A) Total levels of triglyceride (TG), diglyceride (DG) and free fatty acid (FFA) extracted from total lipids from brains of *f/f* and KO mice ($n=6-7$) fed LF or HF diet for 33 weeks are reported. The quantity of individual FFA species (ng/mg tissue) making up brain FFA (C) and TG (E) fractions isolated from *f/f* and KO mice are depicted. Changes in hypothalamic mRNA levels of target genes involved in lipid uptake and storage (B; LPL, CD36, GPAT and DGAT), lipid synthesis (D; FAS, ACC, SCD) and fatty acid oxidation (F; ACO, PDK4, CPT1A, UCP2) were assessed by quantitative real-time PCR. Gene expression levels were normalized to endogenous RPL13A levels and are expressed as group mean \pm SEM relative to the level of the *f/f*, LF diet control group. Statistical significance is designated as ^a ($p<0.05$, *f/f* vs. KO, same diet) and/or ^b ($p<0.05$, LF vs. HF, same genotype), as determined by two-way ANOVA and Bonferroni post test.

storage, glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT) were quantified. On LF diet, hypothalamic mRNA levels of LPL, CD36 and DGAT were similar between f/f and KO groups, while GPAT gene expression increased by 2.4-fold in KO mice relative to the LF fed f/f control group (Figure 26B). Exposure to HF compared to LF diet did not alter expression of lipid uptake genes within either genotype (Figure 26B). On HF fed diet, LPL and CD36 mRNA levels were significantly elevated in KO mice relative to HF fed control levels (Figure 26B).

Levels of individual FFA species were similar between genotypes on LF diet (Figure 26C). In f/f control animals, HF diet increased the prevalence of SFAs, palmitate (16:0) by 1.5-fold and stearate (18:0) by 1.4-fold, the MUFA oleate (18:1) by 1.2-fold, and the PUFA linoleate by 1.5-fold (Figure 26C). PPAR δ deletion prevented HF diet-induced accumulation in these common dietary FFAs in the CNS (Figure 26C). Expression of key lipogenic enzymes, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), as well as stearoyl CoA desaturase 1 (SCD1) were determined to test the possibility that differences in lipogenesis could be involved. On LF diet, FAS gene expression was 3.1-fold higher in KO mice compared to f/f mice (Figure 26D). HF diet exposure increased FAS mRNA levels in f/f mice relative to LF diet levels, reaching levels roughly equivalent to that observed of LF diet fed KO mice (Figure 26D); however, HF diet did not additionally increase gene expression in KO mice relative to LF diet or HF fed f/f mice. Gene expression of SCD1 was also elevated by 2.4-fold in KO mice fed LF diet, compared to f/f controls on LF diet. Levels of SCD1 mRNA were not different between KO and f/f mice fed HF diet (Figure 26D), nor were they different between KO HF fed and KO LF fed mice. ACC mRNA levels did not differ among the groups on either diet (Figure 26D).

In agreement with increased expression of FAS, GPAT and SCD1, KO mice had elevated 18:1 levels in TG when on LF diet (Figure 26E). Transition to a HF diet resulted in significantly reduced 16:0 and 18:1 levels in TG of KO animals (Figure 26E). To determine if increased fatty acid oxidation might contribute to decreased FFA accumulation in brains of KO mice fed HF diet,

mRNA expression levels of key proteins involved in lipid catabolism we quantified. On LF diet, pyruvate dehydrogenase kinase 4 (PDK4) gene expression increased by 1.7-fold in KO mice (Figure 26F). HF feeding increased expression of UCP2 in f/f mice by 2.4-fold, but did not alter UCP2 expression in KO mice (Figure 26F). On HF diet, KO mice had ~2-fold greater expression of two markers of mitochondrial fatty acid oxidation, carnitine palmitoyltransferase 1A (CPT1A) and PDK4, compared to f/f mice fed HF diet. Expression of acyl-CoA oxidase (ACO), a marker of peroxisomal fatty acid beta-oxidation, did not differ among the groups on either diet (Figure 26F). As a whole, these data suggest that PPAR δ contributes to the regulation of expression of lipid metabolism genes in the hypothalamus, thereby affecting both CNS basal lipid metabolism and responses to increased dietary lipid exposure.

CNS PPAR δ KO mice are resistant to diet-induced hypothalamic inflammation

Agonists of PPAR δ prevent lipotoxicity and have anti-inflammatory effects in the CNS (263, 308). In order to determine if loss of PPAR δ increases inflammatory signaling in hypothalamus, mRNA levels of the inhibitor of NF- κ B, I κ B α , and two transcriptional targets of NF- κ B, the pro-inflammatory cytokines, IL-6 and IL-1 β (378) were assessed. On LF diet, I κ B α levels in hypothalamic total cell protein extracts were reduced by 25% in KO mice relative to LF fed f/f mice (Figure 27A). Exposure to HF diet reduced I κ B α protein levels by ~40% in f/f mice,

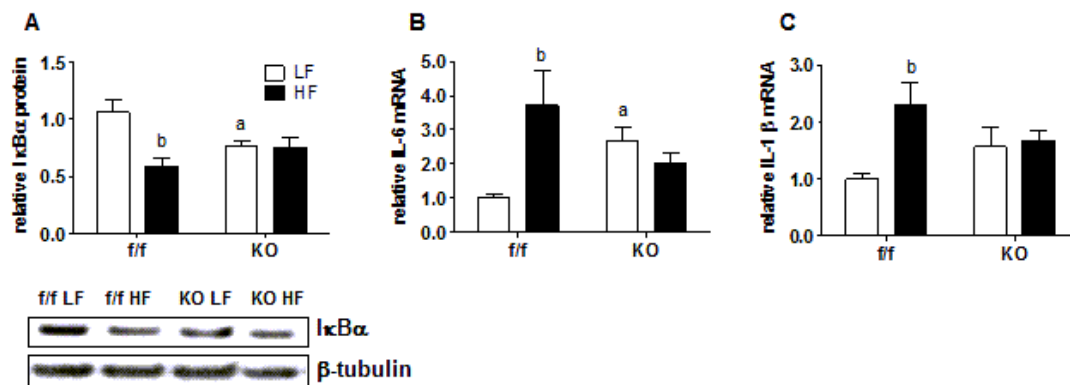


Figure 27. Effects of CNS PPAR δ deletion on diet-induced hypothalamic inflammation.

Protein and mRNA were isolated from bisected sections of mediobasal hypothalamus of f/f and KO mice fed LF or HF diet for 33 weeks. A) Total hypothalamic protein extracts were subjected to Western blot analysis using an antibody directed against total I κ B α . Levels of β -tubulin were determined and used as a loading control. Densitometry of blots yielded relative intensity as a measure of protein levels (n=6). Insert of A shows a representative Western blot. Hypothalamic mRNA levels of inflammatory cytokines IL-6 (B) and IL-1 β (C) in f/f and KO mice measured by RT-PCR after 33 weeks of Lf or HF diet are shown. Target gene mRNA levels were normalized to endogenous RPL13A levels. Values are represented as group mean \pm SEM relative to the LF f/f control group. Statistical significance is designated as ^a (p<0.05, f/f vs. KO, same diet), and/or ^b (p<0.05, LF vs. HF, same genotype), as determined by two-way ANOVA and Bonferroni post test.

as reported in several DIO models (133, 137). HF diet exposure failed to reduce I κ B α protein levels beyond the lower levels found on LF diet in hypothalamus of KO mice (Figure 27A), thus f/f and KO mice both had protein levels of I κ B α on HF diet. Consistent with I κ B α data and activation of pro-inflammatory signaling, KO animals have increased hypothalamic IL-6 and IL-1 β gene expression on LF diet relative to LF fed f/f controls (Figure 27B, C). HF diet increased mRNA levels of IL-6 and IL-1 β in f/f control mice, while KO mice were protected from a further HF diet induced elevation of hypothalamic cytokine gene expression (Figure 27B, C).

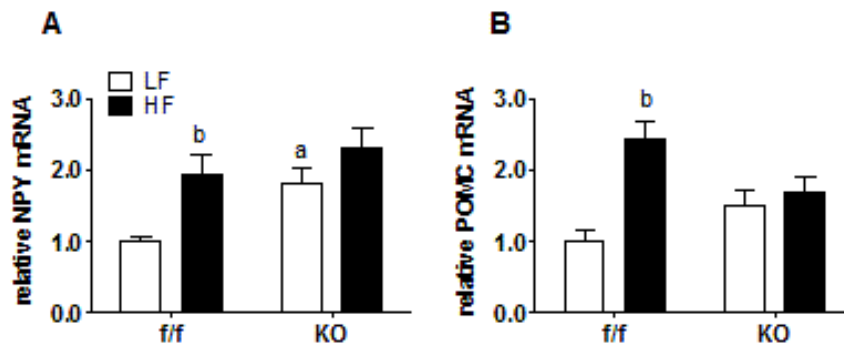


Figure 28. Effects of CNS PPAR δ deletion on hypothalamic neuropeptide mRNA levels.

Hypothalamic mRNA levels of neuropeptides, NPY (A) and POMC (B), in f/f and KO mice (n=6-7) fed LF or HF diet for 33 weeks were assessed by quantitative RT-PCR. Target gene mRNA levels were normalized to endogenous RPL13A levels. Values represent the mean \pm SEM. Statistical significance is designated by ^a (p<0.05, f/f vs. KO, same diet) and/or ^b (p<0.05, LF vs. HF, same genotype), as determined by two-way ANOVA and Bonferroni post test.

CNS PPAR δ deletion alters regulation of hypothalamic neuropeptide gene expression

To identify mechanisms by which loss of neuronal PPAR δ increases DIO susceptibility, hypothalamic mRNA levels of NPY and POMC, key regulatory neuropeptides were determined. On LF diet, NPY gene expression increased by 1.8-fold in KO mice (Figure 28A), which was consistent with the basal phenotype of KO animals on a chow diet (Figure 23). HF diet exposure doubled NPY mRNA levels in f/f mice, but did not further increase NPY mRNA levels in KO mice (Figure 28A). POMC mRNA levels did not differ between KO and f/f mice fed LF diet (Figure 28B). On HF diet, POMC expression increased 2.4-fold in f/f mice, but was not altered by HF diet exposure in KO mice (Figure 28B).

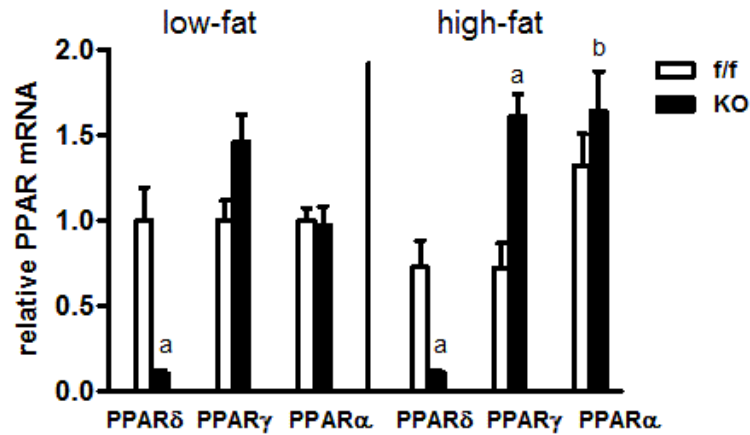


Figure 29. Effects of CNS PPAR δ deletion on hypothalamic PPAR γ and PPAR α gene expression.

Hypothalamic mRNA levels of PPAR isoforms in f/f and KO mice fed LF or HF diet for 33 weeks was assessed by quantitative real-time PCR. PPAR δ , PPAR γ and PPAR α levels were normalized to endogenous RPL13A levels, and expressed relative to that of the f/f, LF fed group. Values represent group mean \pm SEM (n=6-7). Statistical significance is designated by ^a (p<0.05, f/f vs. KO, same diet) and/or ^b (p<0.05, LF vs. HF, same genotype), as determined by two-way ANOVA and Bonferroni post test.

CNS PPAR δ deletion leads to increased hypothalamic PPAR γ and PPAR α expression.

I next evaluated hypothalamic mRNA expression levels of all three PPAR isoforms and mRNA levels of several target genes of these transcription factors to determine if PPAR α and PPAR γ could have altered brain lipid content, inflammation and gene expression in PPAR δ KO mice. Gene deletion of PPAR δ resulted in a ~90% reduction in PPAR δ mRNA levels irrespective of diet (Figure 29). In line with similar CNS lipid content and hypothalamic gene expression of LF diet, expression levels of PPAR α and PPAR γ mRNA were similar between f/f and KO groups on LF diet (Figure 29). Consumption of HF diet increased PPAR α gene expression by 1.6-fold in KO, but not in f/f mice, relative to the LF fed f/f control group (Figure 29). PPAR γ gene

expression was 2.6-fold higher in hypothalamus of HF fed KO mice relative to HF fed f/f mice (Figure 27).

As you may recall in CNS PPAR δ KO mice, mRNA levels of the PPAR δ target gene UCP2, were not increased by fasting (Figure 23C) or HF diet (Figure 26F). In the context of elevated PPAR α mRNA levels in KO mice on HF diet, the PPAR α target gene, CPT1A, increased, although mRNA levels of another target gene of PPAR α , ACO, did not differ between genotypes (Figure 26F). Gene expression of PPAR γ target genes, LPL and CD36, were also elevated in the presence of higher PPAR γ gene expression in HF fed KO mice (Figure 26B).

Summary

In peripheral tissues, PPAR δ is a known regulator of lipid oxidation, and thereby, is thought to reduce inflammation and promote insulin sensitivity (223, 231, 379). Relatively less is known about PPAR δ function in the CNS, where it has been implicated in neuroprotection by reducing inflammation and oxidative stress (reviewed in (323)). The purpose of these experiments was to confirm a role for neuronal PPAR δ in energy homeostasis, and tested the hypothesis that PPAR δ acts to reduce lipid accumulation and inflammation, opposing the development of biochemical resistance to homeostatic signals such as leptin and genetic deletion, would therefore be predicted to lead to obesity. Consistent with this hypothesis, neuronal PPAR δ deletion results in higher adiposity on LF diet and a profound susceptibility to DIO. Surprisingly, the results of these studies did not provide evidence favoring a role for PPAR δ in stimulation of neuronal FFA oxidation and reduction of lipotoxic intermediates. Rather, loss of PPAR δ led to reductions in CNS FFA accumulation accompanied by a similar attenuation in hypothalamic inflammation in response to dietary fat exposure that could not be attributed to alterations in peripheral inflammatory mediators (not shown). Several mechanisms may explain this finding, including a robust upregulation of PPAR γ , and perhaps, PPAR α function. Recently, brain PPAR γ activation has been implicated in hyperphagia, weight gain and obesity in rodents (242, 366), potentially

consistent with these findings. These findings reveal a previously underappreciated role for neuronal PPAR δ in the regulation of body composition, obesity susceptibility, and in suppression of hypothalamic PPAR α and PPAR γ expression.

Neuronal deletion of PPAR δ results in a complex phenotype characterized by increased fat mass and reduced lean mass on a LF diet. KO mice fed LF diet were more efficient at storing fat mass despite lower total food intake. In addition to recapitulating an obesity-like phenotype characterized by higher adiposity on a LF diet, genetic loss of PPAR δ function potentiates fat mass gain on a HF diet. Excess fat mass accrual occurred, while mice were on a HF diet, in the absence of large differences in food intake or energy expenditure (normalized to BW). When these parameters were normalized to lean mass, KO mice consumed more calories and exhibited higher energy expenditure after 20 week on a HF diet. Therefore, preferential disposition of consumed calories towards adipose tissue storage, along with subtle imbalances between food intake and energy expenditure that are below the detection limits of the methods that were used to measure these parameters, contribute to excess fat mass gain in KO mice over many weeks of HF feeding.

Increased brain cytokine gene expression on LF diet and leptin resistance in chow fed mice indicates that PPAR δ has anti-inflammatory activity and supports basal leptin sensitivity. Coinciding with leptin resistance and mild hyperleptinemia, KO mice exhibit elevated basal NPY gene expression on LF diet. A possible explanation for why NPY gene expression is decreased during fasting, while POMC gene expression is not altered, may be a blunted induction of UCP2 expression that results in elevated levels of ROS, which has been shown to repress NPY gene expression while simultaneously promoting POMC gene expression (380). The regulatory effects of ROS on neuropeptide expression are abrogated by UCP2 mediated uncoupling of the mitochondrial proton gradient (373), and published studies implicate a protective role for UCP2 in neuroinflammatory disease and hypothalamic inflammation (381) via its ability to attenuate ROS production. Mice lacking the gene for UCP2 have elevated levels of basal NF- κ B activation

(367) and increased cytokine expression after ischemic injury (382). PPAR δ KO mice also displayed elevated hypothalamic expression of several proinflammatory cytokine genes on LF diet, supporting the hypothesis that PPAR δ has an important anti-inflammatory function.

Ectopic lipid accumulation and low-grade inflammation are linked to insulin and leptin resistance in the CNS (133, 325, 329) and activation of PPAR δ attenuates lipotoxicity in peripheral tissues (231, 234, 236, 238, 308). It was somewhat surprising then, to find that KO mice were resistant to CNS lipid accumulation and further inflammation even after prolonged HF feeding despite a baseline increase in markers of inflammation on LF diet. On LF diet, KO mice displayed a reduction in I κ B α , a key inhibitor of NF- κ B activity, and an increase in two key pro-inflammatory targets of NF- κ B regulation, IL-6 and IL-1 β . This basal hypothalamic inflammatory phenotype was not further altered with HF diet in KO mice. Limited FFA oxidation does occur in the CNS, and may play a particularly important role in homeostatic feeding centers (383). Gene expression of CPT1A, which catalyzes the rate limiting step of beta-oxidation and is implicated in neuronal fatty acid sensing (243), is induced by PPAR α activation, providing a possible explanation for the reduction in CNS FFA accumulation in KO mice fed HF diet.

If changes in PPAR gene expression have biological effects on lipid metabolism and inflammation, we would expect to find alterations in the expression of target genes of these receptors as well. There were consistent changes in the expression of several isoform specific target genes of PPAR α (CPT1A) and PPAR γ (LPL), which are known to be induced by specific chemical agonists of these receptors in the CNS (242, 244). PPAR α expression was slightly elevated, while PPAR γ was more significantly elevated in brains of KO mice on HF diet. An additional target gene of PPAR γ , CD36, also showed elevated expression in KO mice. Deletion of PPAR δ in cardiomyocytes was shown to cause a similar induction of PPAR α and PPAR γ , as well as their target genes involved in fatty acid oxidation (384). These findings are consistent with evidence from in vitro studies that show PPAR δ represses both the expression and activation of these other PPAR isoforms (276, 385). Activation of hypothalamic PPAR α and/or PPAR γ has

been implicated in weight gain and obesity, which is consistent with the elevated adiposity and greater susceptibility to DIO observed with PPAR δ KO mice. Thus, these data reveal a previously underappreciated role for neuronal PPAR δ in the regulation of body composition, neuropeptide expression, leptin sensitivity, obesity susceptibility, and suppression of hypothalamic PPAR α and PPAR γ gene expression.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

The overarching and major themes presented in this dissertation are:

I: Dietary fatty acid composition is an important determining factor of cellular and metabolic responses to dietary fat intake.

II: PPAR δ is a target of MUFAs in neurons and a mediator of cellular and metabolic responses.

III: PPAR δ in neurons has a role in the regulation of energy balance, fuel partitioning, and in homeostatic responses to metabolic challenges, as determined by studies in a neuronal genetic deletion model.

The focus of this chapter will be to place these three themes into a larger context of an obesogenic environment, and cellular mechanisms to protect against metabolic dysfunction.

The ability of dietary fat to promote insulin resistance in peripheral tissues has been appreciated for some time (119, 386, 387). Dietary fat may also contribute to obesity pathogenesis more so than other macronutrients (123). These effects are thought to be mediated primarily by SFAs, via their ability to induce lipotoxicity and inflammation in key regulatory neurons and brain regions (such as mediobasal hypothalamus), which contributes to positive energy balance and weight gain (133, 137, 313, 388). There is extensive epidemiologic evidence that MUFAs improve insulin sensitivity (187, 193, 332, 358, 389), promote weight loss (341, 354, 390) and reduce inflammation (314, 335, 391, 392).

Chapter III explored the role of dietary fat composition in DIO pathogenesis and diet-induced hypothalamic inflammation. A HF diet, rich in MUFAs, compared to a HF diet rich in SFAs promoted less weight gain (Figure 12), and did not activate markers of inflammation, ER or

oxidative stress in the hypothalamus (Figure 13). Similar caloric and fat contents of these two HF diets, coupled with similar levels of food intake between groups of rats consuming the HF-SFA and HF-MUFA diets, suggested that structural differences between MUFAs and SFAs may play an important role in the differential effects. This led to the hypothesis that dietary MUFAs may interact with a fatty acid sensor, potentially in the hypothalamus, that could protect against hypothalamic stress and inflammation, as well as promote insulin sensitivity.

Several lines of evidence suggested that PPAR δ could be involved in the beneficial effects of the HF-MUFA on hypothalamic inflammation and DIO. At the mRNA level, MUFAs, consumed in the diet increased expression of PPAR δ as well as a key neuronal PPAR δ target gene, UCP2 (238), in the hypothalamus. The biological effects of PPARs are mediated via transcriptional regulation of target gene expression (213), therefore it was important that increased expression of a known PPAR δ target gene, UCP2 could be detected. A specific-agonist (GW0742) of PPAR δ was administered via ICV cannula, into the 3rd ventricle of rats to recapitulate the effect of MUFAs, confirming UCP2 as a specific CNS gene target of ligand-activated PPAR δ in the hypothalamus.

The findings from in vitro studies described in chapter IV confirmed PPAR δ as a molecular mediator of the beneficial effects of MUFAs on ER and oxidative stress, inflammation and insulin signaling. In addition to inducing stress and inflammation, antagonism of PPAR δ with GSK0660 prevented ligand induced expression of the PPAR δ target gene UCP2. This data suggested that MUFAs are protective via transcriptional activation of PPAR δ and expression of target genes, such as UCP2, that encode proteins capable of opposing lipotoxicity. Thus, the next important question to address is if this occurs in vivo as well. However, a limitation of the genetic targeting approached used to disrupt PPAR δ function, through deletion of the DBD, may lead to depression of some target genes (276), as well as compensatory upregulation of other PPAR isoforms (276, 385). Therefore, to genetically recapitulate the molecular consequence of the antagonist GSK0660 in an in vivo system, these studies could be performed in mice that

conditionally express a dominant negative transgene lacking the carboxy terminal LBD of PPAR δ (393). If the effects of MUFAs are dependent on transcriptional activation of PPAR δ , consumption of the HF-MUFA diet would be expected to lead to a similar increase of weight gain and adiposity as the more obesogenic HF-SFA diet in mice that express a dominant negative form of PPAR δ .

Evidence from human feeding experiments suggests that dietary fat, more so than carbohydrate or protein, promotes triglyceride storage in adipose tissue (394, 395). However, the findings from cell culture experiments suggested PPAR δ acts to maintain insulin sensitivity in neuronal cells, in the presence of high levels of mixed fatty acids. This raises the possibility that PPAR δ could be involved in the beneficial effects of very low carbohydrate, high fat diets, such as Atkins on weight loss (396). Mice expressing the dominant negative form of PPAR δ could be fed an Atkins style diet containing high levels of protein and fat consisting of a mixture of SFAs and MUFAs with small amounts of carbohydrates, to determine if PPAR δ activation mediates the beneficial effects of low carbohydrate diets on weight loss. At the conclusion of this study, biochemical markers of hypothalamic ER stress (p⁺PERK), oxidative stress (p⁺p66shc) and inflammation (IL-6 mRNA) would also be predicted to be elevated in mice that express the dominant negative form of PPAR δ . Positive findings will support the conclusion that MUFAs protect against SFA induced obesity and hypothalamic stress and inflammation at least in part, through transcriptional activation of PPAR δ (Figure 31B).

Neuronal PPAR δ deletion did not affect brain size, neuronal density or gross CNS morphology, nor did it cause observable behavioral abnormalities. Based on this evidence, CNS development appears to be normal in KO mice. However, nestin mediated deletion occurs at embryonic day (E) 7.5-8.0 and recombination of a floxed allele is detectable at E15.5 (369). Peters et al reported that germ line deletion of the LBD of PPAR δ leads to reduced brain size and myelination defects in the corpus callosum of a small percentage (30%) of male mice (254). To investigate the effects of PPAR δ deletion on CNS development, an inducible system such as the

tetracycline-responsive operator (Tet-O) (397) could be used to control when nestin-cre is expressed, and thus when PPAR δ is deleted.

The neuronal PPAR δ KO model used in these studies revealed a previously unappreciated role of PPAR δ in CNS. The phenotype however, is complex and many questions remain regarding the role of CNS PPAR δ in DIO susceptibility and regulation of fuel partitioning and body composition. A major limitation of the neuronal KO model is the inability to isolate the type of neurons, the neurological system or even the brain regions that are involved in the phenotype. To localize the effects of gene deletion, the floxed allele of PPAR δ could be deleted by stereotaxic delivery of an adeno-associated virus expressing Cre recombinase (398). Several lines of evidence suggested that PPAR δ is critical for the function of hypothalamic neurons involved in energy balance. Therefore a future direction could be to determine if targeted deletion in the hypothalamus can recapitulate the abnormal feeding and neuropeptide responses to fasting found in the neuronal KO mouse. Biochemical analysis could be used to determine if loss of PPAR δ expression in the hypothalamus is sufficient to cause oxidative stress, ER stress, and inflammation, and if so, whether the effects lead to changes in hormone sensitivity. A follow up experiment could determine if chemical modulation of these pathways (ER stress, oxidative stress or inflammation) or conditional overexpression of a PPAR δ target gene, such as UCP2 is sufficient to rescue the phenotype caused by regional deletion of PPAR δ . These proposed experiments will be described in more detail later in this chapter.

In neuronal PPAR δ KO mice on both LF and HF diet, excess fat mass accrual occurred in the absence of large differences in food intake or energy expenditure (normalized to either BW or lean mass). Therefore, preferential disposition of consumed calories towards adipose tissue storage coupled with likely subtle imbalances between food intake and energy expenditure contribute to excess weight gain in these animals over time. Loss of PPAR δ function with GSK0660 led to insulin resistance in mHYPO E42 cells, and leptin resistance in neuronal PPAR δ KO mice. An important question to address in future studies is if PPAR δ modulates insulin and

leptin sensitivity in neuronal circuits outside the hypothalamus that could lead to positive energy balance. The reinforcing aspects of dopamine release on food reward are modulated by peripheral adiposity signals acting in brain regions associated with reward and cognition (70) and resistance to these hormones may contribute to feeding dysregulation in obesity (74). PPAR δ mRNA expression is reduced after HF diet feeding in the striatum (71), which contains postsynaptic neurons involved in the mesolimbic dopamine reward pathway (62, 63). To determine if PPAR δ affects the neurocircuitry involved in reward, neuronal PPAR δ KO mice could be assessed using a conditioned place preference paradigm (399) to determine if the rewarding properties of high-fat diet are increased in the absence of PPAR δ expression.

There is evidence that disruption of the circadian rhythmicity of feeding leads to increased adiposity and weight gain despite similar food intake in mice (400). Therefore, it is possible that neuronal PPAR δ KO mice gain weight due to alterations in feeding patterns, which would not be detected by measurements of cumulative biweekly or daily food intake. Obesity is associated with perturbations in the expression of clock genes controlling circadian rhythms involved in energy homeostasis and feeding patterns within the caudal brainstem nucleus of the solitary tract (401). These changes in circadian gene expression occurred in the presence of increased expression of PPAR α , a gene that is normally repressed by PPAR δ (274). To determine if alterations in the circadian feeding rhythms may contribute to increased adiposity in neuronal PPAR δ KO mice, a meal pattern analysis could be performed using an advanced phenotyping system such as the Promethion System (Sable Systems Inc., Las Vegas, NV) which is available in the Vanderbilt MMPC.

Other regions of the brain which may be affected by loss of PPAR δ could be identified by assessing UCP2 expression by in situ analysis after HF-MUFA diet exposure. These may include the hippocampus, which expresses UCP2 (402) and PPAR δ (248) and where both genes may be involved in protection against oxidative stress (349, 403). Leptin signaling in this region reduces food intake (404) while damage to the hippocampus by lesioning interferes with energy

and body weight regulation, potentially due to dysregulation of food related memory processing (405). Collectively, positive findings following regional deletion of PPAR δ through bilateral stereotaxic delivery of an adeno-associated virus expressing Cre recombinase, along with behavioral analysis studies will help determine potential cell types and neuronal signaling networks involved. Following identification putative targets, specific cre driver lines can be used to localize deletion of the floxed allele to a specific cell type.

Oxidative stress is highly associated with inflammation, via activation of NF- κ B (171, 406) and has been linked to insulin resistance in numerous peripheral tissues (161, 162, 174, 175, 177, 316, 407-411). Oxidative stress is also a common feature of neurodegenerative disease states (164, 165, 167). PPAR δ agonists have neuroprotective qualities that may be attributed to reductions in ROS production and protection from the deleterious effects of oxidative stress (323). Thaler et al observed morphological changes in mitochondria after 1 week of HF feeding (313) suggestive of mitochondrial dysfunction (412), however there are no published reports of oxidative stress measured in hypothalamus of DIO animals. Therefore, superoxide levels were determined in medialbasal hypothalamus of Long Evans rats after 1 week of ad libitum feeding with LF or HF-SFA diet. There was a profound increase in mitochondrial superoxide accumulation in the hypothalamus of rats fed HF-SFA diet, compared to LF diet (Figure 30), as determined with the mitochondrial superoxide specific probe, MitoSOX.

Additional groups of rats received LF or HF-SFA diet as well as a single daily bolus injection of the superoxide scavenger, mitoTEMPO (1.5mg/kg/day, IP) (413), or vehicle during the one week study period to demonstrate that mitochondria were indeed the source of superoxide.

MitoTEMPO did not alter hypothalamic superoxide levels in rats fed LF diet (Figure 30). In rats fed HF-SFA diet, MitoTEMPO treatment prevented hypothalamic mitochondrial superoxide accumulation, compared to HF-SFA fed, vehicle treated rats (Figure 30, $p < 0.05$). Collectively, this data provides evidence that hypothalamic mitochondrial superoxide accumulation is a feature of early DIO and supports a model where SFA induced hypothalamic mitochondrial ROS

accumulation may promote ER stress and inflammation resulting in insulin and leptin resistance, thereby contributing to dysregulated energy homeostasis and DIO (Figure 31A).

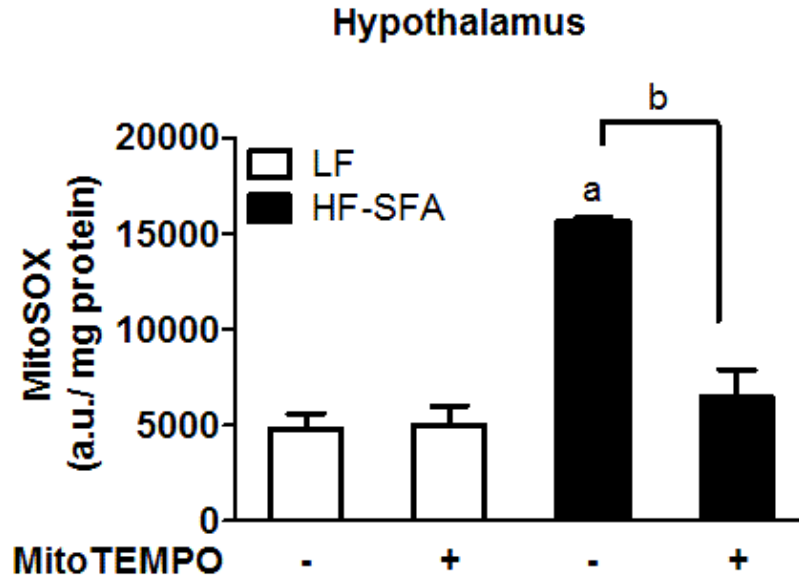


Figure 30. One week of high fat diet leads to hypothalamic mitochondrial oxidative stress.

Mitochondrial oxidative stress was measured in medialbasal hypothalamus of Long Evans rats after 1 week of ad libitum feeding of LF or HF diet with and without MitoTEMPO treatment. ROS levels were quantified using the mitochondria specific superoxide probe MitoSOX. Values represent group mean \pm SEM (n=6). Statistical significance is designated as ^a ($p < 0.05$ vs. LF - MitoTEMPO) and/or ^b ($p < 0.05$ vs. HF-SFA - MitoTEMPO), as determined by one-way ANOVA and post-hoc Tukey's multiple comparison test.

PPAR δ activation with GW0742 reduced palmitate induced IL6 expression in mHYPO E42 cells, while loss of PPAR δ function, in vitro with GSK0660, and in neuronal PPAR δ KO mice, increased IL6 and IL-1 β expression. Changes in cytokine expression were accompanied by similar elevations in activation of the ER stress marker p'PERK and evidence for increased ROS production (not shown). There is some evidence that ER stress and inflammation are intimately linked via the IKK-NF-KB pathway (152). The temporal sequence in which these pathological events occur in DIO is not known, although chemical inhibitors of ER stress (414) or genetic

deletion of IKK, the upstream kinase involved in activation of the NF- κ B pathway (152, 415) improve hypothalamic insulin and leptin sensitivity in DIO animals. Oxidative stress can contribute to insulin resistance through activation of ER stress (416) as well as activation of the IKK-NF- κ B pathway (171). Thus the next key question is whether reducing oxidative stress will prevent ER stress and inflammation caused by loss of PPAR δ function with the PPAR δ inhibitor GSK0660. The role of oxidative stress can be determined in mHYPO E42 cells using polyethylene glycol conjugated superoxide dismutase (PEG-SOD) (417) or MitoTEMPO to reduce cytoplasmic or mitochondrial superoxide levels, respectively.

As shown in chapter III, 1 week of HF diet feeding was associated with elevated IL-6 and slightly lower UCP2 mRNA levels. Superoxide levels were also increased at this time point (Figure 30). Data implicate UCP2 in neuroprotection, reduction of CNS oxidative stress and protection from neuroinflammation and neuronal injury (381, 403, 418). Thus, the next question is whether UCP2 expression is sufficient to restore the protective effects of PPAR δ activation, which could be tested in mHYPO E42 cells treated with a viral expression vector to restore UCP2 expression following exposure to mixed FFAs and GSK0660.

Consistent with a primary role for neuronal PPAR δ to regulate UCP2 in response to fatty acids, fasting failed to increase hypothalamic expression of UCP2 in neuronal PPAR δ KO mice. This was accompanied by dramatic differences in neuropeptide gene expression (both NPY and POMC) at baseline, and a complete absence of compensatory responses to fasting. ROS serve as a nutrient signal and second messenger in hypothalamic neurons, where they are known to repress NPY while simultaneously promoting POMC gene expression (380). The regulatory effects of ROS, to repress NPY neuronal activation and neuropeptide expression, are abrogated by UCP2 mediated mitochondrial uncoupling (116). The blunted fasting induced UCP2 and NPY expression as well as impaired refeeding in this neuronal PPAR δ KO model are similar to effects observed with a global PPAR δ KO model (259). This evidence suggests that PPAR δ regulates neuropeptide expression indirectly, via control of UCP2 expression and ROS production. To

address this critical question, UCP2 expression could be restored through microinjection of a viral vector expressing UCP2 (419) into the hypothalamus, to determine if it could reverse the effects of PPAR δ deletion on neuropeptide expression and feeding responses as well as increased basal stress and inflammation present in KO mice on LF diet. Superoxide levels and UCP2 protein content should also be measured, to confirm that an increase in UCP2 mRNA leads to increased protein and measurable effects on ROS production.

An intriguing observation was that in PPAR δ KO mice, HF diet exposure did not further increase CNS inflammation or ER stress beyond that observed with LF diet. These effects may be attributed to a robust upregulation of PPAR γ which has well documented anti-inflammatory effects (256, 289). In addition to a marked increase in PPAR γ expression, expression of two PPAR γ target genes, LPL (217) and CD36 (420) were also increased. There was also evidence for increased activation of PPAR α on HF diet, as evidenced by increased expression of the target gene CPT1A (244) and reduced lipid accumulation. These findings are consistent with evidence from in vitro studies that show PPAR δ represses both the expression and activation of other PPAR isoforms (276, 385). In order to quantify the effect of PPAR γ upregulation, the following experiments could be done.

Weight gain is a side effect of the TZD class of PPAR γ ligands (421), which has been attributed to effects of activation of PPAR γ in the CNS (241, 242). This evidence suggested that PPAR γ was a potential mediator of elevated adiposity and increased susceptibility to DIO in PPAR δ KO mice. The basal phenotype of increased adiposity in PPAR δ KO mice is greatly augmented upon exposure to HF diet which suggests that PPAR γ activation by dietary fatty acids (242) contributes to obesity in this model. If this theory is correct, treatment of neuronal PPAR δ KO mice with a chemical PPAR γ antagonist should attenuate the rate at which KO mice accrue fat mass on a HF diet.

While hypothalamic inflammation was elevated in the basal state (LF diet), KO mice exhibited resistance to further HF diet induced hypothalamic inflammation. PPAR γ has potent

anti-inflammatory effects via transrepression of NF- κ B and inhibition of cytokine expression (290), raising the possibility that dietary fat provides ligands that activate PPAR γ , which in turn reduces inflammation in KO animals. Based on this, antagonist treatment would be expected to increase inflammation in KO mice on HF diet. Lastly, a double PPAR γ /PPAR δ knockout approach may be required to reveal the full range of the effects of PPAR γ modulation of the PPAR δ phenotype.

A number of dual PPAR agonists have been developed, that retain the beneficial effects of TZDs but cause less weight gain (422-425). This can be achieved with compounds that are dual agonists of PPAR δ , the activation of which opposes weight gain through increased expression of target genes involved in mitochondrial function and lipid oxidation (346). Therefore, based upon several lines of evidence, both in the literature and presented here, PPAR δ may reduce inflammation indirectly, through upregulation of protective target genes that oppose lipotoxicity. In addition to selectively amplifying the expression of protective PPAR δ target genes that also may promote fat oxidation, dual agonists minimize the fat storage promoting potential of PPAR γ by acting as partial agonists, which have weak transactivation potential of adipogenic gene expression (423). The insulin sensitizing effects of partial PPAR γ agonists may therefore be attributed to transrepression of inflammatory gene expression (289). There is some data to suggest that partial loss of PPAR γ transactivation with a dual PPAR γ /RXR antagonist (426) or in heterozygous PPAR γ KO mice (427), is associated with weight loss, resistance to DIO, and improved insulin sensitivity. Posttranslational modification of the PPAR γ LBD by the action of small ubiquitin-like modifier proteins (SUMO), reduces inflammation despite preventing RXR from associating with PPAR γ after ligand binding, which commits it to the transrepression pathway (428). Therefore, development of a dual agonist compound that could promote transactivation of PPAR δ target genes and also selectively commit PPAR γ to the transrepression pathway would have extensive therapeutic potential as an anti-inflammatory weight loss and insulin sensitizing drug. No compounds like this currently exist, although one approach may be to

design a ligand that can block RXR binding to PPAR γ through steric hindrance of the region of the LBD that contains the RXR docking site (283).

The PPAR α target gene CPT1 has been implicated in impaired fuel sensing and hyperphagia in response to HF diet (383, 429) and in peripheral tissues, is known to augment mitochondrial oxidative stress and superoxide production (174). Transient oxidative stress damages mitochondria (430) which may explain lipid accumulation after prolonged HF feeding in wild type animals and as reported previously (133). However, the blunted FFA accumulation found in KO mice on HF diet did not support the inflammation based model (325) that also suggests toxic lipid accumulation is involved in DIO pathogenesis (133). Lack of inflammation and blunted lipid accumulation in neuronal PPAR δ KO mice on HF diet, are consistent with an alternative model that suggests that HF diet may lead to loss of hypothalamic fuel sensing which promotes obesity, possibly through alterations in neuropeptide expression and neuronal energy metabolism (383, 429). While the hypothalamic gene expression profile of PPAR δ KO mice suggests increased mitochondrial fatty acid oxidation, peroxisomal fatty acid oxidation would also explain blunted FFA accumulation in KO animals (196). DIO leads to peroxisome proliferation in POMC neurons which has been attributed to PPAR γ activation in DIO (380). This model is also consistent with upregulation of UCP2 expression in wild type mice on HF diet, which would be predicted to contribute to increased food intake (117). Collectively, the findings in neuronal PPAR δ KO mice on HF diet support a model where protective responses that restrain stress, inflammation and lipid accumulation may contribute to obesity pathogenesis, and that these effects may be mediated by acquired loss of PPAR δ repression and upregulation and/or activation of PPAR α and PPAR γ .

An integrated model, based upon the individual findings presented in this dissertation, and discussed in this chapter, for the roles of PPAR, UCP2, ROS and compensatory upregulation of other PPAR isoforms will be described next (Figure 31). The inability to upregulate UCP2 in response to elevated FFA levels may augment ROS production that acutely acts to disrupt

neuropeptide expression causing abnormal feeding responses. Chronic exposure to ROS results in hypothalamic oxidative stress, which then activates IKK-NF- κ B and promotes ER stress, which may contribute to insulin and leptin resistance in neuronal PPAR δ KO animals on LF diet. Basal inflammation promotes leptin and insulin resistance, which could then contribute to higher basal adiposity in KO mice. Exposure to circulating FFAs, during fasting or HF feeding, may lead to unregulated activation of PPAR α , and increased fatty acid oxidation and blunted lipid accumulation. Without the protective effects of PPAR δ , the effects of FFAs on oxidative and ER stress and inflammation are augmented. However, PPAR δ is also a known repressor of the PPAR γ , which becomes activated and may act to reduce inflammation but also could promote DIO through a yet to be determined mechanism. Thus, in the absence of PPAR δ , the normally protective responses of these two PPAR isoforms appear to be unregulated, effectively preventing lipotoxicity while promoting obesity.

Conclusions

Collectively, my findings show that neuronal PPAR δ expression is critical to the function of regulatory neurons involved in energy homeostasis. Profound dysregulation of homeostatic responses to fasting and refeeding, an experimental maneuver to amplify potential defects in the system, reveal UCP2 as a potential molecular mediator of the phenotype. The inability to upregulate UCP2 in response to normal physiological stressor, and after feeding, suggests that PPAR δ may play a pivotal role, regulating the delicate balance between redox control of feeding regulation and protection from oxidative stress (Figure 31). More work is needed to identify the brain regions and neuronal cell types involved in the phenotypic abnormalities and also, to determine if oxidative stress contributes to hypothalamic inflammation and leptin resistance in DIO.

Future studies will be required to understand compensatory changes in other PPAR isoforms in this complex phenotype. Given multiple complex modes of regulation of multiple target genes,

including other PPAR isoforms (269, 431), an understanding of the relevance of isoform compensation and crosstalk will require studying mice with genetic deletion of PPAR δ and PPAR γ . PPARs are transcriptional targets of dietary lipids (or metabolites thereof) and are likely to shed important new light upon plausible mechanisms by which a changing dietary environment may multi-factorally enhance susceptibility to obesity. More work is clearly needed to understand the role of these complex receptors in the brain.

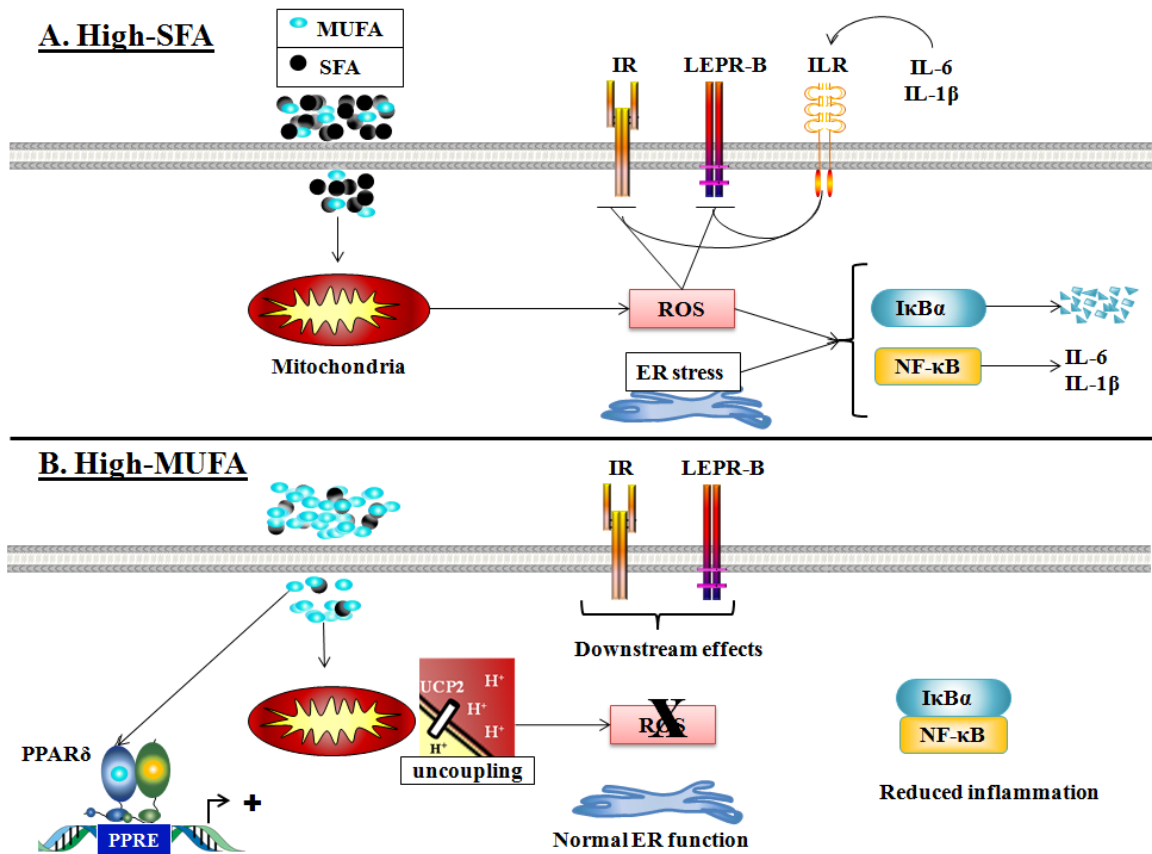


Figure 31 Model: PPAR δ is a protective monounsaturated fatty acid sensor in neurons

A) During HF-SFA feeding, cells are flooded with SFA from plasma, which promote mitochondrial ROS production and ER stress leading to activation of inflammatory pathways including NF- κ B. NF- κ B is normally retained in the cytoplasm by I κ B α . SFAs promote ER stress and mitochondrial ROS production leading to degradation of cytoplasmic I κ B α resulting in transcriptional activation of proinflammatory NF- κ B cytokine target genes that further promote inflammation and insulin and leptin resistance. B) MUFAs from HF-MUFA diet activate PPAR δ causing transcriptional upregulation of UCP2 and mitochondrial uncoupling such that mitochondrial oxidation of FFAs does not promote ROS formation. MUFAs do not promote ER stress. Collectively, reduced ER and oxidative stress result in low levels of NF- κ B activation, thereby maintain insulin and leptin sensitivity despite HF diet consumption.

ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ACO	acyl-CoA oxidase
BAT	brown adipose tissue
CD36	cluster of differentiation 36
CNS	central nervous system
CPT1A	carnitine palmitoyltransferase-1A
DGAT2	diacylglycerol acyltransferase 2
DIO	diet-induced obesity
DBD	DNA binding domain
EE	energy expenditure
FAS	fatty acid synthase
FFA	free fatty acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPAT	glycerol-3-phosphate acyltransferase
HF	high-fat
HSC70	heat shock cognate protein 70
ICV	intracerebroventricular
IL-1 β	interleukin-1 beta
IL-6	interleukin-6
I κ B α	inhibitor of NF- κ B alpha
KO	knockout
LBD	ligand binding domain
LF	low-fat
LPL	lipoprotein lipase
MUFA	monounsaturated fatty acid
NF- κ B	nuclear factor-kappa B
NPY	neuropeptide Y
p66shc	p66 Src homology 2 domain containing transforming protein
PDK4	pyruvate dehydrogenase kinase 4
PERK	protein kinase RNA-like endoplasmic reticulum kinase
POMC	Proopiomelanocortin
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
RPL13A	ribosomal protein L13A
SCD1	stearoyl CoA desaturase 1
STAT3	signal transducer and activator of transcription 3
TG	triglyceride

TNF α	tumor necrosis factor alpha
TZD	thiazolidinedione
UCP2	uncoupling protein 2
WAT	white adipose tissue

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